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ACCELERATED COMMUNICATION

Identification of Novel and Selective K_V2 Channel Inhibitors

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ABSTRACT

Identification of selective ion channel inhibitors represents a critical step for understanding the physiological role that these proteins play in native systems. In particular, voltage-gated potassium (K_V2) channels are widely expressed in tissues such as central nervous system, pancreas, and smooth muscle, but their particular contributions to cell function are not well understood. Although potent and selective peptide inhibitors of K_V2 channels have been characterized, selective small molecule K_V2 inhibitors have not been reported. For this purpose, high-throughput automated electrophysiology (IonWorks Quattro; Molecular Devices, Sunnyvale, CA) was used to screen a 200,000-compound mixture (10 compounds per sample) library for inhibitors of $K_V2.1$ channels. After deconvolution of 190 active samples, two compounds (A1 and B1) were identified that potently inhibit $K_V2.1$ and the other member of the K_V2 family, $K_V2.2$ (IC_{50}, 0.1–0.2 μM), and that possess good selectivity over $K_V1.2$ (IC_{50} >10 μM). Modeling studies suggest that these compounds possess a similar three-dimensional conformation. Compounds A1 and B1 are >10-fold selective over Na_V channels and other K_V channels and display weak activity (5–9 μM) on Ca_V channels. The biological activity of compound A1 on native K_V2 channels was confirmed in electrophysiological recordings of rat insulinoma cells, which are known to express K_V2 channels. Medicinal chemistry efforts revealed a defined structure-activity relationship and led to the identification of two compounds (RY785 and RY796) without significant Ca_V channel activity. Taken together, these newly identified channel inhibitors represent important tools for the study of K_V2 channels in biological systems.

Introduction

Voltage-gated potassium (K_V) channels open in response to membrane depolarization and are present in many cell types. In excitable cells, K_V channels serve as the primary mechanism of repolarization of action potentials, whereas in nonexcitable cells, K_V channels control the cell resting potential. Given the role of K_V channels, it is not surprising that they regulate many fundamental physiological processes and are therefore considered important therapeutic targets for treatment of autoimmune, metabolic, neurological, and cardiovascular disorders, as well as cancer (Wulff et al., 2009). Despite these facts, there has been limited success in the clinical development of therapeutic agents that target K_V channels. One reason for this is that many of the small molecules identified to date lack true molecular selectivity across members of the K_V and other ion channel families, which could significantly compromise their therapeutic index. The lack of ion channel selectivity seems to be due to binding of compounds to highly conserved regions across channels (Hanner et al., 1999, 2001; Rolf et al., 2000; Decher et al., 2004, 2006; Eldstrom et al., 2007; Karczewski et al., 2009; Zimin et al., 2010). Another reason for the slow progress in drug development is the difficulty in screening large compound libraries with assays that measure channel function (i.e., K conduction) directly, although the development of automated elec-

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ABBREVIATIONS: K_V channel, voltage-gated potassium channel; Na_V channel, voltage-gated sodium channel; Ca_V channel, voltage-gated calcium channel; CHO, Chinese hamster ovary; C-1, 3-(4-(benzo[*d*][1,3]dioxol-5-yl)butyl)-7-methyl-3,7-diazabicyclo[3.3.1]nonan-9-yl 4-chlorobenzoate; DMSO, dimethyl sulfoxide.

trophysiology platforms is beginning to address some of these issues (Dunlop et al., 2008).

