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RESEARCH PAPER

Inhibitory actions of the phosphatidylinositol 3-kinase inhibitor LY294002 on the human Kv1.5 channel

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Background and purpose: Kv1.5 channels conduct the ultra-rapid delayed rectifier potassium current (I_{Kur}), and in humans, Kv1.5 channels are highly expressed in cardiac atria but are scarce in ventricles. Pharmacological blockade of human Kv1.5 (hKv1.5) has been regarded as effective for prevention and treatment of re-entry-based atrial tachyarrhythmias. Here we examined blockade of hKv1.5 channels by LY294002, a well-known inhibitor of phosphatidylinositol 3-kinase (PI3K).
Experimental approach: hKv1.5 channels were heterologously expressed in Chinese hamster ovary cells. Effects of LY294002 on wild-type and mutant (T462C, H463C, T480A, R487V, A501V, I502A, I508A, L510A and V516A) hKv1.5 channels were examined by using the whole-cell patch-clamp method.
Key results: LY294002 rapidly and reversibly inhibited hKv1.5 current in a concentration-dependent manner (IC_{50} of 7.9 μmol·L^{-1}). In contrast, wortmannin, a structurally distinct inhibitor of PI3K, had little inhibitory effect on hKv1.5 current. LY294002 block of hKv1.5 current developed with time during depolarizing voltage-clamp steps, and this blockade was also voltage-dependent with a steep increase over the voltage range for channel openings. The apparent binding (k_{+1}) and unbinding (k_{-1}) rate constants were calculated to be 1.6 μmol·L^{-1-1}·s^{-1} and 5.7 s^{-1} respectively. Inhibition by LY294002 was significantly reduced in several hKv1.5 mutant channels: T480A, R487V, I502A, I508A, L510A and V516A.
Conclusions and implications: LY294002 acts directly on hKv1.5 currents as an open channel blocker, independently of its effects on PI3K activity. Amino acid residues located in the pore region (Thr480, Arg487) and the S6 segment (Ile502, Ile508, Leu510, Val516) appear to constitute potential binding sites for LY294002.
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Keywords: Kv1.5; LY294002; PI3K; open channel block; I_{Kur}; site-directed mutagenesis; binding site

Abbreviations: AF, atrial fibrillation; APD, action potential duration; I_{Kr}, rapidly activating component of delayed rectifier potassium current; I_{Ks}, slowly activating component of delayed rectifier potassium current; I_{Kur}, ultrarapid delayed rectifier potassium current; PI3K, phosphatidylinositol 3-kinase; Kv, voltage-dependent potassium channel; CHO, Chinese hamster ovary; WT, wild-type; DMSO, dimethyl sulphoxide; GFP, green fluorescent protein; MAP kinase, mitogen-activated protein kinase; PCR, polymerase chain reaction

Introduction

Atrial fibrillation (AF) is the most frequent cardiac arrhythmia that can result in serious morbidity and a doubling of mortality in elderly persons (Chugh *et al.*, 2001). It is well known that AF or atrial flutter is caused, or at least maintained, by re-entrant wavelets. A well-established method of extinguishing or preventing such re-entries is prolongation of the myocardial refractoriness, primarily determined by the action potential duration (APD). A major determinant of APD is the amount of repolarizing outward potassium currents, particularly provided by the three (ultra-rapid, rapid and slow) delayed rectifier potassium currents (I_{Kur}, I_{Kr} and I_{Ks} respectively). Therefore, blockers of these currents can be expected to exert anti-arrhythmic actions against re-entrant-based tachyarrhythmias (Knobloch *et al.*, 2002). However, because I_{Kr} and I_{Ks} are present in both the atrium and ventricle, undesired effects such as excess prolongation of ventricular action potentials can be caused by blockers of I_{Kr} and I_{Ks}, which limits their use for the treatment of atrial arrhythmias (Bril, 2002; Knobloch *et al.*, 2004; Vos, 2004; Regan *et al.*, 2007). In

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contrast, I_{Kur} is expressed predominantly in atrial myocytes but is scarce in ventricular myocytes, in humans (Wang *et al.*, 1993; Li *et al.*, 1996). I_{Kur} may therefore be a promising molecular target for anti-arrhythmic drugs to treat re-entrant-based atrial tachyarrhythmias. Experimental evidence has also been presented to show that I_{Kur} blockade increases the plateau phase and thereby prolongs APD in atrial trabeculae obtained from patients with long-standing AF associated with electrical remodelling (Wettwer *et al.*, 2004).

