Title: Dual mechanism for inhibition of inwardly rectifying Kir2.x channels by quinidine involving direct pore block and PIP$_2$-interference

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Running Title Page

Running title: Inhibitory effects of quinidine on hKir2.x channels

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Abstract

Class IA antiarhythmic drug quinidine was one of the first clinically used compounds to terminate atrial fibrillation and acts as multichannel inhibitor with well-documented inhibitory effects on several cardiac potassium channels. In the mammalian heart, heteromeric assembly of Kir2.1–2.3 channels underlies I_{K1} current. While a low-affinity block of quinidine on Kir2.1 has already been described, a comparative analysis of effects on other Kir2.x channels has not been performed to date. Therefore, we analyzed the effects of quinidine on wild type and mutant Kir2.x channels in the Xenopus oocyte expression system. Quinidine exerted differential inhibitory effects on Kir2.x channels with the highest affinity towards Kir2.3 subunits. Onset of block was slow and solely reversible in Kir2.2 subunits. Quinidine inhibited Kir2.x currents in a voltage-independent manner. By means of comparative Ala-scanning mutagenesis we further found that residue E224, F254, D259 and E299 are essential for quinidine block in Kir2.1 subunits. Analogously, quinidine mediated Kir2.3 inhibition by binding corresponding residues E216, D247, D251 and E291. In contrast, Kir2.2 current block merely involved corresponding residue D260. Using channel mutants with altered PIP_{2} affinities, we were able to demonstrate that high PIP_{2} affinities (i.e. Kir2.3 I214L) correlate with low quinidine sensitivity. Inversely, mutant channels interacting only weakly with PIP_{2} (i.e. Kir2.1 K182Q and L221I) are prone to a higher inhibitory effect. Thus, we conclude that inhibition of Kir2.x channels by quinidine is mediated by joint modes of action involving direct cytoplasmic pore
block and an impaired channel stabilization via interference with PIP$_2$. 
Introduction

Originally derived from cinchona bark, class IA antiarrhythmic drug quinidine was the first clinical used compound to terminate atrial fibrillation and still is in widespread use today (Grace and Camm, 1998). Nevertheless, compared with novel class IC antiarrhythmics quinidine has been associated with an increased mortality and unfavorable side effects. Consequently, novel compounds such as class IC and class III antiarrhythmics have been developed and replaced quinidine in the management of atrial fibrillation. Adverse side effects, however, were regularly based on a compromised renal and biliary clearance of digoxin as quinidine competitively inhibits multi drug resistance protein 1 (MDR1) (Doering, 1979; Fromm et al., 1999). Thus, proarrhythmic side effects of quinidine may be circumvented when its administration is combined with verapamil. Only recently, quinidine has regained clinical interest as one of the most effective agents in the pharmacological management of inherited malignant arrhythmias such as Brugada syndrome, idiopathic ventricular fibrillation and short QT syndromes (Kaufman, 2007; Viskin et al., 2007). Electrophysiologically, quinidine acts as a broad multichannel blocker with well-documented inhibitory effects on several potassium channels including $I_{to}$, $I_{Kur}$, $I_{KATP}$, $I_{Kr}$, $I_{Ks}$, $I_{KAC}$ and $I_{K1}$ (Tamargo et al., 2004).

Cardiac inwardly rectifying current $I_{K1}$ is essential for stabilizing the resting membrane potential of cardiomyocytes (Lopatin and Nichols, 2001). Several lines of evidence suggest that at the molecular level heteromeric assembly of inwardly
rectifying potassium channels Kir2.1, Kir2.2 and Kir2.3 underlie human cardiac \( I_{K1} \) currents (Schram et al., 2003). Kir2.1 subunits are abundantly expressed in the entire myocardium and heteromeric Kir2.1/Kir2.2 channels underlie human ventricular \( I_{K1} \) current (Schram et al., 2003). In contrast, Kir2.3 subunits are stronger expressed in human atria (Wang et al., 1998; Melnyk et al., 2005).

Cardiac \( I_{K1} \) may constitute a promising target for pharmacological suppression of atrial and ventricular fibrillation. For instance, increased \( I_{K1} \) outward currents due to gain-of-function mutations of Kir2.1 underlie Short-QT-Syndrome Type 3 (SQTS3) (Priori et al., 2005). These patients are characterized by a shortened QT interval, a strong predisposition for atrial or ventricular fibrillation and can effectively be treated with quinidine. In line with this pathophysiology, it has been demonstrated that familial atrial fibrillation (FAF) is linked to a gain-of-function mutation of Kir2.1 subunits (Xia et al., 2005). Furthermore, Noujaim et al. (2007) provided direct evidence that increased \( I_{K1} \) currents might stabilize reentrant tachycardias as overexpressing Kir2.1 subunits generated fast and stable rotors in a mouse model.

In silico approaches identified several aromatic residues within the cytoplasmic pore region of Kir2.1 (E224, E299, D255, D259 and F254) to serve as potential binding positions of cationic amphipathic drugs which has been experimentally validated by Ala-scanning mutagenesis (Rodriguez-Menchaca et al., 2008; Noujaim et al., 2010; Lopez-Izquierdo et al., 2011b; van der Heyden et al., 2013). Noujaim et al. (2011) demonstrated that the inhibitory effect of quinidine on Kir2.1 is mediated by an acute pore block of Kir2.1 subunits involving amino acid
residue E224, F254 and D259.