The K_V2 channel family consists of two members, $K_V2.1$ and $K_V2.2$. $K_V2.1$ is prominently expressed in the brain, notably in pyramidal neurons of the hippocampus and cortex, where it regulates excitability (Misonou et al., 2005). In rodents, $K_V2.1$ channels are present in cardiac ventricular myocytes (Nerbonne and Kass, 2005). $K_V2.1$ also regulates insulin secretion from the pancreatic β cell (Jacobson et al., 2007). $K_V2.2$ is expressed in brain (Hwang et al., 1992), smooth muscle (Schmalz et al., 1998), and somatostatin secreting δ-cells of the pancreatic islet (Yan et al., 2004; Wolf-Goldberg et al., 2006); however, little is known about the role of $K_V2.2$ channels in these tissues. The assessment of the roles of $K_V2.1$ and $K_V2.2$ channels in tissues where they are expressed, and the consequences of channel modulation in vivo, has been hampered by the lack of selective pharmacological tools. Gating modifier peptides highly selective for K_V2 channels have been identified in the venoms of tarantulas (for review, see Swartz, 2007). However, the limited availability of these peptides has often hampered their use in the study of physiological systems. Highly selective, small-molecule inhibitors of K_V2 channels would be useful in this regard, but the reported number of these molecules is quite limited. For example, although the antiarrhythmics propafenone and flecainide seem to block $K_V2.1$ channels more potently than K_V1 channels (Rolf et al., 2000), these compounds have actions on other channels as well. 3-(4-(Benzo[*d*][1,3]dioxol-5-yl)butyl)-7-methyl-3,7-diazabicyclo[3.3.1]nonan-9-yl 4-chlorobenzoate (C-1), a besipirdine derivative, has been shown to have some selectivity for $K_V2.1$ channels over other K_V channels (MacDonald et al., 2002). Thus, there is a need for identifying novel and selective K_V2 inhibitors with which to investigate the role of these channels and develop their pharmacology.

The IonWorks Quattro automated electrophysiology instrument functions in a 384-well format that is well suited for screening compound libraries for activity on K_V channels, and in a previous work, we reported on the development of a robust assay for $K_V2.1$ channels using this platform (Ratliff et al., 2008). In this study, we apply this assay to screen a 200,000-compound library for inhibitors of $K_V2.1$ channels. We report the discovery and optimization of two series of compounds with striking selectivity for K_V2 channels over other K_V, and Ca_V, and Na_V channels.

Materials and Methods

Materials. CHO cells stably expressing human $K_V2.1$ were obtained from Dr. O. Pongs (Institut fuer Neurale Signalverarbeitung, Hamburg, Germany). CHO cells stably expressing human $K_V1.2$ were prepared at Merck Research Laboratories (Rahway, NJ). INS-1 cells (clone 832/13) were supplied by Dr. C. Newgard (Duke University, Durham, NC). Compounds were synthesized by the Department of Medicinal Chemistry, Merck Research Laboratories. All tissue culture media and additives were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. Chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Cell Culture. $hK_V2.1$.CHO cells were maintained in minimal essential media α with nucleosides supplemented with 10% certified fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 0.29 mg/ml L-glutamine, and 2 μg/ml blasticidin S HCl. $hK_V1.2$.CHO cells were maintained in Iscove's modified Eagle's medium supplemented with 10% certified fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 0.29 mg/ml L-glutamine, 1× hypoxanthine-thymidine supplement, and 0.5 mg/ml G418. $hK_V2.1$.CHO were grown in the presence of 10% CO_2, whereas $hK_V1.2$.CHO cells were grown at 5% CO_2. INS-1 cells were cultured as described previously (Hohmeier et al., 2000).

Automated 384-Well Electrophysiology. $K_V2.1$ currents were recorded using the IonWorks Quattro system (Molecular Devices, Sunnyvale, CA) in population patch clamp mode as described previously (Ratliff et al., 2008). The standard voltage pulse protocol was a series of forty 100-ms pulses at a frequency of 5 Hz. The prepulse holding potential was −80 mV, and the steps were to +50 mV. Currents were sampled at a rate of 1.25 kHz. After an initial read, compound (or vehicle) was added for ~3 min, and a second voltage train was applied. Ten point concentration dilution series were created by serially diluting a 2 mM DMSO stock 1:3 in DMSO. The upper final concentration applied to cells was 20 μM. The final concentration of DMSO (1%) had no effect on control current recordings.

Conventional Patch Clamp Electrophysiology. Membrane currents were recorded at room temperature (23–25°C) using standard dialyzed, whole-cell voltage clamp techniques as described previously (Herrington et al., 2005). The internal solution was 100 mM potassium aspartate, 40 mM KCl, 10 mM EGTA, 10 mM HEPES, 4 mM MgATP, pH 7.2 with KOH. The external solution was 150 in mM NaCl, 4 in mM KCl, 1.8 mM $CaCl_2$,0.5 mM $MgCl_2$, 10 mM HEPES, and 3 mM glucose, pH 7.4 with NaOH. Compounds were diluted in external solution from 10 to 20 mM stocks in DMSO. The final concentration of DMSO did not exceed 0.1%.

