Antiarrhythmic Drug-Induced Internalization of the Atrial-Specific K⁺ Channel Kv1.5

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Abstract—Conventional antiarrhythmic drugs target the ion permeability of channels, but increasing evidence suggests that functional ion channel density can also be modified pharmacologically. Kv1.5 mediates the ultrarapid potassium current \(I_{\text{Kur}}\) that controls atrial action potential duration. Given the atrial-specific expression of Kv1.5 and its alterations in human atrial fibrillation, significant effort has been made to identify novel channel blockers. In this study, treatment of HL-1 atrial myocytes expressing Kv1.5-GFP with the class I antiarrhythmic agent quinidine resulted in a dose- and temperature-dependent internalization of Kv1.5, concomitant with channel block. This quinidine-induced channel internalization was confirmed in acutely dissociated neonatal myocytes. Channel internalization was subunit-dependent, activity-independent, stereospecific, and blocked by pharmacological disruption of the endocytic machinery. Pore block and channel internalization partially overlap in the structural requirements for drug binding. Surprisingly, quinidine-induced endocytosis was calcium-dependent and therefore unrecognized by previous biophysical studies focused on isolating channel–drug interactions. Importantly, whereas acute quinidine-induced internalization was reversible, chronic treatment led to channel degradation. Together, these data reveal a novel mechanism of antiarrhythmic drug action and highlight the possibility for new agents that selectively modulate the stability of channel protein in the membrane as an approach for treating cardiac arrhythmias. (Circ Res. 2009;104:1390-1398.)

Key Words: potassium channel • trafficking • cardiac • antiarrhythmic

Atrial fibrillation is the most common cardiac arrhythmia, and a major risk factor for increased stroke, heart failure, and cardiovascular morbidity. The preferred therapy for atrial fibrillation is sustained sinus rhythm control; however, the efficacy of currently used antiarrhythmic drugs is diminished by adverse side effects resulting from a lack of ion channel selectivity and nonspecific ventricular activity.1,2 Because of the frequency of atrial fibrillation and its associated morbidity, development of atrial-specific therapies is a major focus of both industrial and academic research efforts.3,4 Kv1.5 (KCNA5) has emerged as a promising pharmacological target for treatment of atrial fibrillation. In humans, Kv1.5 is selectively expressed in atrial myocytes, where it mediates the ultrarapid delayed rectifier current \(I_{\text{Kur}}\) that contributes to cellular repolarization and controls action potential duration.5,6 Although significant effort has been made to identify novel blockers of Kv1.5, compounds with both atrial selectivity and clinical efficacy remain elusive and highlight the need for new potential therapeutic strategies or targets.

Conventional antiarrhythmic drugs generally target the ion permeability of channels. Increasing evidence, however, suggests that functional ion channel density can be modified pharmacologically in that a drug may both directly block an ion channel and indirectly disrupt normal protein trafficking.7 In fact, 1 report shows that nearly half of hERG channel pore blockers tested also decrease anterograde delivery of the channel to the cell surface.8 In addition, 2 reported cases of disrupted protein trafficking leading to drug-induced prolongation of the cardiac action potential highlight that this pleiotropic drug action can modulate cardiac excitability.9,10 Research into the therapeutic value for antiarrhythmic agents that affect channel trafficking has focused almost exclusively on hERG channel blockers that stabilize misfolded channels and rescue hERG trafficking mutants. To date, no studies have addressed the potential properties of antiarrhythmic drugs to acutely modulate surface density of functional channels that exist on the cardiac myocyte membrane. Here we report, for the first time, a novel paradigm for antiarrhythmic pharmacology in the control of the cell surface stability of Kv1.5 in atrial myocytes and present a new mechanism for the inhibition of ion current through drug-stimulated endocytosis of channel protein.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Immunocytochemistry

Immunocytochemistry was performed, and all images were collected, quantified, and analyzed as reported previously11 and as described in the online data supplement.
Electrophysiology
Whole-cell voltage-clamp experiments were performed on HL-1 cells stably expressing Kv1.5-pHluorin as described previously. All experiments were performed at room temperature.

Neonatal Myocyte Isolation and Electroporation
Cardiomyocytes from neonatal mice were isolated and cultured according to methods adapted from Zlochiver et al. Electroporation was performed on acutely isolated myocytes before plating as described in supplement.

Results

The Antiarrhythmic Drug Quinidine Stimulates Rapid Kv1.5 Internalization in HL-1 Atrial Myocytes
Multiple antiarrhythmic drugs have been shown to inhibit Kv1.5, including the class I antiarrhythmic drug quinidine, which, like many Kv channel inhibitors, causes an activity-dependent, open-channel block of Kv1.5 (Figure I in the online data supplement). The present study was designed to investigate the potential pleiotropic effects of antiarrhythmic agents on Kv1.5 using this well-characterized drug as a prototype. Our studies were initiated in the HL-1 immortalized mouse atrial myocyte cell line in which we previously demonstrated that Kv1.5 undergoes constitutive internalization and recycling to maintain steady-state ion channel surface levels. Using an extracellular GFP epitope–tagged Kv1.5 construct that mimicked wild-type channel function, we discovered that quinidine triggered Kv1.5 internalization concomitant with block of channel current (Figure 1A and Online Figure I). Exposure to increasing concentrations of quinidine stimulated a dose-dependent increase in internalized Kv1.5 with a corresponding loss of surface protein (Figure 1B). This quinidine-induced internalization culminated in an 80±13% (n=143; P<0.001) increase over constitutive channel endocytosis at 100 μmol/L quinidine (EC50≈1 μmol/L). Quinidine-induced internalization of Kv1.5 was rapid and achieved a maximum within 10 minutes of treatment (Online Figure I). Electrophysiology experiments confirmed that 10 minutes quinidine treatment represents steady-state channel block (Online Figure I). Together, these data indicate that the rate-limiting step for this process is equilibration of quinidine across the cell membrane. Following 10 minutes of vehicle treatment, the level of internalized Kv1.5 is minimal and consistent with constitutive endocytosis as previously reported (Figure 1A). Importantly, at the 10-minute time point, quinidine-stimulated internalization was statistically greater compared to control (P<0.001) and could be reliably separated from constitutive endocytosis and was therefore used, unless otherwise stated, in our later studies.