To date, there are no data available regarding other potential mechanisms of action of quinidine or further effects on other Kir2.x subtypes. Therefore, we studied the effects of quinidine on all cardiac wild type and mutant Kir2.x potassium channels in the Xenopus oocyte expression system in order to fully elucidate the molecular properties that may underlie the inhibition of I\textsubscript{K1} current by quinidine.
Methods

DNA constructs and heterologous expression

Kir2.x cDNA constructs were kindly provided by Dr. Barbara Wible (Cleveland, Ohio, USA) and Dr. Carol Vandenberg (Santa Barbara, California, USA). Complementary mRNA was synthesized from Kir2.x cDNA with the mMESSAGE mMACHINE in vitro transcription kit (Ambion) using T7 Polymerase (Kir2.1 and Kir2.2 constructs) or T3 Polymerase (Kir2.3 constructs). Kir2.x mutants were generated by inverse polymerase chain reaction (PCR) with synthetic mutant oligonucleotide primers using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) as previously described (Karle et al., 2002). The Kir2.x-Kir2.y hetero-concatemers (Fig. 1) used in this study were kindly provided by Dr. Regina Preisig-Müller (Marburg, Germany). All mutations and DNA constructs were confirmed by sequencing (SeqLab Goettingen, Germany).

Using a Nanoject automatic injector (Drummond, Broomall, USA) 50 nl per oocyte of cRNA (50 to 500 ng/μl) was injected into stage V and VI defolliculated oocytes. Experiments were performed 1 to 3 days after injection. All experiments performed in this study are in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996).

Electrophysiology and data analysis

Currents were recorded using the two-microelectrode voltage-clamp technique in
Xenopus laevis oocytes using a commercially available amplifier (Warner OC-725A, Warner Instruments, Hamden, USA) and pCLAMP software (Axon Instruments, Foster City, USA) for data acquisition and analysis. Data were low-pass filtered at 1 to 2 kHz (−3 dB, four-pole Bessel filter) prior to digitalization at 5 to 10 kHz. During the experiments no leak subtraction was performed. Statistical data are presented as mean ± S.E.M. In all figures, n represents the number of cells recorded. Statistical significance was evaluated using Student’s t test. For more than 2 groups analysis of variance (oneway ANOVA) was used instead. Differences were considered to be significant if the p value was <0.05. For statistical analysis and figures OriginPro 2015 (b9.2.214, OriginLab Corp., Northampton, MA) was used. The concentration-response curves were fitted with the Hill equation: \( \frac{I}{I_0} = \frac{I_0}{(1+X/IC_{50})^n} \), \( \frac{I}{I_0} \) being the relative current, \( I_0 \) the unblocked current amplitude, \( X \) the drug concentration, \( IC_{50} \) the concentration for half maximal block and \( n \) the Hill coefficient.

Solutions and drug administration

Voltage clamp measurements of Xenopus oocytes were performed in a K\(^+\) solution containing (in mM) 5 KCl, 100 NaCl, 1.5 CaCl\(_2\), 2 MgCl\(_2\), and 10 HEPES. The pH of this solution was corrected to 7.4 with 1 M NaOH. Electrodes were filled with 3 M KCl solution. All measurements were carried out at room temperature (20 °C). Xenopus oocytes were incubated in the drug solution. Recordings were made prior to incubation and 30 min afterwards. Quinidine (Sigma-Aldrich, Steinheim, Germany) was diluted in DMSO to obtain a 100 mM
stock solution and stored at −20 °C. Aliquots of the stock solution were dissolved in the bath solution to the desired concentrations on the day of the experiments.
Results

*Quinidine inhibits Kir2.3 currents*

We initially characterized the effects of quinidine on homomeric Kir2.3 subunits. Channels were heterologously expressed in *Xenopus laevis* oocytes and currents were recorded using the voltage-clamp technique. In order to measure Kir2.x currents, a standardized voltage protocol was applied. From a holding potential of -80 mV, 400 ms test pulses ranging from -120 mV to +40 mV were applied in 10 mV increments. Inward current amplitudes at -120 mV were used to quantify inhibitory effects, unless stated otherwise. In Figure 2 representative current traces under control conditions (Fig. 2A) and after 30 minutes of incubation with 1 mM quinidine (Fig. 2B) are shown. The corresponding current-voltage (IV) curves are shown in Fig. 2C. Under control conditions we observed a slight current run-up by 9 ± 5.9 % (n=7, data not shown) as previously described (Karle et al., 2002). Dose-response curves were obtained accordingly. Relative effects of quinidine at concentrations ranging from 5 μM to 1 mM were compared in *Xenopus* oocytes heterologously expressing Kir2.3 channels and yielded an IC$_{50}$ of approximately 72 μM (n=4–16, Fig. 2D).