Results

A ~200,000-compound library was screened on $hK_V2.1$ channels stably expressed in CHO cells using the IonWorks Quattro 384-well automated electrophysiology platform. The details of this assay have been described previously (Ratliff et al., 2008). In brief, a 40-pulse train of voltage steps was applied at 5 Hz. This protocol is designed to detect use-dependent block (i.e., greater inhibition of current at the 40th pulse compared with the 1st pulse). To maximize the throughput of the screen and to contain the cost of consumables, each compound well contained a mixture of 10 compounds. Initial studies revealed that a screening concentration of 1 μM per compound (10 μM total in the well) was optimal for achieving a modest hit rate. Higher screening concentrations produced too many active wells, presumably as a result of the additive effects of 10 compounds in each well. Figure 1A shows the number of active wells for the 56 384-well plates used in the screen. Using a cutoff of 40% inhibition at pulse 40, 7.8 ± 0.6 active wells were detected per plate. From the primary screen of the library, 190 wells were selected for deconvolution, yielding 1894 compounds. These compounds were tested in isolation in two different $K_V2.1$ paradigms: IonWorks and a fluorescence assay measuring changes in membrane potential. Results from testing in IonWorks Quattro (in triplicate) and the resulting histogram are shown in Fig. 1B. Based on the 40% inhibition cutoff at pulse 40, 180 compounds were confirmed as active, yielding an overall hit rate for the IonWorks Quattro screen of approximately 0.1%.

Evaluation of the 1894 compounds at 4 μM in the membrane potential assay (data not shown) identified 31 compounds that were chosen for retesting on $K_V2.1$ in the IonWorks assay. As an initial test for selectivity, the 31 compounds were tested in parallel on $K_V1.2$ using the same IonWorks assay protocol. Data from these experiments are illustrated in Fig. 1C, where the percentage inhibition of

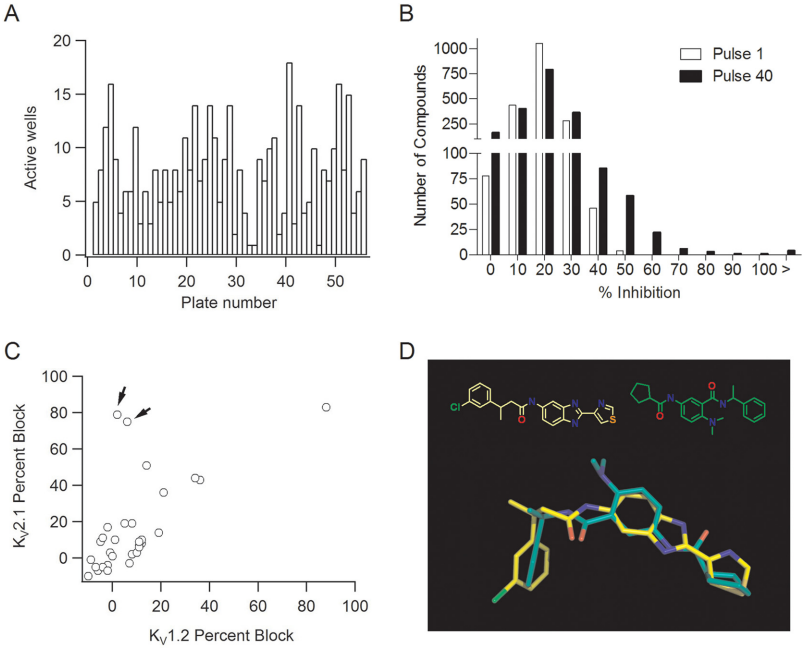


Fig. 1. Identification of $K_V2.1$ inhibitors by high-throughput automated electrophysiology screening. A, plot of the number of active wells per screening plate versus plate number for the screen. Wells with >40% inhibition of pulse 40 current were considered active. B, histogram of percentage inhibition at pulse 1 (open bars) and pulse 40 (solid bars) for 1894 compounds from the deconvolution of the original 190 active mixture wells. C, plot of the percentage inhibition of $K_V2.1$ versus percentage inhibition of $K_V1.2$ for 31 compounds when tested at 3 μM. The arrows point to two compounds (compounds A1 and B1) with apparent selectivity for $K_V2.1$ over $K_V1.2$. D, overlay of three-dimensional conformations of compounds A1 and B1. The color scheme for various atoms is shown in the inset.