The molecular component that underlies I_{Kur} in the human atrium is the Kv1.5 channel (Fedida *et al.*, 1993; Li *et al.*, 1996; Nattel *et al.*, 1999: channel nomenclature conforms to Alexander *et al.*, 2008), which is encoded by KCNA5 gene. The Kv1.5 channels belong to the super-family of voltage-gated potassium channels, comprising four pore-forming subunits, each containing six transmembrane segments (S1 to S6). The segment between S5 and S6 forms the external part of the ion conduction pathway. The flanking S5 and S6 segments may contribute to the presumably wider intracellular mouth of the ion channel (Yeola *et al.*, 1996). During the past decade, considerable efforts have been made to develop novel blockers of Kv1.5 channels (Brendel and Peukert, 2003; Varro *et al.*, 2004; Trotter *et al.*, 2006) and to characterize their binding sites in the channel. A few of the known Kv1.5 channel blockers have been tested in humans (Crijns *et al.*, 2006; Dorian *et al.*, 2007). However, newly developed compounds are not so highly selective for Kv1.5 channels and thus, currently, the clinical data to validate the effectiveness of Kv1.5 channel blockade for the treatment of AF are lacking. Mutational analyses have indicated that some residues located near the pore helix as well as in the S6 domain of Kv1.5 channels are important for the binding of the channel blockers (Decher *et al.*, 2004; 2006; Herrera *et al.*, 2005; Rezazadeh *et al.*, 2006).

The compound LY294002 is derived from the naturally occurring bioflavinoid quercetin and potently inhibits phosphatidylinositol 3-kinase (PI3K) activity (IC_{50} = 1.40 μmol·L^{-1}) via competitive inhibition of an ATP binding site on the p85α subunit (Vlahos *et al.*, 1994). This compound has been used in studies of neuronal, cardiovascular, immune and diabetes-related cellular functions for more than a decade (Knight *et al.*, 2004). In recent years, it has been reported that LY294002 can inhibit Kv channel in MIN6 insulinoma cells through a PI3K-independent mechanism (El-Kholy *et al.*, 2003).

The present study was designed to investigate the effect of LY294002 on human Kv1.5 (hKv1.5) channels, heterologously expressed in Chinese hamster ovary (CHO) cells by using the whole-cell patch-clamp technique. Our findings indicate that LY294002 interacts with hKv1.5 channels in a PI3K-independent manner and directly inhibits hKv1.5 currents as an open channel blocker. The putative binding site for this compound is found to be located at the base of the pore helix (Thr480), in the outer pore region (Arg487) and in the S6 domain (Ile502, Ile508, Leu510 and Val516) of hKv1.5 channels.

Methods

Cell preparation, site-directed mutagenesis and transfection

CHO cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum and antibiotics (100 IU·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin) under a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were passaged twice weekly by harvesting with trypsin-EDTA, and a part of treated cells were seeded onto glass coverslips (5×3 mm^2) for the later transfection.