Onset and wash-out of the inhibitory effect of quinidine on Kir2.x subunits were investigated using a repetitive voltage clamp protocol at start-to-start intervals of 30 seconds over a time period of 45 min. Starting from a holding potential of -80 mV test pulses to -120 mV and -110 mV for 400 ms were applied to elicit large inward currents. Relative inhibition of Kir2.3 currents during wash-in and wash-
out of quinidine at a concentration of 100 μM are shown in Fig. 2E and Fig. 2F, respectively. Onset of block was slow and approximated steady-state conditions after 45 min (32.8 ± 2.4 % of initial current amplitudes, n=10). Correspondingly, recovery from inhibition was also slow. Even after 45 min of reperfusion with bath solution the current block still persisted as seen in Fig. 2F (71.9 ± 6.3 % of initial current amplitudes, n=10). For control experiments the chamber was continuously perfused with extracellular control solution (n=4) which resulted in a relative current amplitude of 104.9 ± 4.2 % (n=4) and 105.8 ± 3.8 % (n=4) after 45 min and 90 min, respectively.

Inhibition of Kir2.1 and Kir2.2 currents by quinidine

Next, we studied the effects of quinidine on homomeric Kir2.2 and Kir2.1 currents in order to compare electrophysiological characteristics in different cardiac Kir2.x subunits. Concentration-response relations were obtained analogously to those of Kir2.3 as described above. Under control conditions Kir2.1 and Kir2.2 channels showed a current run-up by 2.4 ± 7 % (n=7) and 6.5 ± 8.3 % (n=5) respectively (data not shown). Representative IV relations of current recordings before and after administration of 2 mM quinidine are shown in Fig. 3A for Kir2.1 subunits and in Fig. 3E for Kir2.2 subunits. Dose-response characteristics were acquired as described above and yielded IC$_{50}$ values of approximately 290 μM for Kir2.1 (n=5–13) and 370 μM for Kir2.2 (n=4–7) channels (Fig. 3B and F). Contrary to Kir2.3, neither Kir2.1 nor Kir2.2 currents reached steady-state conditions after 45 min. We did not lengthen incubation times, as according to
our experience *Xenopus* oocytes do not tolerate longer experiments. Kir2.1 currents and Kir2.2 currents were reduced to 46.7 ± 4.1 % (n=4) and 63.9 ± 6.4 % (n=4), respectively (Fig. 3 C and G). Interestingly, quinidine-mediated current inhibition was solely reversible in Kir2.2 subunits and reached 122.4 ± 9 % of initial current amplitudes (n=4) (Figure 3 H). In contrast, inhibitory effects on Kir2.1 current persisted even after 45 min of quinidine wash-out as shown in panel D (66.4 ± 8.8 %, n=4). Oocytes were continuously perfused with extracellular bath solution to obtain time-matched controls. After 45 min and 90 min relative currents increased to 100.1 ± 0.1 % and 103.3 ± 0.07 % or 103.0 ± 1.4 % and 107.8 ± 0.08 % (n=4 each) for Kir2.1 or Kir2.2, respectively.

**Inhibition of hetero-concatemeric Kir2.x-2.y channels**

As stated earlier, heterotetrameric assembly of Kir2.1 and Kir2.2 is the main molecular correlate of human ventricular *I*\(_{K1}\) current, while Kir2.3 is more abundantly expressed in atrial myocardium (Liu et al., 2001; Gaborit et al., 2007). Co-expression of Kir2.1 and Kir2.2 subunits in *Xenopus* oocytes results in distinctive biophysical current characteristics more closely resembling human *I*\(_{K1}\) (Schram et al., 2003). In order to minimize random association artefacts of co-injected subunits, we solely injected mRNA of hetero-concatemeric Kir2.x-2.y channels (Fig. 1) as previously described (Preisig-Muller et al., 2002). Under control conditions, Kir2.1-2.2, Kir2.1-2.3 and Kir2.2-2.3 currents increased by 13.6 ± 5.4 %, 18 ± 7 % and 16.6 ± 2.6 %, respectively (n=4 each, data not shown). IV curves of representative current recordings before and after
administration of quinidine are shown in Fig. 4A, C and E. Dose-response relationships were assessed analogously to the experiments described above and yielded IC$_{50}$ values of 470 µM, 250 µM and 190 µM for Kir2.1-2.2 (n=4–6), Kir2.1-2.3 (n=4–8) and Kir2.2-2.3 (n=4–8) subunits, respectively (Fig. 4B, D and F).

*Comparison of maximum inhibitory effects of quinidine on Kir2.x channels*

The maximum subunit-specific and relative inhibition of Kir2.x or Kir2.x-2.y currents at supramaximal concentrations of quinidine are listed in Table 1. In comparison, Kir2.3 currents were blocked to the greatest extent, followed by Kir2.2 and Kir2.1 currents. The effects of quinidine on Kir2.x-2.y heteroconcatemers were far less pronounced. Hetero-concatemeric channels containing the Kir2.3 subunits, however, were prone to a greater block when compared to Kir2.1-2.2 hetero-concatemers.