$K_V2.1$ (40th pulse) is plotted versus percentage inhibition of $K_V1.2$ (40th pulse). It is noteworthy that two compounds, identified by arrows, showed apparent selectivity for $K_V2.1$ over the $K_V1.2$ channel. The structures of these compounds (compounds A1 and B1) are shown in Fig. 1D. Superposition of three-dimensional conformations of A1 and B1 predicted reasonable overlap. Good overall overlap can be maintained by superimposing the center benzene ring of both A1 and B1 with the imidazole ring of A1 mapping onto the aniline amide of B1. This overlay also places the thiazole ring of A1 on top of the cyclopentane of B1.

The activities of A1 and B1 on $K_V2.1$ were re-confirmed by purification or re-synthesis of the compounds. Inspection of the recordings from the IonWorks assay revealed that the compounds are use-dependent inhibitors of $K_V2.1$ (Fig. 2A, top). For compound A1, the potency was 10-fold higher at

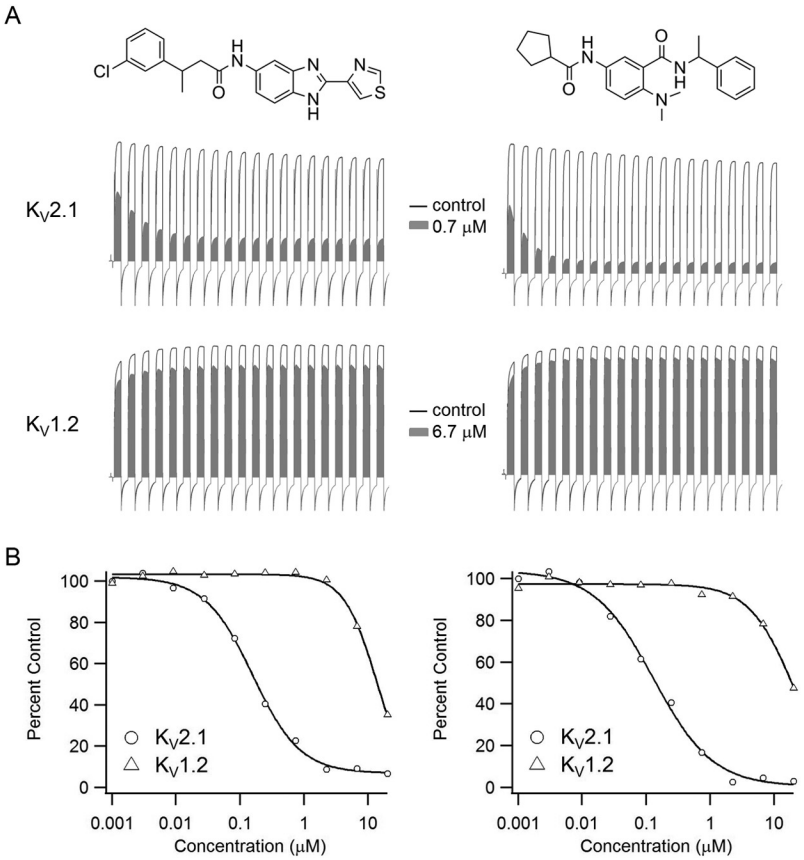


Fig. 2. Compounds A1 and B1 are use-dependent inhibitors of $K_V2.1$ with selectivity over $K_V1.2$. A, representative IonWorks recordings of $K_V2.1$ (top) and $K_V1.2$ (bottom) before (control, solid lines) and after addition of A1 (left) or B1 (right) (gray filled traces). Note the differences in concentrations (0.7 μM for $K_V2.1$; 6.7 μM for $K_V1.2$) and the use-dependent inhibition of $K_V2.1$. B, concentration-response relationships for A1 (left) and B1 (right). Pulse 40 current expressed as percentage of control is plotted versus compound concentration for $K_V2.1$ (○) and $K_V1.2$ (△). The solid lines are fits of the Hill equation to the data. For compound A1, parameters of the fits are $K_V2.1$: IC_{50}, 0.16 μM; n_H, 1.2; $K_V1.2$: IC_{50}, 13.4 μM; n_H, 1.7. For compound B1, parameters of the fits are $K_V2.1$: IC_{50}, 0.13 μM;, n_H, 0.9; $K_V1.2$: IC_{50}, 19.6 μM; n_H, 1.3.