The mammalian expression vector pcDNA3.1 containing hKv1.5 cDNA (kindly provided by Dr D Fedida, University of British Columbia, Vancouver, Canada) was used for expression of all constructs in this study (Eldstrom *et al.*, 2003). Polymerase chain reaction (PCR)-based site-directed mutagenesis was applied to introduce mutations into hKv1.5 cDNA by using Quikchange Kit (Stratagene, La Jolla, CA, USA). All PCR-products were fully sequenced (ABI3100, Applied Biosystems, Foster City, CA, USA) to ensure the fidelity of the PCR reactions. Wild-type (WT) hKv1.5 cDNA and hKv1.5 mutants (T462C, H463C, T480A, R487V, A501V, I502A, I508A, L510A and V516A cDNA) were transiently transfected into CHO cells together with green fluorescent protein (GFP) cDNA (0.5 μg WT or mutant hKv1.5 + 0.5 μg GFP) by using Lipofectamine (Invitrogen Life Technologies, Carlsbad, CA, USA). Patch-clamp experiments were conducted 2–3 days after transfection on GFP-positive cells.

Electrophysiological recordings and data analysis

Whole-cell membrane currents (Hamill *et al.*, 1981) were recorded with an EPC-8 patch-clamp amplifier (HEKA, Lambrecht, Germany), and data were low-pass filtered at 1 kHz, acquired at 5 kHz through an LIH-1600 analogue-to-digital converter (HEKA) and stored on a hard disc drive, by using Pulse/PulseFit software (HEKA). For experiments to measure the activation time course of hKv1.5 currents, the data were low-pass filtered at 10 kHz and sampled at 50 kHz. Patch electrodes had a resistance of 2.5–3.0 MΩ when filled with the pipette solution containing (in mmol·L^{-1}) 70 potassium aspartate, 40 KCl, 10 KH_2PO_4, 1 $MgSO_4$, 3 Na_2-ATP (Sigma Chemical Co., St. Louis, MO, USA), 0.1 Li_2-GTP (Roche Diagnostics GmbH, Mannheim, Germany), 5 EGTA and 5 HEPES (pH adjusted to 7.2 with KOH). In the whole-cell configuration, average series resistances were 5.6 ± 0.1 MΩ. Because the average current was 4.9 nA at +30 mV, the voltage drops induced by the series resistances can be calculated to be 27 mV. The series resistances were usually compensated by 80%. After compensation, the voltage drops were considered to be less than 5.4 mV. Cells attached to glass coverslips were transferred to a recording chamber (0.5 mL in volume) mounted on the stage of an inverted microscope (ECLIPSE TE2000-U, Nikon, Tokyo, Japan). The chamber was maintained at 25°C and was perfused continuously at a rate of 1–2 mL·min^{-1} with Tyrode solution containing (in mmol·L^{-1}) 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.33 NaH_2PO_4, 5.5 glucose and 5.0 HEPES (pH was adjusted to 7.4 with NaOH).

hKv1.5 channel currents were elicited by applying 300 ms depolarizing steps from a holding potential of –80 mV to various levels. Except for frequency-dependent experiments, the interval between voltage-clamp steps was 10 s or longer to allow channels to recover fully from possible inactivation between voltage steps. Drug-induced inhibition was measured at the end of a 300 ms depolarizing step to +30 mV unless

otherwise indicated. Voltage-dependent activation of hKv1.5 channels was assessed by fitting the normalized *I–V* relationship of the tail currents to a Boltzmann equation: $I_{tail} = 1/(1 + \exp((V_{1/2} - V_m)/k))$, where I_{tail} is the tail current amplitude normalized with reference to the maximum value measured at +50 mV; $V_{1/2}$ is the voltage at half-maximal activation; V_m is the test potential and *k* is the slope factor. The concentration–response curve for inhibition of hKv1.5 current by LY294002 was drawn by a least-squares fit of a Hill equation: $\%Control = 1/(1 + (IC_{50}/[D])^{nH})$, where %Control represents the current in the presence of the drug normalized with reference to the control amplitude (expressed as a percentage); IC_{50} is the concentration of LY294002 causing a half-maximal inhibition; n_H is the Hill coefficient and [D] is drug concentration. A first-order blocking scheme was used to describe the drug–channel interaction (Snyders and Yeola, 1995; Yeola *et al.*, 1996). The apparent rate constants for binding (k_{+1}) and unbinding (k_{-1}) were obtained from fitting the equations: $\tau_D = 1/(k_{+1}[D] + k_{-1})$, where τ_D is the drug-induced time constant, which was calculated from single exponential fits to the traces of current decay during depolarizing step to +30 mV. The apparent dissociation constant K_D is expressed as $K_D = k_{-1}/k_{+1}$. The deactivation kinetics was determined by fitting a single exponential function to the tail current trace.