We also measured a temperature dependence for the quinidine-induced internalization of Kv1.5 (Figure 1C). The drug-induced internalization was greater than 2-fold higher at the physiological temperature of 37°C compared to room temperature (80±13% [n=143] at 37°C versus 45±11% [n=130] at 25°C; P<0.001). Importantly, the EC50 values were identical and only the extent of quinidine-induced internalization was temperature-dependent. It is noteworthy that previous biophysical studies measuring pore block with...
quinidine were performed at room temperature where the effects of internalization would be inadvertently reduced.

To negate the possibility of an antibody-induced artifact, we generated a Kv1.5 construct in which GFP was replaced with pHluorin, a GFP variant whose fluorescent properties are sensitive to the pH of the immediate environment. Using live-cell imaging of HL-1 cells expressing Kv1.5-pHluorin, we found no difference in the time course, extent, or subcellular localization of quinidine-induced internalized channel compared to results from our antibody-labeling internalization assay (Online Figure II). Together, these results suggest that antiarrhythmic drugs may modulate retrograde trafficking of Kv1.5 as a mechanism contributing to their inhibition of outward K⁺ current.

Constitutive and Quinidine-Induced Internalization of Kv1.5 Is Conserved in Native Dissociated Mouse Myocytes

We investigated the validity of the quinidine effect in native dissociated mouse myocytes. The live-cell internalization assay was performed on acutely dissociated neonatal mouse myocytes expressing Kv1.5-GFP to probe for alterations in Kv1.5 distribution on quinidine treatment. Using this technique, we found levels of constitutive and quinidine-induced channel internalization similar to those observed in the HL-1 model (Figure 2A). This drug-induced internalization culminated in an 84 ± 19% (n = 45) (P < 0.001) increase over constitutive channel endocytosis at 100 μmol/L quinidine (n = 52), with a corresponding decrease in surface levels (Figure 2B). These data confirm that the endogenous machinery and mechanistic requirements for constitutive and quinidine-induced Kv1.5 internalization are conserved in native cardiac tissue.

Specificity of Quinidine-Induced Internalization

We investigated the subunit specificity by measuring quinidine effects on internalization of 2 other prominent cardiovascular potassium channels, Kv4.2 and Kv2.1, expressed in HL-1 cells. Although ion permeability of both channels is blocked by quinidine (IC₅₀ of 10 μmol/L and 20 μmol/L quinidine, respectively), neither Kv4.2 nor Kv2.1 internalized in response to any drug concentration tested over the time course studied (Figure 3A and Online Figures III and IV). Together, these data demonstrate a subunit dependence for the quinidine-induced internalization of Kv1.5.

Quinidine-induced internalization occurred at resting membrane potentials suggesting that the drug-induced trafficking effects may be conduction-independent. To address this possibility, we measured internalization of the pore–dead mutant Kv1.5-W472F, which efficiently traffics to the myocyte membrane but is incapable of conducting current. For both the mutant and wild-type controls, the quinidine-induced internalization resulted in an approximately 90 ± 20% (n = 90) (P < 0.001) increase over constitutive endocytosis, with no difference observed in the EC₅₀ value (Figure 3B). These data confirm that the quinidine-induced internalization of Kv1.5 is independent of channel ion conductance.

Structural Requirements of Quinidine-Induced Internalization

To further examine specificity and gain possible insight into the pharmacophore responsible for drug-induced internalization of Kv1.5, we tested quinine, the diastereomer of quinidine that also causes a dose-dependent block of Kv1.5 current. We found that, contrary to quinidine, a maximal concentration of quinine was not able to enhance Kv1.5 internalization above constitutive levels (Figure 3C). Quinine was, however, capable of inducing significant, dose-dependent block of Kv1.5 that was reversible on drug washout (Online Figure V). The inability of quinine to induce channel internalization, despite effective pore block, demonstrates that quinidine-induced internalization of Kv1.5 is stereospecific.

The subunit dependence and stereospecificity of quinidine-induced internalization of Kv1.5 indicates a reliance of this internalization on the structure of the channel. To determine whether the protein structural requirements for quinidine-induced internalization were the same as those for pore block, we used the live-cell internalization assay to investigate the effect of quinidine on several mutants of Kv1.5. Kv1.5-T480A contains a single point mutation in the putative binding site for quinidine, resulting in a >90% reduction in sensitivity to open-channel blockers of Kv1.5. We found
that, as with pore block, quinidine-induced internalization was abolished with the T480A mutation (Figure 4A). To further probe the amino acid requirements, we expanded our Ala-scanning mutagenesis to include 3 additional amino acid residues (Ile508, Leu510, and Val512), which are considered part of the highly conserved antiarrhythmic drug binding site within Kv channels.21,22 We found that as reported for channel block, quinidine-induced internalization was abolished by the Kv1.5-I508A mutation. Surprisingly, however, unlike channel block, internalization was near wild type for both Kv1.5-L510A and Kv1.5-V512A (Figure 4B). Additionally, we tested Kv1.5-P532L, a naturally occurring mutation reported to cause a significant rightward shift in the dose-response curve for quinidine block of channel current.23 We observed a similar decrease in sensitivity to quinidine-induced channel internalization with a shift in the EC50 from approximately 1 μmol/L for wild-type channel to 104 μmol/L for Kv1.5-P532L (n=90) (Figure 4C). These data show that quinidine is acting directly on the channel and not through off-target effects that may influence trafficking. Importantly, these data also indicate that antiarrhythmic drugs may both block channel current and disrupt protein endocytosis concurrently to alter the functional ion channel density in the cell membrane and that these 2 effects share partial overlap in the structural requirements for drug binding, but the necessary amino acids are not are not identical.