*Inhibition of Kir2.x currents by quinidine is voltage-independent*

In order to examine whether quinidine inhibits Kir2.x currents in a voltage-dependent manner, inhibitory effects were compared at voltages ranging from -120 mV to 0 mV. Inhibitory effects between -80 and -60 mV were excluded from the analysis due to small current amplitudes. We found that the inhibition of Kir2.x currents by quinidine is not significantly different at the analyzed voltages (Fig. 5). Relative current inhibition by 1 mM quinidine ranged from 72.7 ± 8.6 % at -50 mV to 50.6 ± 9.7 % at 0 mV in Kir2.2 currents (n = 6) and 64.5 ± 7 % at -50
mV to 45.3 ± 17.3 at -20 mV in Kir2.1 currents (n = 4). The relative inhibitory effect of 0.1 mM quinidine on Kir2.3 channels was 68.2 ± 4.2 % at -50 mV and 59.5 ± 2.9 % at -100 mV (n = 7), respectively.

Functional significance of the cytoplasmic pore domain for the inhibitory effects of quinidine

Several negatively charged amino acid residues within the cytoplasmic pore domain of Kir2.1 (E224, F254, and D259) have been associated with an acute block by quinidine (Noujaim et al., 2011). Furthermore, chloroquine, structurally similar to quinidine, blocks the permeation pathway by interacting electrostatically with residues Kir2.1 D255 and E299 (Rodriguez-Menchaca et al., 2008; Noujaim et al., 2010). We performed sequence alignments in order to identify corresponding residues in other Kir2.x subunits (Fig. 6). Via Ala-scanning mutagenesis we experimentally validated their role in quinidine-mediated Kir2.x inhibition. Unfortunately, the influence of Kir2.1 D255 on channel inhibition could not be verified, as heterologous expression in Xenopus oocytes resulted in non-functional currents. Summary data of inhibitory effects of 1 mM, 0.5 mM and 0.1 mM quinidine on Kir2.1, Kir2.2 and Kir2.3 pore mutants compared to corresponding effects in wild type channels (Kir2.1 wild type: 49 ± 2.1 %, n=13; Kir2.2 wild type: 51.6 ± 3.3 %, n=7; Kir2.3 wild type: 58.6 ± 2 %, n=15) are shown in Fig. 7. The inhibitory effect of quinidine was significantly reduced in all Ala-mutated Kir2.1 and Kir2.3 channels (Fig. 7A and C). Relative current was inhibited by 22.3 ± 2.2 % in Kir2.1 E224A (n=5) and by 5.1 ± 4.8 % in Kir2.1
D259A (n=4). Furthermore, in Kir2.1 E299A and in Kir2.1 F254A, current increases by 8.2 ± 4.5 % (n=6) and by 3.8 ± 3.3 % (n=5) were observed, respectively. Also, all studied Ala-mutated Kir2.3 subunits exhibited significantly reduced effects of quinidine, resulting in relative current reductions by 33.9 ± 1.4 % (n=6) in Kir2.3 E216A and 45.1 ± 2.7 % (n=6) in Kir2.3 D247A and in a current increase of 0.07 ± 2.6 % (n=4) in Kir2.3 D251A and 2.3 ± 2.9 % (n=6) in Kir2.3 E291A channels. Interestingly, only mutation D260A altered the effect on Kir2.2 channels with a current increase of 11.5 ± 5.6 % (n=5) after quinidine application (Fig. 7B).

\textit{PIP}_2-medi\textit{ated inhibition of} Kir2.x \textit{subunits by quinidine}

Glycerophospholipid PIP$_2$ is essential for membrane-associated signaling. As an important cofactor for channel function and lipid rafts (Suh and Hille, 2008), it has previously been recognized to participate in high-affinity interactions with inwardly rectifying ion channels and particularly with Kir2.1 subunits (Rohacs et al., 2003; Logothetis et al., 2007). Various Kir2.x inhibitors possess cationic amphiphilic properties thereby interfering with membranous PIP$_2$ distributions and effectively abrogating channel stabilization (Ponce-Balbuena et al., 2009; Lopez-Izquierdo et al., 2011a; Lopez-Izquierdo et al., 2011b). As a cationic amphiphilic drug we postulated that quinidine interferes with PIP$_2$-mediated channel activation. Thus, we generated channel mutants with low (Kir2.1 K182Q and L222I) and high (Kir2.3 I214L) endogenous PIP$_2$ affinities (Xie et al., 2008) and compared the extent of quinidine-mediated blocks as shown in Figure 8.
Mutation K182Q as well as L222I of Kir2.1 increased its sensitivity towards 200 µM quinidine as the wild-type current reduction of 18.2 ± 3.8% (n=5) is further increased to 68.9 ± 3.3% (n = 6) and 31 ± 1.1% (n =5), respectively. In contrast, the inhibitory effect of 100 µM quinidine in Kir2.3 wild-type channels (58.6 ± 1.9%, n=16) was completely abolished in Kir2.3 I214L channels with a total current increase of 9.2 ± 3.6 % (n=7).
Discussion