pulse 40 versus pulse 1 (pulse 40 IC_{50}, 0.20 μM; pulse 1 IC_{50}, 2.0 μM, $n = 4$). The potency of compound B1 shifted similarly (pulse 40 IC_{50}, 0.15 μM; pulse 1 IC_{50}, 2.1 μM, $n = 4$). Compound B1 was further resolved to its enantiomers by chiral high-performance liquid chromatography. The *S*-enantiomer [compound B1 (S)] and the *R*-enantiomer [compound B1 (R)] had similar potency on $K_V2.1$ (IC_{50}, 0.15 and 0.20 μM, respectively). Further profiling showed that these compounds are equipotent inhibitors of $K_V2.1$ and $K_V2.2$ channels (Table 1).

The initial observation concerning the selectivity of these compounds for $K_V2.1$ over $K_V1.2$ channels was confirmed in detailed concentration-response measurements (Fig. 2B). Both compounds displayed weak activity as inhibitors of $K_V1.2$ at either pulse 40 or pulse 1 (Fig. 2, Table 1). For example, compound A1 inhibited $K_V1.2$ at pulse 40 with an IC_{50} of 12.1 μM ($n = 3$), which is 50-fold higher than the IC_{50} for inhibition of $K_V2.1$.

The two $K_V2.1$ inhibitors were also tested on a variety of voltage-gated channels, using primarily functional assays. For comparison with other K_V channels, the identical IonWorks electrophysiology assay of $K_V2.1$ was used to allow direct comparison with K_V2 channel data. Both compounds displayed weak activity on the K_V channels $K_V1.5$ and $K_V3.2$. The compounds also displayed weak activity on the human *ether-à-go-go*-related gene channel ($K_V11.1$) based on a radioligand binding assay as well as on Na_V and Ca_V channels in functional assays. In general, compound B1 showed greater selectivity for K_V2 channels over other channels (average, 75-fold) compared with compound A1 (average, 35-fold). Compound B1 was screened on 163 additional targets in a panel of enzyme and radioligand binding assays (performed by MDS Pharma Services, King of Prussia, PA). This panel included 10 additional ion channel targets. At 10 μM, compound B1 displayed significant activity (>50% inhibition) on only three targets: adenosine receptor A3 (IC_{50}, 0.84 μM, radioligand binding), 5-lipoxygenase (IC_{50}, 2.0 μM, enzyme activity), and serotonin receptor 2B (IC_{50}, 6.5 μM, radioligand binding).

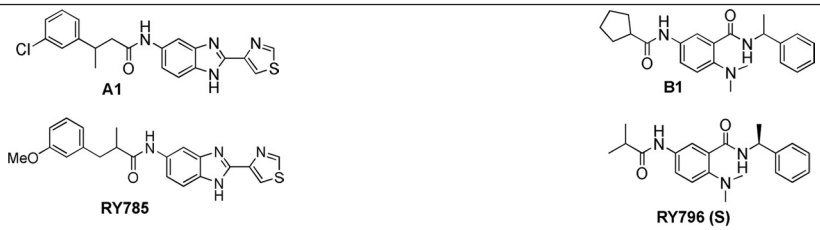
Despite the selectivity of A1 and B1 for many ion channels, however, both compounds display moderate activity on $Ca_V1.2$ and $Ca_V2.3$ channels (Table 1). Because functional block of Ca_V channels will limit the utility of these compounds in the evaluation of certain physiological systems, medicinal chemistry efforts were aimed at identifying analogs of these compounds with reduced activity on Ca_V channels. Two analogs were found that retained potency on K_V2 channels, but had much reduced activity on Ca_V2 channels. These compounds were termed RY785 and RY796 (Table 1).