Channel Internalization Is Prevented by Disruption of Endocytic Machinery

Our previous studies demonstrated that constitutive Kv1.5 internalization occurs via a microtubule-dependent, dynemin-mediated endocytic pathway.11 To further address specificity and determine whether quinidine-induced internalization shares a similar mechanism, we used pharmacological and dominant-negative methods to disrupt the endocytic machinery before measuring quinidine-stimulated Kv1.5 internalization. Dynasore, a cell-permeant, potent small molecule inhibitor of dynamin, prevents the budding and pinching off of endocytic vesicles,24,25 and has been used in neurons to block
Is Calcium-Dependent

Quinidine-Induced Internalization

Synaptic Vesicle Endocytosis

Acute pretreatment with this pharmacological inhibitor abolished quinidine-induced internalization of Kv1.5 in atrial myocytes (Figure 5A). We also observed a nearly 100% increase in surface levels of Kv1.5 over untreated controls, indicating that Dynasore prevents constitutive endocytosis of the channel (Figure 5B and 5C). Additionally, we found that p50-dynamitin overexpression, which we and others have reported blocked constitutive Kv1.5 endocytosis, also blocked quinidine-induced internalization with a corresponding increase in surface levels similar to the inhibition measured with Dynasore (Online Figure VI). These data indicate that quinidine-induced internalization of Kv1.5 occurs via a microtubule-dependent, dynein-mediated pathway as previously demonstrated for constitutive endocytosis.

Differential Effects of Acute Versus Chronic Treatment With Quinidine

Previously we reported that, following constitutive internalization, a population of Kv1.5 originating on the atrial myocyte cell surface recycled back to the plasma membrane. Electrophysiologically, we showed that quinidine-induced block of Kv1.5 current is reversible on drug washout (online Figure I). On quinidine stimulation, internalized Kv1.5 also colocalized with the early endosomal marker EEA1 (data not shown). Therefore, we measured the intracellular fate of the quinidine-induced, internalized Kv1.5 on drug washout. Using a modified form of the recycling assay developed previously within our laboratory, and HL-1 cells transiently expressing this process. The biophysical properties of Kv1.5 pore block by quinidine have been previously characterized. Our survey of the literature revealed that all of these electrophysiological studies included a calcium-chelating agent in the pipette solution to isolate the drug-channel interaction. Because several intracellular trafficking pathways are known to be calcium-dependent, inclusion of a calcium-chelating agent could inhibit the endogenous or drug-induced trafficking pathways. To investigate this possibility, we performed whole-cell patch-clamp recordings on cells stably expressing Kv1.5 both in the presence and absence of the calcium-chelating agent BAPTA in the pipette solution. In response to a single depolarizing pulse from −80 to +60 mV, perfusion of cells for 10 minutes with 6 μmol/L quinine reduced the peak current and accelerated the time course of inactivation under both conditions. Surprisingly, however, exclusion of BAPTA resulted in a much larger effect on peak current and revealed a significant calcium-dependent decrease (Figure 6A). This decrease in Kv1.5 current in the absence of BAPTA was observed over a range of voltages (Online Figure VII). Furthermore, in the absence of BAPTA in the pipette solution, the dose–response for quinidine was significantly leftward shifted with a 3-fold decrease in the IC_{50} (IC_{50} = 13 μmol/L with BAPTA and 3.5 μmol/L without BAPTA) (n=5) (Figure 6B). Conversely, this calcium-dependent decrease in current density did not occur with 20 μmol/L quinine, the diastereomer of quinidine (Online Figure VIII). This is in agreement with our finding that quinine did not induce channel internalization. Together, these data provide functional evidence for quinidine-induced channel internalization through a calcium-dependent mechanism.

To determine whether these functional measurements are supported by fluorescence imaging data, we measured the quinidine-induced internalization of Kv1.5 in the presence of the cell-permeant compound BAPTA-AM to chelate intracellular calcium. Using this method, pretreatment with 10 μmol/L BAPTA-AM completely blocked quinidine-induced internalization of Kv1.5 with a corresponding increase in surface levels (Figure 6C). These results were nearly identical to those determined after endocytic disruption with Dynasore and p-50 dynamitin (Figure 5C and Online Figure VI). Together, these data demonstrate that quinidine-induced internalization is calcium-dependent and therefore unrecognized by previous biophysical studies focused on isolating channel–drug interactions.
Kv1.5-GFP, we found that a population of Kv1.5 undergoing quinidine-induced internalization recycled back to the plasma membrane with the same time constant as constitutive recycling ($\tau =$ 29.06 minutes and $\tau =$ 25.72 minutes, respectively) (Figure 7A). This channel recycling also occurred on a time scale nearly identical to recovery from current block. This suggests that the rate-limiting step for recovery of Kv1.5 current is not quinidine dissociation from channel and indicates that quinidine is acting on the internalization pathway and not on the recycling pathway. Importantly, over the time course studied, we detected no loss in total protein, as measured by total GFP fluorescence, indicating that quinidine-induced internalization recycled back to the plasma membrane with the same time constant as constitutive recycling and that acute quinidine treatment leads to channel internalization that is reversible on drug washout, whereas chronic quinidine treatment leads to channel degradation.

**Discussion**

Here, we report a previously unrecognized mechanism of antiarrhythmic drug action in the acute modulation of surface channel density. Using quinidine, an antiarrhythmic agent that has both class Ia actions and class III actions in mammalian atrium and ventricle, we demonstrate that channel blockers can both inhibit ion conduction and regulate the stability of the channel protein within the membrane. These pleiotropic actions, which may be independent, have important implications for antiarrhythmic drug therapy, as well as drug safety testing.