Inhibitory effects of quinidine on cardiac $I_{K1}$ current are well known and may contribute to its antifibrillatory efficacy (Salata and Wasserstrom, 1988). However, apart from previous investigations focusing on Kir2.1 subunits, no further analyses of the drug’s inhibitory effect on other Kir2.x channels were performed prior to this study. Here, we show that quinidine differentially inhibits Kir2.x channels with the highest potency towards Kir2.3 subunits, being approximately 5-fold more sensitive than homomeric Kir2.1 or Kir2.2 channels. In addition, quinidine inhibits Kir2.3 currents by a 3–4-fold greater degree when compared to homomeric Kir2.1 or Kir2.2 currents. In line with these results, hetero-concatemeric Kir2.1-2.3 and Kir2.2-2.3 currents exhibit an approximately 2-fold higher sensitivity towards quinidine compared to Kir2.1-2.2 hetero-concatemers. Biophysical current characteristics, however, are not altered by quinidine. Onset of inhibition is slow and effects are solely reversible in Kir2.2 currents. Furthermore, quinidine inhibits Kir2.x currents in a voltage-independent manner. By application of channel mutants with altered PIP$_2$ affinities and Ala-scanning mutagenesis of amino acids within the channel permeation pathway, we provide evidence that the inhibitory effect of quinidine is dependent on a dual mode of inhibitory action: centrally blocking the channel permeation pathway as well as interfering with PIP$_2$-mediated channel stabilization.

Pharmacological $I_{K1}$ inhibition might constitute a novel approach for antiarrhythmic treatment strategies (Rees and Curtis, 1993). Patients suffering
from SQTS3 and FAF might benefit from a selective $I_{K1}$ or even Kir2.x-specific inhibition. However, the lack of selective Kir2.x inhibitors simultaneously suited for in vivo application renders the validation of potential benefits difficult. Interestingly, with β3-adrenoceptor antagonist SR 59230A our group could recently identify a selective Kir2.x inhibitor which may serve as a potential model substance for the development of $I_{K1}$ modulators (Kulzer et al., 2012).

Chronic atrial fibrillation, chronic AV block and ischemic heart disease are linked to complex regional changes in $I_{K1}$/Kir2.x expression profiles (Mustroph et al., 2014). It has been shown that the increased expression of Kir2.1 contributes to reentrant tachycardias by stabilizing high frequency atrial and ventricular rotors (Noujaim et al., 2007; Girmatsion et al., 2009; Sekar et al., 2009). Interestingly, the inhibition of $I_{K1}$ might constitute an effective therapeutic approach to the treatment of reentrant arrhythmias (Warren et al., 2003; Pandit et al., 2005; Kharche et al., 2014).

However, $I_{K1}$ dysfunction as a result of chronic heart failure or LQTS has been recognized to destabilize the resting membrane potential of cardiomyocytes and increase their susceptibility for tachyarrhythmias. Furthermore, animal studies provide evidence that pulmonary vein cardiomyocytes exhibit reduced $I_{K1}$ and Kir2.3 current densities (Ehrlich et al., 2003; Melnyk et al., 2005), which may at least partly explain their increased arrhythmogenic potential (Cherry et al., 2007). Quinidine was the first antiarrhythmic drug used clinically to terminate atrial fibrillation. As a multichannel blocker, quinidine may display a favorable electrophysiological profile by normalizing the effective refractory period while
simultaneously preventing EADs by reducing Ca$^{2+}$ and Na$^{+}$ inward currents. Due to distinctive intrinsic affinities towards different ion channels, quinidine primarily prolongs the cardiac action potential at low concentrations by inhibiting $I_{Kr}$. As the concentration further increases, additional effects on other cardiac potassium channels and sodium channels are observed which consequently shorten the action potential duration (Paul et al., 2002; Tamargo et al., 2004; Wu et al., 2008).

Blocking mechanisms of several cationic amphiphilic drugs such as chloroquine have been deciphered by means of molecular modeling and Ala-scanning mutagenesis. Several amino acid residues within the central pore of Kir2.1 channels have been identified as critical binding sites for these compounds (Rodriguez-Menchaca et al., 2008). Kir2.1 E224, F254, D259 have been verified experimentally to be crucial for the inhibitory effect of quinidine (Noujaim et al., 2011). Here we demonstrate that, analogously to chloroquine, amino acid residue E299 is involved in the quinidine-mediated inhibition of Kir2.1 channels. Additionally, the effect of quinidine on Kir2.2 and Kir2.3 channels was evaluated. Increased $I_{K1}$ density in patients with chronic atrial fibrillation (Bosch et al., 1999; Workman et al., 2001; Zhang et al., 2005) may render atrial Kir2.3 subunits as promising novel targets for antiarrhythmic therapies. We identified homologous residues in Kir2.2 and Kir2.3 subunits and consequently assessed their role in quinidine-mediated current inhibition. Inhibitory effects of quinidine were altered in all Kir2.3 mutants. These data resemble the situation in Kir2.1 channels and may either be based on a decentralization of quinidine within the permeation
pathway or a sterically intact blocking position but weakened interactions with the remaining residues. Regardless of the molecular basis, we here demonstrate that Kir2.3 E216, D247, D251 and E291 are essential amino residues for quinidine-mediated block. Interestingly Kir2.2 subunits fundamentally differ from this pattern. Solely Kir2.2 D260A was found to abrogate quinidine block and may directly interact with quinidine. Further investigations with multiple substituted residues are needed to clarify whether the unchanged residues in the remaining Kir2.2 mutants or yet unidentified residues are involved in their unaltered inhibition.