Statistical analysis

All of the averaged data are presented as mean ± s.e.mean, with the number of experiments shown in parentheses. Statistical comparisons were evaluated by using either Student's *t*-test or ANOVA with Dunnett's post hoc test, as appropriate. Differences were considered to be statistically significant if a *P* value of <0.05 was obtained.

Materials

LY294002, LY303511 (Calbiochem, San Diego, CA, USA) and wortmannin (Sigma) were dissolved in dimethyl sulphoxide (DMSO; Sigma) to yield stock solutions of 50 mmol·L^{-1}. The concentration of DMSO in the final solution was <0.1% (V/V), which had no effect on hKv1.5 currents.

Results

Inhibitory action of LY294002 on hKv1.5 current

Figure 1 demonstrates a representative experiment to examine the effects of LY294002 on the hKv1.5 channels heterologously expressed in a CHO cell. The hKv1.5 current was evoked by 300 ms depolarizing voltage-clamp steps given from a holding potential of –80 mV to various test potentials (–50 to +50 mV) with a return potential of –40 mV, before (Control, Figure 1A) and during (Figure 1B) exposure to 10 μmol·L^{-1} LY294002. In control conditions, hKv1.5 current activated rapidly upon depolarization to reach a peak and then remained stable during moderately depolarized test steps (≤+30 mV) but decayed minimally during strongly depolarized test potentials (≥+40 mV), consistent with previous studies (Snyders *et al.*, 1993; Feng *et al.*, 1998; Choi *et al.*, 2000; Herrera *et al.*, 2005; Rezazadeh *et al.*, 2006). The activation time constant of 1.46 ± 0.11 ms ($n = 6$) was obtained by fitting a single exponential function to the current traces during the initial 30 ms of depolarizing step to +30 mV. The decaying outward tail currents were detected on return to –40 mV.

Bath application of 10 μmol·L^{-1} LY294002 did not significantly affect the activation time constant (1.45 ± 0.16 ms at +30 mV; $n = 6$, $P > 0.05$) while modestly reducing the peak amplitude of hKv1.5 current. However, this compound caused a marked, time-dependent decline in outward currents during depolarizing test potentials, which was more prominent at more positive potentials. Figure 1C illustrates *I–V* relationships for late currents (measured at the end of 300 ms clamp steps) in the absence and presence of LY294002. The hKv1.5 current was blocked by LY294002 over the whole potential range for activation. The voltage-dependent activation of hKv1.5 current in the absence and presence of the drug was evaluated by fitting a Boltzmann equation to the amplitude of tail current elicited on return to –40 mV following depolarizing voltage steps to various test potentials (Figure 1D). In a total of seven cells, $V_{1/2}$ averaged –13.4 ± 1.1 mV in control and –22.6 ± 2.0 mV ($P < 0.01$) in the presence of LY294002, while *k* was 10.3 ± 0.6 mV in control and 6.2 ± 1.1 mV ($P < 0.05$) in the presence of the compound. Thus, LY294002 significantly shifted the voltage dependence of channel opening to more hyperpolarized potentials, as reported for the action of two other drugs (mibefradil, Perchenet and Clement-Chomienne, 2000; papaverine, Choe *et al.*, 2003) on hKv1.5 channels.