The manipulation of ion channel trafficking pathways, particularly those that target functional channels existing in the myocyte membrane, represents an alternative and potentially beneficial new therapeutic strategy. There is a clear need for the development of new, longer-term antiarrhythmic drugs to successfully maintain normal atrial sinus rhythm without risking the occurrence of potentially life-threatening, proarrhythmic ventricular side effects. The inhibition of $I_{to}$ current, through block of Kv4.2/Kv4.3 channels in the atria and ventricle, is a frequent side effect of putative Kv1.5-selective agents. Our data demonstrate that quinidine-induced internalization of Kv1.5 is dose- and temperature-dependent and, although it inhibited current, did not stimulate internalization of Kv2.1 or Kv4.2 channels. Ultimately, the development of new compounds and their efficacy to selectively modulate trafficking pathways is dependent on the ability to separate the pleiotropic actions and to isolate the pharmacophore responsible for block and/or internalization. In the

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*Figure 6. Quinidine-induced internalization occurs via a calcium-dependent mechanism. Whole-cell voltage-clamp experiments were performed on HL-1 cells stably expressing Kv1.5-pHluorin. A single depolarizing pulse to +60mV was applied as described in Materials and Methods. A, Current traces are shown for a single cell before (black) and following (gray) 10 minutes exposure to 6 μmol/L quinidine in the presence (left) or absence (right) of BAPTA in the pipette solution (n=10 cells). B, Dose–response curve on treatment with increasing concentrations of quinidine for 10 minutes at room temperature in the presence or absence of BAPTA in the pipette solution (IC$_{50}$ = 13 μmol/L with BAPTA and 3.5 μmol/L without BAPTA; n=5 cells; Hill slope=1.042 with BAPTA and 1.135 without BAPTA). C, HL-1 cells expressing Kv1.5-GFP were pretreated for 1 hour with 10 μmol/L BAPTA-AM before surface labeling with anti-GFP antibody. Quantification of internalized (left) and surface (right) Kv1.5 following treatment with 100 μmol/L quinidine for 10 minutes at 37°C in the continued presence of BAPTA-AM. Below each bar graph is a representative image for that condition.*

Scale bars=10 μm. *P<0.05, **P<0.01, ***P<0.001 as determined by one-way ANOVA with Tukey post test.
Figure 7. Acute quinidine-induced internalization is reversible, whereas chronic treatment results in channel degradation. A, Quantification of recycled Kv1.5 at 10, 20, 30, and 60 minutes at 37°C posttreatment with 100 μmol/L quinidine for 10 minutes at 37°C. Vehicle and quinidine treated data sets were each normalized to their own baseline (no washout) and maximum. Corresponding total GFP levels are provided for 0 and 60 minutes of recycling (inset). Below are representative images showing the increase in recycled Kv1.5-GFP with time, after quinidine treatment. Scale bar=10 μm. B, HL-1 cells stably expressing Kv1.5-pHluorin were treated with 10 μmol/L quinidine for 0, 12, or 48 hours at 37°C. Quantification of total Kv1.5 protein levels normalized to actin and control (48-hour DMSO) levels. Below is a representative image of a Western blot for 0, 12, and 48 hours of quinidine treatment. C, HL-1 cells stably expressing Kv1.5-pHluorin were treated with vehicle, 10 μmol/L quinidine, 10 μmol/L leupeptin, 500 nmol/L ALLN, quinidine and leupeptin, or quinidine and ALLN for 48 hours at 37°C. Quantification was performed as in B. *P<0.05, **P<0.01 as determined by 1-way ANOVA with Tukey post test.

The potential for drugs to modulate surface density also raises important issues for drug safety screening. It is well known that nonantiarrhythmic drugs can have proarrhythmic liabilities through the off-target inhibition of ion channels in the heart. This issue has received considerable attention from pharmaceutical companies and regulatory agencies that now mandate cardiac ion channel testing as part of drug safety profiling. Attention to the potential problem of nonantiarrhythmic drugs affects both pore block and channel trafficking ( Figures 1 and 6). In contrast, open channel block of Kv1.5 was voltage- and activity-dependent (Online Figure I). In addition, there are several pieces of data in this study in which pore block occurred without channel internalization. As mentioned above, both Kv4.2 and Kv2.1 exhibit significant quinidine-mediated current block; however, neither underwent drug-induced internalization, despite constitutive endocytosis of Kv4.2 nearly identical to Kv1.5 (Online Figure III). Importantly, drug-induced internalization of Kv1.5 was stereospecific in that quinine, the diastereomer of quinidine, caused current block of Kv1.5 but was incapable of inducing channel internalization even at a maximal dose (Figure 3). Interestingly, Ala-scanning mutagenesis of the conserved Kv channel drug-binding site showed that not all residues were conserved for both pore block and channel internalization (Figure 4). To facilitate the development of new antiarrhythmic drugs that selectively modulate channel density, future detailed structure/activity studies are required to identify the essential features within the channel and as part of the drug molecule responsible for the multiple activities of quinidine. The mechanism of drug-induced internalization most likely involves a conformational change in the channel protein; however, not all of the mutagenesis results in this study can be reconciled with the model that drug block and drug-induced internalization can be separated. For example, one considers the effects of the T480A mutation, which occurs within the pore helix and diminishes both block and internalization, compared to our results that drug-induced internalization occurs when cells are clamped at −80 mV, it is difficult to envision how quinidine can reach a binding site in the conduction pathway to initiate internalization if the cytoplasmic gate of Kv1.5 is firmly closed. An alternative strategy is the identification of the molecular machinery and further elucidation of the proteins involved in channel targeting, such as the dynein motor complex (Online Figure VI), which may reveal novel, selective auxiliary therapeutic targets. Nonetheless, this study highlights the potential for development of new agents that can selectively affect either ion conduction and/or ion channel trafficking pathways as a new means to gain therapeutic specificity.