In initial structure-activity relationship studies, analogs of both A and B series were found to display similar structure-activity relationship at the corresponding overlapping regions, as shown in Table 2. It is thus likely that the two compound series bind at overlapping sites on K_V2 channels. A stereochemical preference for binding to $K_V2.1$ was present in some analogs in the B series. For compound B2, the *S*-enantiomer (RY796) is 5-fold more potent than the *R*-enantiomer. For the A series compounds, a stereochemical preference for $K_V2.1$ inhibition did not seem to exist. For example, RY785 is the first (fast) eluting enantiomer (IC_{50}, 0.05 μM) from the chiral column separation of a racemic mixture. The slow eluting enantiomer was equally active as an inhibitor of $K_V2.1$ (IC_{50}, 0.07 μM). Similar results were observed when the *m*-MeO group in RY785 was replaced with

TABLE 1

Summary of activity on selected voltage-gated ion channels

For all potassium channels, reported IC_{50} values were measured by automated electrophysiology (IonWorks Quattro) at pulse 40 of a 5-Hz train, except that Kv7.1/KCNE1 (IK_s) values were determined from an automated electrophysiology (PatchXpress) assay and $K_V11.1$ values were determined from a ^{35}S-MK-499 radioligand displacement assay. For all calcium channels, values are from fluorescence-based functional FLIPR assays (Dai et al., 2008). For all sodium channels, values are from fluorescence membrane potential-based assay (Felix et al., 2004). Data for compound B1 on $K_V2.1$, $K_V1.2$, $K_V1.5$, and $K_V3.2$ were obtained using the racemic mixture; all other data were obtained using the pure *S*-enantiomer. Pure *S*- and *R*-enantiomers of compound B1 had equal potency on $K_V2.1$.



Channels	IC_{50}			
	A1	B1	RY785	RY796
	μM			
$K_V2.1$	0.20	0.15	0.05	0.25
$K_V2.2$	0.41	0.17		0.09
$K_V1.2$	12.1	17	>10	>10
$K_V1.5$	9.5	>20		
$K_V3.2$	>20	>20		
$K_V7.1$			>30	>30
$K_V11.1$	2.9	>10		
$Ca_V1.2$	6.6	8.9	17	>20
$Ca_V2.1$	>10	>10	>10	>10
$Ca_V2.2$	>10	>10	>10	>10
$Ca_V2.3$	5.7	4.6	>10	>10
$Na_V1.5$	>10	>10		
$Na_V1.7$	8.0	8.2		

TABLE 2
$K_V2.1$ activities of analogs of Compound A1 and B1

R	Compound	$K_V2.1$ IC_{50}
		μM
	A2	0.18
	B1 (S)	0.15
	B1 (R)	0.20
	A3	0.47
	RY796 (S)	0.25
	B2 (R)	1.3
	B3 (S)	0.18
	B3 (R)	1.0
	A4	0.22
	B4	0.15
	B5	0.15
	B6	0.33
	A5	0.23
	A6	0.22

o-chlorine (IC_{50}, 0.12 and 0.14 μM for inhibition of $K_V2.1$ by the enantiomers).

Pancreatic β cells are known to express K_V2 channels (for review, see MacDonald and Wheeler, 2003). The rat insulinoma cell line INS-1 expresses both $K_V2.1$ and $K_V2.2$ channels (Su et al., 2001). The majority of K_V current in INS-1 cells probably arises from K_V2 channels, based on its sensitivity to the K_V2 gating modifier peptide GxTX-1E (Herrington, 2007). Thus, we tested the newly identified K_V2 inhibitors on the K_V current in INS-1 cells. Compound A1 inhibited the majority of current in these cells (Fig. 3), and inhibition seems to be reversible upon washing out the compound. Based on recordings from three cells, compound A1 blocked INS-1 K_V current an average of 71% at 0.3 μM and 84% at 3 μM (n = 2 per concentration), providing further evidence that the compounds identified by the automated electrophysiology screen are indeed inhibitors of native K_V2 channels.