Onset of current inhibition was slow. Solely the effect on Kir2.3 currents reached steady state conditions after 45 min. Interestingly, upon wash-out inhibition of Kir2.2 currents was fully reversed and it is intriguing to speculate that this observation might be due to fewer binding sites of quinidine and/or weakened interaction of the drug within the channel pore. Nevertheless, the significance of homomer-specific inhibiting kinetics in vivo is questionable considering the low myocardial expression of Kir2.2 subunits on the one hand and the heteromeric nature of human I_k1 on the other (Wang et al., 1998; Schram et al., 2002; Melnyk et al., 2005). In line with these considerations Salata and Wasserstrom (1988) were able to demonstrate that quinidine inhibits canine I_k1 currents irreversibly for a period of 45 min.

Noujaim et al. (2011) reported a diminished current inhibition of outward currents after heterologous expression of Kir2.1 channels in HEK293 cells. This observation not only contradicts our presented detailed analysis of Kir2.x
channels but also earlier reports from animal models suggesting a voltage-independent $I_{K1}$ block (Salata and Wasserstrom, 1988). We here provide evidence that endogenous polyamines and quinidine mediate Kir2.x current blocks by interacting with the same residues that line the functional channel pore (Baronas and Kurata, 2014). Contrary to polyamines, quinidine, however, inhibits Kir2.x subunits in a voltage-independent manner. This discrepancy may suggest an additional mode of action by which quinidine can facilitate current inhibition. Considering the subunit-specific sensitivities and kinetics, we hypothesized a combined mechanism for Kir2.x inhibition by quinidine involving direct pore block of the permeation pathway and additional inhibition by interference with PIP$_2$-mediated channel stabilization. To validate this hypothesis, we generated previously described Kir2.x mutants with altered PIP$_2$ affinities (Xie et al., 2008). We noticed that high PIP$_2$ affinities (Kir2.3 I214L) correlate with smaller inhibitory effects. Inversely, channels interacting weakly with PIP$_2$ (Kir2.1 K182Q and L221I) are prone to a strong inhibitory effect. These data suggest that quinidine inhibits Kir2.x channels by a combined mechanism involving direct pore block and impaired open channel gating by PIP$_2$-interference. Although concordant changes in Kir2.1 K182Q/L221I and Kir2.3 I214L currents might suggest that the intrinsic affinity in these channels towards quinidine is unaltered, we acknowledge the fact that these data are obtained at single concentrations. Lopez-Izquierdo et al. (2011b), noted a rightward shift in mefloquine inhibition in corresponding Kir2.3 I213L channels, suggesting a decreased IC$_{50}$ value. The corresponding dose-response curve shows simultaneously a change of slope.
that might be an indicator of altered mefloquine binding characteristics. A similar alteration could complicate the interpretation of our presented results. Further experiments at multiple quinidine concentrations are needed to fully exclude the possibility of an altered Hill coefficient in mutant Kir2.x channels.

A similar blocking mechanism has previously been proposed for anti-malarial agent quinacrine (Lopez-Izquierdo et al., 2011a). However, to the best of our knowledge, quinidine is the first classical \( I_{K1} \)-inhibiting antiarrhythmic compound in which this complex mode of action may be an essential part of its proven antifibrillatory efficacy.

*Xenopus* oocytes are ideally suited for longer current recordings as performed in this study. Due to their low leakage of currents as well as their vigorous nature, they offer a couple of advantages over mammalian cells rendering them useful to study pharmacological properties of ion channels. However, anatomical characteristics such as yolk sacks and vitelline membranes may reduce effective drug concentrations within the cell. Hence, \( IC_{50} \) values may differ up to 30-fold compared to native cardiomyocytes or transfected cell lines (Madeja et al., 1997). As recordings of native \( I_{K1} \) in cardiomyocytes require pharmacological inhibition of other ion channels, non-specific interactions with intrinsic posttranslational pathways cannot be excluded and are a general concern when interpreting native electrophysiological data. However, it has been demonstrated that quinidine blocks human atrial \( I_{K1} \) currents in a voltage-dependent manner with \( IC_{50} \) ranging from 4.1–42.6 \( \mu \)M (Nenov et al., 1998). Noujaim et al. (2011) reported that quinidine inhibits Kir2.1 in mammalian cells in a dose-dependent
manner with an IC$_{50}$ of 57 μM, being approximately 6-fold lower than our result from *Xenopus* oocytes. In humans, antiarrhythmic plasma concentrations of quinidine fall within the range of 2–5 μg/ml corresponding to 6–15 μM (Fremstad et al., 1979; Yang et al., 2009) and may even be higher in patients with an individual susceptibility based on renal or hepatic dysfunction, drug interactions, sex, age and pharmacogenomics. Thus, on the data presented here, we hypothesize that an antifibrillatory $I_{K_1}$ block within therapeutic plasma concentrations may primarily be based on the compound’s predominant influence on atrial Kir2.3 subunits. In conjunction with other highly sensitive atrial-specific potassium currents like $I_{to}$, $I_{kur}$ and $I_{KAC_h}$ (Tamargo et al., 2004), quinidine may exhibit a favorable pharmacological profile for the suppression of supraventricular tachyarrhythmias mainly by prolonging refractory periods in human atria.