present study, several lines of evidence indicate that these 2 processes may be separable. For instance, quinidine-induced internalization was voltage- and activity-independent in that it occurs at resting membrane potentials during immunocytochemistry and when cells are voltage clamped at −80 mV during electrophysiology measurements (Figures I and 6). In contrast, open channel block of Kv1.5 was voltage- and activity-dependent (Online Figure I). In addition, there are several pieces of data in this study in which pore block occurred without channel internalization. As mentioned above, both Kv4.2 and Kv2.1 exhibit significant quinidine-mediated current block; however, neither underwent drug-induced internalization, despite constitutive endocytosis of Kv4.2 nearly identical to Kv1.5 (Online Figure III). Importantly, drug-induced internalization of Kv1.5 was stereospecific in that quinine, the diastereomer of quinidine, caused current block of Kv1.5 but was incapable of inducing channel internalization even at a maximal dose (Figure 3). Interestingly, Ala-scanning mutagenesis of the conserved Kv channel drug-binding site showed that not all residues were conserved for both pore block and channel internalization (Figure 4). To facilitate the development of new antiarrhythmic drugs that selectively modulate channel density, future detailed structure/activity studies are required to identify the essential features within the channel and as part of the drug molecule responsible for the multiple activities of quinidine. The mechanism of drug-induced internalization most likely involves a conformational change in the channel protein; however, not all of the mutagenesis results in this study can be reconciled with the model that drug block and drug-induced internalization can be separated. For example, if one considers the effects of the T480A mutation, which occurs within the pore helix and diminishes both block and internalization, compared to our results that drug-induced internalization occurs when cells are clamped at −80 mV, it is difficult to envision how quinidine can reach a binding site in the conduction pathway to initiate internalization if the cytoplasmic gate of Kv1.5 is firmly closed. An alternative strategy is the identification of the molecular machinery and further elucidation of the proteins involved in channel targeting, such as the dynein motor complex (Online Figure VI), which may reveal novel, selective auxiliary therapeutic targets. Nonetheless, this study highlights the potential for development of new agents that can selectively affect either ion conduction and/or ion channel trafficking pathways as a new means to gain therapeutic specificity.

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*P<0.05, **P<0.01 as determined by 1-way ANOVA with Tukey post test.
focus almost entirely on the capacity of a drug to block ion conduction. The calcium dependence of quinidine-induced internalization of Kv1.5 is important when considering the in vivo effects of this drug and most likely many others. Our results demonstrate that use of compounds that deplete free intracellular calcium block a major component of quinidine action on Kv1.5. This calcium-dependent component is responsible for a significant fraction of the quinidine-mediated decrease in current density and can explain the leftward shift in the IC_{50} for quinidine from 13 μmol/L in the biophysical studies including BAPTA to 3.5 μmol/L in the absence of BAPTA (Figure 6B). However, it is important to note that the free calcium concentration is likely very high in our electrophysiological experiments performed in the absence of any calcium buffer, whereas large changes in free calcium are not expected in immunocytochemistry experiments. In addition, the 2 experiments were performed at different temperatures; therefore, the 2 conditions may not be identical. It is also possible that what is marked as calcium-dependent channel internalization (Figure 6A) is a mixture of fast block and channel internalization. However, separation of these 2 mechanisms is complicated by our finding that the rate-limiting step for the onset of drug action is equilibration across the membrane and both block and internalization recover on washout of the drug. Nevertheless, this work implies that antiarrhythmic agents such as quinidine, which affect channel trafficking pathways, may show greater efficacy and potency in the in vivo condition where calcium-dependent pathways are uninhibited. Screens for pore block may simply miss channel-trafficking effects and dramatically underestimate drug actions.

Another issue that may compound these concerns is the acute versus chronic effects of altering channel surface density. Our results show that chronic quinidine treatment results in a significant decrease in Kv1.5 channel protein by diverting channel from a recycling to degradation pathway. Recent work suggests that a fraction of internalized Kv1.5 enters proteasomal compartments.  

This is supported by data in this report showing that inhibition of the proteasomal, but not lysosomal, degradation machinery prevented the chronic quinidine-induced decrease in total Kv1.5. The time course of recovery from this depression may precipitate drug-withdrawal side effects, whereas long-term suppression of channel expression may contribute to remodeling of heart tissue. The alternative is that chronic suppression may overcome current antiarrhythmic drug limitations of acute cardiovascular and result in the benefit of maintained rhythm control. Nonetheless, together these data give further credence to concerns regarding the comprehensiveness of current ion channel drug safety tests.

In summary, this report reveals a novel mechanism of antiarrhythmic drug action in the modulation of surface channel density. Results of this study highlight the possibility for development of new agents that selectively modulate ion conduction and/or the stability of channel protein in the membrane as an alternative or complementary strategy for treating atrial fibrillation and other potential cardiac arrhythmias.

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Disclosures

None.

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Supplemental Material

Experimental Procedures:

Materials – Kv1.5-GFP and Kv1.5-mCherry constructs were generated from human Kv1.5 as previously described 1. Polyclonal anti-GFP, monoclonal anti-V5, and AlexaFluor secondary antibodies were from Invitrogen (Carlsbad, CA). Polyclonal Anti-DsRed antibody was obtained from BD Biosciences Clontech (Palo Alto, CA). Anti-troponin I was obtained from Millipore (Billerica, MA). Horseradish peroxidase-conjugated secondary antibodies were from Zymed (San Francisco, CA). HL-1 cells were a generous gift from Dr. William Claycomb (Louisiana State University Health Sciences Center, New Orleans, LA). Dynasore was a generous gift from Dr. Tomas Kirchhausen (Department of Cell Biology, Harvard Medical School and the CBR Institute for Biomedical Research, Inc. Boston, Massachusetts). Quinidine and quinine from Sigma (St. Louis, MO) were dissolved in DMSO and diluted 1:1000 into culture medium. All vehicle was DMSO 1:1000 in culture medium equivalent to drug addition.

Western Blot – HL1 cells were harvested in PBS containing Complete® protease inhibitors (Roche Applied Science, Indianapolis, IN) and lysed in SDS-PAGE sample buffer. Proteins were then separated by SDS-PAGE on a NuPAGE, Novex 4-12% Bis-Tris gel (Invitrogen), transferred to nitrocellulose, and probed with the indicated primary antibody for 1 hr at 4°C. Blots were then incubated with secondary antibodies conjugated to horseradish peroxidase (1:5000) and
visualized using the Western Lightning enhanced chemiluminescent reagent according to the manufacturer’s protocol (Perkin-Elmer Life Sciences, Wellesley, MA). Images were captured using the EpiChemi3 Darkroom (UVP, Inc., Upland, CA).