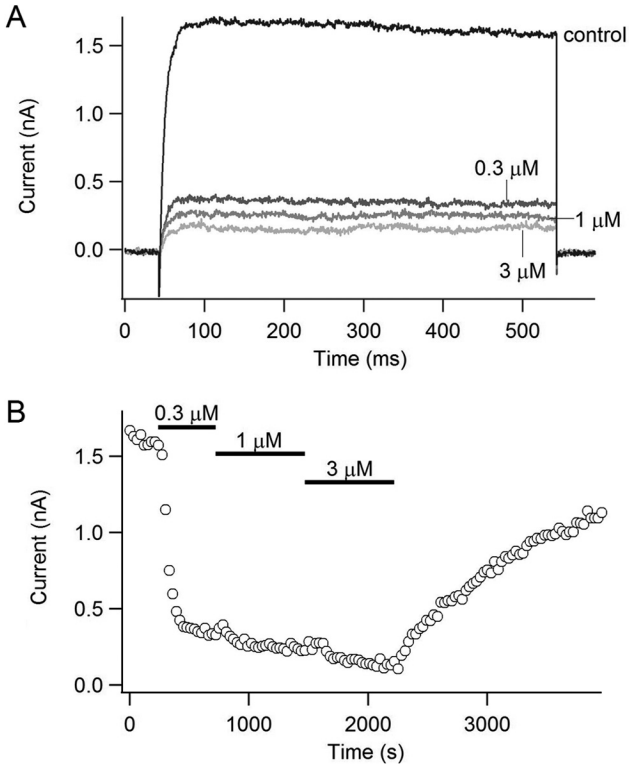


Fig. 3. Inhibition of K_V current in rat insulinoma INS-1 cells by compound A1. A, currents activated in response to a 500-ms step to +20 mV from a holding potential of −80 mV are shown. Currents before (control) and after application of 0.3, 1, and 3 μM compound A1 are shown. B, plot of the peak current versus time for the recording in A. The period of application of compound A1 is denoted by the solid bars.

Discussion

The aim of the present study was to identify novel inhibitors of K_V2 channels with improved selectivity over other available small molecule tools. We chose to use automated electrophysiology as the primary assay because it provides a direct measurement of channel activity and is the method best suited to identify compounds that interact with K_V channels in a state-dependent manner. The IonWorks Quattro 384-well platform allowed sufficient throughput to screen a library of approximately 200,000 compounds. Despite the higher density format of IonWorks compared with other automated electrophysiology devices, we needed to test compounds in mixtures to maximize throughput and minimize consumable costs. This approach, however, did not allow a screening concentration higher than 1 μM per compound (10 μM total) to achieve manageable hit rates. Nevertheless, our data provide convincing evidence that this approach has utility for the screening of large compound libraries by automated electrophysiology. It is noteworthy that the use of a single instrument and voltage protocol allowed the unbiased measurement of selectivity of the newly identified K_V2 inhibitors across K_V channel subtypes.

The successful identification of small molecules that specifically target K_V2 channels using automated electrophysiology suggests areas for future work. In addition, further studies with the K_V2 inhibitors concerning their site of interaction with the channel need to be pursued. These compounds may in fact represent a novel pharmacophore in K_V2 channels that should be exploited for designing new ion channel modulators. Few mapping studies of other K_V2 channel inhibitors exist. Flecainide and propafenone, two antiarrhythmics with broad ion channel activity, are micromolar $K_V2.1$ inhibitors with weaker potency on $K_V1.2$ (Rolf et al., 2000). Flecainide and propafenone are thought to interact with residues at the interface of the P-helix of one subunit and the inner S6 helix of an adjacent subunit and to block ion permeation from within the central cavity (Madeja et al., 2003, 2010). Because the residues at the subunit interface are less conserved than those that line the inside of the

central cavity, such a binding site may provide a degree of selectivity across families of channels. It is noteworthy that the molecules identified in this study are electroneutral, whereas flecainide and propafenone possess cationic groups. Zimin et al. (2010) proposed a mechanism for block of K_V channels by electroneutral molecules. Thus, it will be interesting to determine the binding site(s) and mechanism of block of the K_V2 inhibitors identified in this study.

The future identification of novel molecules specifically targeting distinct K_V channels holds considerable promise for the discovery of therapeutics to treat a spectrum of diseases. Such agents, in addition to their selectivity for other ion channels, will need to be optimized for favorable pharmacokinetic and drug metabolism profiles, as well as other parameters, before they can be considered to enter clinical development. Medicinal chemistry efforts will be needed to determine whether the new classes of K_V2 inhibitors described in the present study can be modified to accomplish such a goal. In the meantime, these agents may prove to be useful to evaluate the role that K_V2 channels play in native tissues.

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Authorship Contributions

Participated in research design: Herrington, Solly, Li, Zhou, Howard, Kiss, Garcia, McManus, Desai, Xiong, and Kaczorowski.

Conducted experiments: Herrington, Solly, Ratliff, Li, and Desai.

Contributed new reagents or analytic tools: Desai.

Performed data analysis: Herrington, Solly, Ratliff, Garcia, Deng, and Xiong.

Wrote or contributed to the writing of the manuscript: Herrington, Zhou, Garcia, McManus, Deng, Xiong, and Kaczorowski.

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