In the present study we characterized the molecular basis of quinidine’s inhibitory effects on human Kir2.x channels. We found that quinidine exerts differential effects on Kir2.x channels with the highest affinity towards Kir2.3 subunits. Inhibition of Kir2.x channels is mediated by joint modes of action involving direct cytoplasmic pore block and impaired channel gating by interference with PIP$_2$ stabilization. These data add to the understanding of the antifibrillatory efficacy of quinidine and may contribute to the development of new antiarrhythmic $I_{K_1}$ antagonists.
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Authorship Contributions

Participated in research design: Koepple, Scherer, Seyler, Scholz, Thomas, Katus, Zitron

Conducted experiments: Koepple

Performed data analysis: Koepple, Seyler

Wrote or contributed to the writing of the manuscript: Koepple, Zitron

Conflict of interest

The authors declare that they have no conflict of interest.
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Footnotes

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Legends for Figures

Figure 1. Schematic illustration of Kir2.x–Kir2.y hetero-concatemers.

Kir2.x-2.y hetero-concatemers were generated by concatenating the mRNA of different Kir2 subunits. C-terminal domains of Kir2.x subunits were fused to the N-Terminus of Kir2.y subunits (Preisig-Muller et al., 2002). Corresponding hetero-concatemeric Kir2.x-2.y constructs were heterologously expressed in the oocyte expression system. For a functional channel pore the association of two hetero-concatemers is required.

Figure 2. Inhibition of Kir2.3 currents by quinidine.

Representative Kir2.3 current traces under control conditions and after 30 minutes of incubation with 1 mM quinidine are shown in panel A and B. Corresponding current-voltage curves are displayed in panel C. Dose-response experiments revealed a half-maximal inhibiting concentration of quinidine at approximately 72 µM for Kir2.3 subunits (D, n=4–16). The relative effects of quinidine on inward current amplitudes are plotted as a function of time in panel E and F. Cells were treated with 100 µM quinidine (E, n=10) and were subsequently reperfused with extracellular bath solution for 45 minutes respectively (F, n=10). Control experiments were performed accordingly with extracellular K⁺ solution (n=4, each).

Figure 3. Inhibition of Kir2.1 and Kir2.2 currents by quinidine.

Typical current-voltage relations of Kir2.1 and Kir2.2 current traces before and after a 30-minute incubation period with 2 mM quinidine are shown in panel A.
and E, respectively. Kir2.1 and Kir2.2 dose-response experiments yielded IC$_{50}$ values of approximately 290 μM (B, n=5–13) and 370 μM (F, n=4–7). Summary data of wash-in/wash-out experiments with 1 mM quinidine are shown in panel C/D and G/H for Kir2.1 and Kir2.2 currents, respectively. Extracellular bath solution was used as control (n=4, each).

Figure 4. Inhibitory effects of quinidine on hetero-concatemeric Kir2.x-2.y channels

Representative current-voltage curves of the quinidine block on hetero-concatemeric Kir2.x-2.y channels are shown in panel A, C and E. Dose-response analysis revealed a half-maximal inhibitory concentration of approximately 470 μM for Kir2.1-2.2 (B, n=4–6), 250 μM for Kir2.1-2.3 (D, n=4–8) and 190 μM for Kir2.2-2.3 hetero-concatemers (F, n=4–8).

Figure 5. Voltage independent effect of quinidine on Kir2.x channels.

Current measurements before and after 30 minutes of incubation with quinidine were compared to determine voltage dependency of the quinidine block. Results are shown for the voltages between -120 mV and 0 mV. Currents of 80±10mV were excluded from the analysis due to small and unrepresentative amplitudes. Relative current block of Kir2.x channels did not show significant differences in the analyzed voltage range (n=4–7).

Figure 6. Alignment of amino acid sequences of human Kir2.x subunits.

Specific amino acid residues that line the cytoplasmic pore of Kir2.1 subunits interact with cationic amphiphilic drugs. Residues reported to be involved in quinidine- and chloroquine-mediated Kir2.1 current inhibition are marked by...
asterisks (Kir2.1 E224, F254 and D259) and obelisks (D255 and E299), respectively. Sequence alignments indicate that they are highly conserved within the Kir2.x subfamily.

Figure 7. Quinidine exerts differential effects on Kir2.x cytoplasmic pore mutants. Inhibitory effects of quinidine were significantly reduced in all Kir2.1 (A) and Kir2.3 pore mutants (C). Interestingly, Kir2.2 channels differed from these patterns. As shown in panel B only in Kir2.2 D260A mutants the inhibitory effect was significantly altered compared to wild-type channels with a current increase of 11.5 ± 5.6 % (n=5). Quinidine concentration used for Kir2.1, Kir2.2 and Kir2.3 pore mutants were 1 mM, 0.5 mM and 0.1 mM, respectively.