Immunocytochemistry –

For analyzing fluorescence data, background fluorescence was subtracted for each cell, prior to normalizing surface or internalized signal to the total GFP fluorescence of that cell. Normalized values for drug treated conditions were then expressed as percent of vehicle or, in some cases, normalized to maximal drug response. These values were calculated by the following equations. \( \lambda \) = wavelength at which the data were collected; \( \lambda_1 \) = fluorophore 1, \( \lambda_2 \) = fluorophore 2.

For % Vehicle:

\[
100 \times \left\{ \frac{(\text{Internalized}_{\text{drug}} \lambda_1 - \text{Background}_{\text{drug}} \lambda_1)}{(\text{GFP}_{\text{drug}} \lambda_2 - \text{Background}_{\text{drug}} \lambda_2)} \right\} \frac{(\text{Internalized}_{\text{vehicle}} \lambda_1 - \text{Background}_{\text{vehicle}} \lambda_1)}{(\text{GFP}_{\text{vehicle}} \lambda_2 - \text{Background}_{\text{vehicle}} \lambda_2)}
\]

For % Max Internalization: 100 ×

\[
\left\{ \frac{(\text{Internalized}_{\text{drug}} \lambda_1 - \text{Background}_{\text{drug}} \lambda_1)}{(\text{GFP}_{\text{drug}} \lambda_2 - \text{Background}_{\text{drug}} \lambda_2)} \right\} - \left\{ \frac{(\text{Internalized}_{\text{vehicle}} \lambda_1 - \text{Background}_{\text{vehicle}} \lambda_1)}{(\text{GFP}_{\text{vehicle}} \lambda_2 - \text{Background}_{\text{vehicle}} \lambda_2)} \right\}
\]
Immunocytochemistry was performed as previously described \(^1\). Briefly, 48 hr post transfection, HL-1 cells transiently expressing Kv1.5-GFP were live cell stained with a polyclonal anti-GFP antibody (1:500) in 2% goat serum for 30 min on ice to minimize internalization of membrane proteins. For surface labeling, cells were then incubated with goat anti-rabbit AlexaFluor secondary antibody (1:500) in 2% goat serum for 30 min on ice, fixed with 4% paraformaldehyde for 10 min on ice, and mounted with ProLong Gold anti-fade reagent (Invitrogen). For labeling surface and internalized protein following live-cell staining with anti-GFP, cells were treated with vehicle or drug for the specified amount of time prior to incubation with secondary antibody. Cells were then incubated with goat anti-rabbit AlexaFluor 405 antibody (1:200) in 2% goat serum for 30 min on ice to saturate remaining surface channel populations, fixed with 4% paraformaldehyde for 10 min on ice, permeabilized with 0.1% Triton X-100 in 2% goat serum for 10 min on ice, incubated with goat anti-rabbit AlexaFluor 647 antibody (1:500) in 2% goat serum for 30 min on ice, and mounted with ProLong Gold. Images of transfected cells displaying fluorescent signals were acquired on an Olympus Fluoview 500 confocal microscope with a 60 by 1.35 N.A. oil objective. Signal intensities were adjusted so that the maximal pixel intensities were at least half saturation. Images were obtained by taking a series of images every 0.5 µm through the cell (generally 3–4 µm) and combining the images into a composite stack. To minimize effects of overexpression on Kv1.5-GFP localization, we analyzed only cells with low to mid levels of expressed protein. Images were analyzed with ImageJ software (NIH, Bethesda,
MD), and statistics were performed with Prism 5 software from Graphpad Prism Software (San Diego, CA). The resolution obtained in these imaging experiments was 512 by 512 pixels with a z resolution of 0.5µm for each filter set. Regarding the laser configuration, alexa fluor 405 was excited using a 405nm laser diode, reflected off of an SDM 490nm dichroic mirror, and passed through a BA 430-460nm bandpass filter. Kv1.5-GFP was excited using a 488nm laser, reflected off of an SDM 560nm dichroic mirror, and passed through a BA505-525nm bandpass filter. Alexa fluor 594 was excited using a 543nm laser and passed through a BA560IF bandpass filter. Alexa fluor 647 was excited using a 633nm laser and passed through a BA660IF bandpass filter. Fluorescent signals of compressed Z-stacks were quantified using NIH ImageJ software (NIH, Bethesda, MD). To determine specific fluorescence, the background signal from neighboring untransfected cells was subtracted from the total fluorescence for all quantified signals. Surface and internalized Kv1.5 fluorescent signals were then normalized to total Kv1.5-GFP fluorescence for each cell.

Recycling Assay – The recycling assay was performed as previously described with the following modifications. 48 hr post-transfection, HL-1 cells transiently expressing Kv1.5-GFP were live-cell stained with an anti-GFP antibody (1:500) for 30 min on ice. Following this incubation, the cells were returned to 37°C for 10 minutes with vehicle or drug, removed, and stained with goat anti-rabbit AlexaFluor 594 secondary antibody (1:200) for 30 min on ice to saturate remaining surface channels. Cells were then returned to 37°C for the indicated
times, removed, and incubated with goat-anti-rabbit AlexaFluor 405 (1:500) for 30 min on ice to detect the recycled channel population. Additionally, cells were fixed and permeabilized as described above and internalized channel was detected with goat-anti-rabbit AlexaFluor 647 (1:500) for 30 min on ice before mounting with ProLong Gold.

Live-cell Imaging – Kv1.5-pHluorin was generated by replacing the extracellular GFP tag with the pH sensitive variant of GFP, pHluorin. 48 hr post transfection HL-1 cells transiently expressing Kv1.5-pHluorin were imaged in a heated perfusion chamber using an Olympus FluoView 500 confocal microscope. Both the chamber and perfusion solutions were maintained at 37°C throughout the experiment. Cells were perfused with Liebovitz’s medium from Gibco (Carlsbad, CA) pH7.4 or Liebovitz’s medium containing 0.1%DMSO for 10 minutes, followed by quinidine treatment for 10 minutes. Images were collected using Olympus FluoView 500 (every 2.5 minutes at 1% laser intensity) and quantified as described above.