To quantify the voltage dependence of current inhibition, the relative amplitude of late currents in the presence and absence of LY294002 (% Control) was measured at each test potential and plotted, together with the activation curve obtained in control conditions, (Figure 1E). The hKv1.5 channel activated with a threshold potential of –40 mV and the channel conductance peaked near +20 mV. The current reduction steeply increased at potentials between –30 and 0 mV, which corresponded to the voltage range of channel opening. At potentials positive to +20 mV where channel conductance was nearly saturated, current reduction exhibited a shallower voltage dependence. This observation is consistent with the premise that LY294002 preferentially affected the open state of the hKv1.5 channels.

Concentration-dependent inhibition of hKv1.5 induced by LY294002

The inhibitory effect of LY294002 on hKv1.5 was examined at various concentrations between 1 and 50 μmol·L^{-1}. The hKv1.5 current was elicited every 15 s by 300 ms depolarizing step to +30 mV, before (Control) and during exposure to increasing concentrations of LY294002 in a cumulative manner after the inhibition due to the previous concentration reached a steady state (Figure 2A). It is evident that at any given concentration of LY294002, the late current level at the end of the 300 ms clamp steps was more potently reduced than initial peak current level, which supports a gradual development of channel inhibition during the open state of the channel. Figure 2B illustrates the concentration–