Figure 8. Quinidine blocks Kir2.x currents in a PIP2-dependent manner. Channel mutants which have less affinity to endogenous PIP2 were significantly more sensitive to the inhibitory effect of 200 µM quinidine (68.9 ± 3.3 %, n=6 for Kir2.1 K182Q and 31 ± 1.1 %, n=5 for Kir2.1 L221I) compared to Kir2.1 wild-type channels (18.2 ± 3.8 %, n=5). Kir2.3 I214L enhances the affinity to PIP2 and the effects of quinidine on these channels were similar to those under control conditions.
Tables

Table 1. Maximum inhibitory effect of quinidine on homomeric Kir2.x and heteroconcatemeric Kir2.x-2.y channels. Data given in mean ± S.E.M. for maximum relative inhibitions of Kir currents after 30 minutes of incubation with quinidine (see text for details).

<table>
<thead>
<tr>
<th>channel</th>
<th>concentration</th>
<th>relative current to control</th>
<th>S.E.M.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir2.1</td>
<td>2 mM</td>
<td>42 %</td>
<td>3.9 %</td>
<td>5</td>
</tr>
<tr>
<td>Kir2.2</td>
<td>2 mM</td>
<td>29 %</td>
<td>5.2 %</td>
<td>4</td>
</tr>
<tr>
<td>Kir2.3</td>
<td>1 mM</td>
<td>10 %</td>
<td>1.4 %</td>
<td>4</td>
</tr>
<tr>
<td>Kir2.1-2.2</td>
<td>2.5 mM</td>
<td>34 %</td>
<td>3 %</td>
<td>5</td>
</tr>
<tr>
<td>Kir2.1-2.3</td>
<td>2.5 mM</td>
<td>22 %</td>
<td>1.8 %</td>
<td>5</td>
</tr>
<tr>
<td>Kir2.2-2.3</td>
<td>2 mM</td>
<td>31 %</td>
<td>4.6 %</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 1

Kir2.x

Kir2.y

M1

M2

N

C
Figure 2

A. Kir2.3: control

B. Kir2.3: quinidine [1 mM]

C. Graph showing the effect of potential on current with different treatments.

D. Graph showing the relative normalized Kir2.3 current against quinidine concentration with IC50=0.072 mM.

E. Graph showing the effect of quinidine wash-in [100 µM] and K+ solution on relative Kir2.3 current.

F. Graph showing the effect of quinidine wash-out [100 µM] and K+ solution on relative Kir2.3 current.
Figure 3

**A**
- Kir2.1 control
- Kir2.1 quinidine [2 mM]

**B**
IC$_{50}$=0.29 mM

**C**
- quinidine wash-in [1 mM]
- K$^+$ solution

**D**
- quinidine wash-out [1 mM]
- K$^+$ solution

**E**
- Kir2.2 control
- Kir2.2 quinidine [2 mM]

**F**
IC$_{50}$=0.37 mM
Figure 4

A

- Kir2.1-2.2 control
- Kir2.1-2.2 quinidine [2.5 mM]

potential [mV]

-120 -80 -40 40

current [µA]

B

IC$_{50}$=0.47 mM

relative normalized Kir2.1-2.2 current

quinidine concentration [mM]

0.1 1

0.0 1

0.5 1

C

- Kir2.1-2.3 control
- Kir2.1-2.3 quinidine [2.5 mM]

potential [mV]

-120 -80 -40 40

current [µA]

D

IC$_{50}$=0.25 mM

relative normalized Kir2.1-2.3 current

quinidine concentration [mM]

0.1 1

0.0 1

0.5 1

E

- Kir2.2-2.3 control
- Kir2.2-2.3 quinidine [2 mM]

potential [mV]

-120 -80 -40 40

current [µA]

F

IC$_{50}$=0.19 mM

relative normalized Kir2.2-2.3 current

quinidine concentration [mM]

0.1 1

0.0 1

0.5 1
Figure 5

- Kir2.1 after quinidine 1 mM
- Kir2.2 after quinidine 1 mM
- Kir2.3 after quinidine 0.1 mM

The graph shows the relative reduction of Kir2.x current as a function of potential (in mV) after exposure to quinidine at different concentrations. The data is represented with error bars indicating variability.
Figure 7

A

Kir2.1 wt
Kir2.1 E224A
Kir2.1 F254A
Kir2.1 D259A
Kir2.1 E299A

relative current reduction [1 mM]

B

Kir2.2 wt
Kir2.2 E225A
Kir2.2 F255A
Kir2.2 D256A
Kir2.2 D260A
Kir2.2 E299A

relative current reduction [0.5 mM]

C

Kir2.3 wt
Kir2.3 E216A
Kir2.3 D247A
Kir2.3 D251A
Kir2.3 E291A

relative current reduction [0.1 mM]
This image represents a bar graph showing the relative change in current amplitude for different Kir2.1 and Kir2.3 variants in response to 200 µM and 100 µM quinidine. The graph includes the following variants:

- Kir2.1 wt
- Kir2.1 K182Q
- Kir2.1 L222L
- Kir2.3 wt
- Kir2.3 I214L

The y-axis represents the relative change in current amplitude, and the x-axis lists the different Kir variants. The graph indicates the effects of quinidine at two different concentrations on the current amplitude. The data points are accompanied by error bars, indicating variability in the measurements.