Electrophysiology - For electrophysiological studies, recordings were performed on HL-1 cells stably expressing Kv1.5-pHluorin and whole-cell voltage clamp experiments were performed as described previously. The intracellular pipette solution contained (in mmol/L): Potassium aspartate 110, KCl 20, NaCl 8, HEPES 10, K2ATP 4, CaCl2 1, and MgCl2 1, either in the presence or absence of 10 mmol/L K2BAPTA; and was adjusted to pH 7.2 with KOH. The bath solution
contained (in mmol/L): NaCl 110, KCl 4, MgCl$_2$ 1, CaCl$_2$ 1.8, HEPES 10, and glucose 1.8; and was adjusted to pH 7.35 with NaOH. All currents were normalized to the peak current measured at +60 mV for each cell tested.

_Neonatal Myocyte Isolation and Electroporation_ - Cardiomyocytes from neonatal mice were isolated and cultured according to methods adapted from Zlochiver, et al. Briefly, hearts from 1-3 day-old mice were removed and collected in Ca$^{2+}$- and Mg$^{2+}$-free Hank’s balanced salt solution (HBSS) from Gibco. Finely minced cardiovascular tissue was digested in 0.05% trypsin (Invitrogen) and 0.001% pancreatin (Sigma) at 37°C in consecutive 10 min steps. Supernatant cells suspensions were collected in an equal volume of medium M199 (Lonza BioWhittaker, Basel, Switzerland), containing 10% fetal bovine serum (FBS) from Gibco, 20 units/mL penicillin, 20 µg/mL streptomycin. Following 8 digestion steps, collected cell suspensions were centrifuged at 800 rpm for 5 min and the supernatant was aspirated and discarded. The dissociated tissue was then resuspended in 10 mL medium and filtered through a 70 µm mesh filter into a 100mm tissue culture dish. Fibroblasts were depleted through 2 hour preplating at 37°C with 5% CO$_2$. Myocytes were collected off the dish by gentle agitation, filtered through a 40 µm mesh filter, and spun down for 1 min at 4,000 rpm. Myocytes were then resuspended in 100µL Nucleofector solution from Amaxa (Gaithersburg, MD) and 2.5 µg of the desired DNA, per reaction. This solution was then transferred to an Amaxa-certified cuvette, inserted into the cuvette holder of an Amaxa biosystems Nucleofector II, and program G-09 was run.
500µL of pre-warmed medium was then added to the solution in the cuvette and the myocytes were plated onto collagen-coated coverslips in medium containing Bromodeoxyuridine (Sigma) and Fungizone (Gibco) and incubated in a humidified tissue culture incubator for 36-48 to allow protein expression.

**Statistical Analysis** - Statistics were performed using GraphPad Prism 5 Software (San Diego, CA). All data are expressed as the mean ± SEM of n cells. Comparisons between groups were made using a Student's *t* test (paired or unpaired) or One-way ANOVA as indicated. Values of *p*<0.05 were considered significant.

**Results:**

The antiarrhythmic drug, quinidine, stimulates rapid Kv1.5 internalization in HL-1 atrial myocytes.

One potential concern regarding our internalization assay is that the treatment with primary antibody prior to initiating internalization could cause an antibody-induced artifact. To negate this possibility we generated a Kv1.5 construct in which GFP was replaced with pHluorin, a GFP variant whose fluorescent properties are sensitive to the pH of the immediate environment. Internalization of cell surface channel to endosomes with acidic pH quenches the pHluorin signal as measured by reduced fluorescence. Internalized channel was protected from photobleach and its perinuclear localization revealed by
neutralization with ammonium chloride (Online Figure II B). Using live-cell imaging of HL-1 cells expressing Kv1.5-pHluorin, we found no difference in the time course, extent, or subcellular localization of quinidine-induced internalized channel compared to results from our antibody-labeling internalization assay (Online Figure IIA).

**Specificity of quinidine-induced internalization.**

Despite differences in surface levels of channel, the surface to total protein ratio for each subunit did not differ from Kv1.5 (Online Figure IIIA and IVB), indicating that the absence of quinidine-induced internalization is not due to a lack of surface channel. In addition, Kv4.2 was found to undergo constitutive endocytosis similar to Kv1.5 (Online Figure IIIB), indicating that detection of internalized channels is not problematic at the low expression levels observed with Kv4.2 and that the lack of quinidine-induced internalization is not due to an inability of the channel to access the endocytic pathway. Interestingly, we observed that Kv2.1 was sequestered in plaque-like clusters in agreement with previous reports in native atrial, but not ventricular, myocytes \(^5\). This finding further validates that the molecular machinery for channel localization is intact in the HL-1 atrial myocyte model (Online Figure IVA).

**Channel internalization is prevented by disruption of endocytic machinery.**
Another major component of the endocytic machinery is the microtubule-dependent retrograde motor complex consisting of dynein and dynactin connected by the adaptor protein p50-dynamitin\(^6,7\). Previously, we and others reported that overexpression of p50-dynamitin, which results in the uncoupling of the retrograde motor complex from its cargo to disrupt retrograde trafficking, blocked constitutive Kv1.5 endocytosis\(^1,8\). In our current studies, we found that p50-dynamitin overexpression also blocked quinidine-induced internalization with a corresponding increase in surface levels similar to the inhibition measured with Dynasore (Online Figure VI).

**Differential effects of acute versus chronic treatment with quinidine.** Recycled channel levels 60 minutes post quinidine-induced internalization were approximately 33% greater than those post constitutive endocytosis (Online Figure IXA). This difference is most likely attributed to an increased pool of internalized Kv1.5 available for recycling following drug treatment. To clarify this difference, we labeled surface channel 60 minutes post washout of both constitutive endocytosis and quinidine-induced internalization, and found no difference in steady-state cell surface levels of channel (Online Figure IXB).

**References:**


**Figure Legends:**

**Online Figure I:** Quinidine treatment causes a reversible, dose-dependent open-channel block of Kv1.5. (A) Whole-cell voltage clamp experiments were performed on HL-1 cells stably expressing Kv1.5-pHluorin. Recordings were taken prior to and following 10 min perfusion with 1, 6, 10, or 100µmol/L quinidine and following 20 min of drug washout. Representative traces for sequential dosing of a single cell are shown. (B) Cells were treated as above and I-V curves were generated for cells prior to treatment and following 10 min perfusion with 6, 10, and 100µmol/L quinidine. (C) I-V curves are shown for cells prior to
treatment, and following 100µmol/L quinidine, and 20 min washout of quinidine. (D) A single depolarizing pulse to +60mV was applied as described in methods. Representative traces are shown for a single cell prior to and following 10, 12, and 15 min perfusion with 100 µmol/L quinidine. (E) Quantitation of internalized Kv1.5-GFP (overexpressed in HL-1 cells) in response to 10 µmol/L quinidine for 5, 10, 15, 30, or 60 min at 37°C. *** indicates p < 0.001 as determined by one-way ANOVA with Tukey post-test.

**Online Figure II:** *Quinidine-induced internalization is independent of antibody labeling.* (A) Quantification of surface Kv1.5-pHluorin following perfusion at 37°C for 10 min with 0.1% DMSO followed by 10 min with 100µmol/L quinidine and a brief pulse with 50 mmol/L NH₄Cl. Representative images for each of these conditions are shown. (B) HL-1 cells expressing Kv1.5-pHluorin were perfused for 10 min with vehicle as described above. Remaining cell surface Kv1.5-pHluorin was photobleached at high laser intensity, followed by rapid application of NH₄Cl, to reveal foci of intracellular, endosomal Kv1.5-pHluorin. Scale bar = 20µm. *** indicates p < 0.001 as determined by one-way ANOVA with Tukey post-test.

**Online Figure III:** *Surface to total protein ratio is similar for Kv1.5 and Kv4.2.* HL-1 cells overexpressing Kv1.5-GFP or Kv4.2-GFP were (A) surface-labeled and the ratio of surface (red) to total protein (green) was quantified. Representative images are shown for both channel subunits. (B) Representative
images of Kv1.5-GFP (top) or Kv4.2-GFP (bottom) showing total GFP signal (left), surface channel as detected by surface labeling with anti-GFP followed by goat anti-rabbit Alexa Fluor 405 (middle), and internalized channel detected by labeling with goat anti-rabbit Alexa Fluor 647 (right), after 10 min at 37°C with vehicle (0.1% DMSO). (C) Quantification of internalized Kv4.2 following treatment with increasing concentrations of quinidine for 10 min at 37°C. Scale bar = 10 µm.

Online Figure IV: Kv2.1 differs from Kv1.5 in expression pattern; however, surface to total protein ratios are similar. (A) Representative images showing total-GFP fluorescence patterns for Kv1.5 with an extracellular GFP tag, Kv2.1 with a C-terminal GFP tag, and Kv2.1 with an extracellular GFP tag. (B) HL-1 cells overexpressing Kv1.5-GFP or Kv2.1-GFP were surface-labeled and the ratio of surface (red) to total protein (green) was quantified. Representative images are shown for both channel subunits. (C) Quantification of internalized Kv2.1 following treatment with increasing concentrations of quinidine for 10 min at 37°C. Scale bar = 10 µm.

Online Figure V: Quinine treatment causes a reversible, dose-dependent block of Kv1.5. (A) Whole-cell voltage clamp experiments were performed on HL-1 cells stably expressing Kv1.5-pHluorin. Recordings were taken before and after 10 min perfusion with 20 or 100 µmol/L quinine, and following 20 min of drug washout. Representative traces for sequential dosing of a single cell are shown. (B) Cells were treated as described above and I-V curves are shown for cells
prior to treatment and following 10 min perfusion with 20 and 100µmol/L quinine. (C) I-V curves are shown for cells prior to treatment, and following 10 min perfusion with 100µmol/L quinine and 20 min washout of quinine.

**Online Figure VI:** *Channel internalization is prevented by dominant-negative disruption of the endocytic machinery.* (A) HL-1 cells co-expressing Kv1.5-mCherry and dynamitin-GFP were live-cell labeled with anti-DsRed followed by treatment with increasing concentrations of quinidine for 10 min at 37°C, and internalized Kv1.5 was quantified. (B) Quantification of surface Kv1.5 for cells treated as described in (A). * indicates p < 0.05; *** indicates p < 0.001 as determined by one-way ANOVA with Tukey post-test.

**Online Figure VII:** *Quinidine-induced internalization occurs via a calcium-dependent mechanism.* Whole-cell voltage clamp experiments were performed on HL-1 cells stably expressing Kv1.5-pHluorin. I-V curves are shown for recordings taken in the presence (left) or absence (right) of BAPTA before and after 10 min perfusion with 6µmol/L quinidine.

**Online Figure VIII:** *Quinine-mediated block of Kv1.5 current is calcium-independent.* Whole-cell voltage clamp experiments were performed on HL-1 cells stably expressing Kv1.5-pHluorin. (A) I-V curves for recordings taken prior to treatment and following 10 min perfusion with 20µmol/L quinine in the presence or absence of BAPTA. (B) Quantification of Kv1.5 current following
quinine treatment in the presence or absence of BAPTA. *** indicates p < 0.001 as determined by one-way ANOVA with Tukey post-test.

**Online Figure IX:** *Recovery of surface Kv1.5 following acute, but not chronic, quinidine treatment.* (A) Quantification of recycled Kv1.5 at 0, 10, 20, 30, and 60 min at 37°C post treatment with 100 µmol/L quinidine for 10 min at 37°C. (B) HL-1 cells expressing Kv1.5-GFP were surface labeled with anti-GFP antibody, incubated 10 min in 100µmol/L quinidine at 37°C, and incubated 60 min in drug-free medium at 37°C, and surface Kv1.5 was quantified. (C) Quantification of surface Kv1.5, in HL-1 cells stably expressing Kv1.5, following treatment for 0, 12, 24, 36, or 48 hours at 37°C with 10µmol/L quinidine. *** indicates p < 0.001 as determined by one-way ANOVA with Tukey post-test.
Online Figure II
Online Figure III
Online Figure IV
Online Figure V
Online Figure VI
Online Figure VII
Online Figure VIII
Online Figure IX