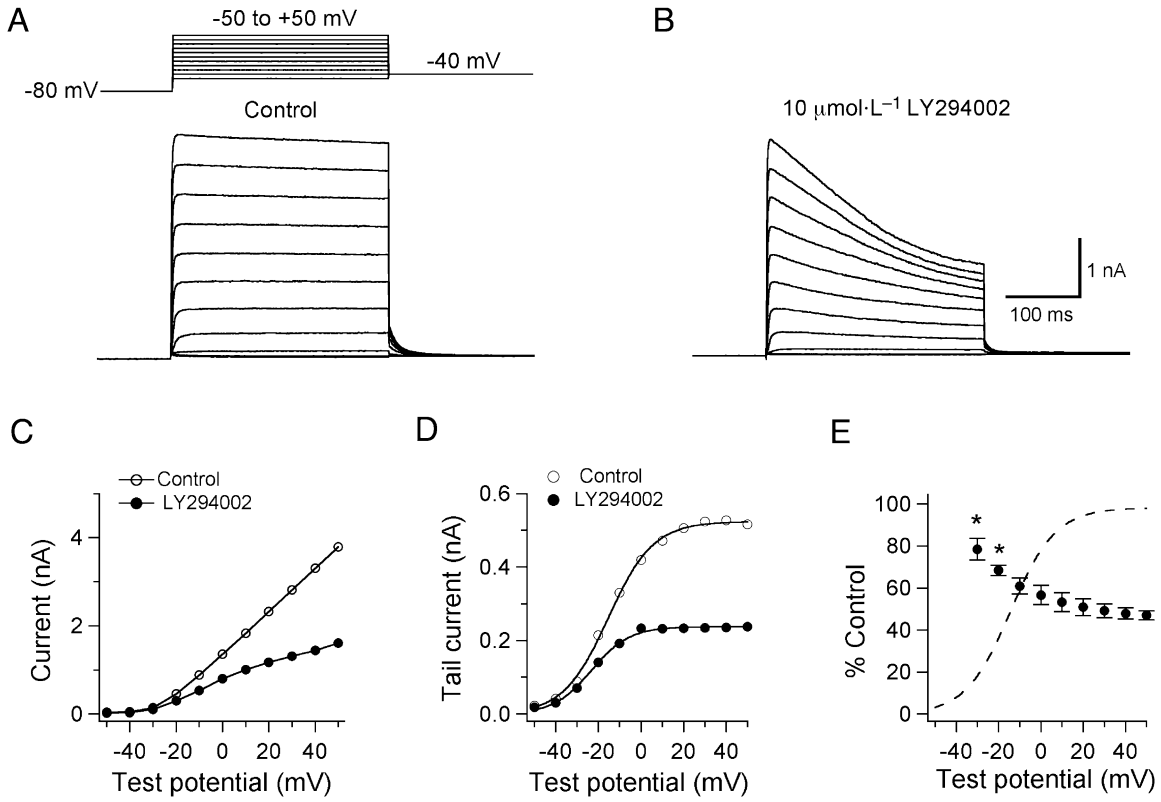

Figure 1 Inhibitory action of LY294002 on human Kv1.5 channels heterologously expressed in a Chinese hamster ovary cell. (A and B) Superimposed human Kv1.5 channel currents elicited during 300 ms depolarizing steps given from a holding potential of –80 mV to potentials between –50 and +50 mV in 10 mV increments, followed by a 200 ms repolarization step to –40 mV before (A, Control) and 3 min after exposure to 10 μmol·L^{-1} LY294002 (B). (C) *I–V* relationships for late currents measured at the end of test steps in control and during exposure to 10 μmol·L^{-1} LY294002. The solid lines were drawn by eye. (D) *I–V* relationships for tail currents in control and during exposure to 10 μmol·L^{-1} LY294002. The smooth curves through the data points represent the least-squares fit to a Boltzmann equation, and the derived $V_{1/2}$ was –14.2 mV in control and –23.2 mV in the presence of LY294002, while *k* was 10.6 mV in control and 8.9 mV in the presence of the compound. The data shown in panels C and D were obtained from the records in panels A and B. (E) Late current amplitude in the presence of LY294002 is plotted as a percentage of control amplitude in the absence of the compound (% Control, filled circles). Data points represent mean ± s.e.mean of six different cells. The dashed curve represents the activation curve obtained in control conditions (see Figure 1D). **P* < 0.05 compared with the values at +50 mV.

response relationship for the inhibition of hKv1.5 current by LY294002, measured at the end of the depolarizing step to +30 mV in seven different cells. The mean data were reasonably well fitted with a Hill equation with an IC_{50} of 7.9 ± 0.5 μmol·L^{-1} and n_H of 1.3 ± 0.2 ($n = 7$).

LY294002 also caused a concentration-dependent acceleration of hKv1.5 current decay during the depolarizing step (to +30 mV). The time constant (τ_D) of block development was measured by fitting a single exponential function to the current trace in the presence of each concentration of LY294002. However, current decay in the presence of lower concentrations (1 and 5 μmol·L^{-1}) of LY294002 was too small to obtain meaningful fits. We therefore omitted the time constant values obtained at low concentrations (1 and 5 μmol·L^{-1}). In Figure 2C, τ_D (measured at +30 mV) was plotted against concentration of LY294002, and data points were fitted to a hyperbolic equation ($\tau_D = 1/(k_{+1}[D] + k_{-1})$, yielding apparent binding (k_{+1}) and unbinding (k_{-1}) rate constants that averaged 1.6 μmol·L^{-1-1}·s^{-1} and 5.7 s^{-1} respectively. The theoretical K_D value derived by k_{-1}/k_{+1} was 3.6 μmol·L^{-1}, which is reasonably close to the value for IC_{50} (7.9 μmol·L^{-1}) derived from a Hill fit of the concentration–response relationship (Figure 2B). This correlation between K_D and IC_{50} may support the hypothesis that LY294002 binds preferentially to hKv1.5 channels in the open state.

Reversible inhibition of hKv1.5 by LY294002

The reversibility of the effect of LY294002 on hKv1.5 current was examined by applying the drug over several consecutive cycles. Figure 3 shows a representative time course of changes in amplitude of hKv1.5 current measured during 300 ms depolarizing step to +30 mV when 10 μmol·L^{-1} LY294002 was applied three times. The hKv1.5 current started to be inhibited within approximately 10 s by each exposure to LY294002, and the steady-state effect (approximately 70% inhibition) was attained about 60 s after the application. The inhibitory effect of LY294002 on hKv1.5 current was fully reversed within approximately 40 s following superfusion with drug-free bath solution. LY294002 was thus found to be a rapidly reversible blocker of the hKv1.5 channel.

We also examined the effect of LY294002 on the deactivation kinetics of the hKv1.5 current. Deactivation time constants, measured by fitting a single exponential function to

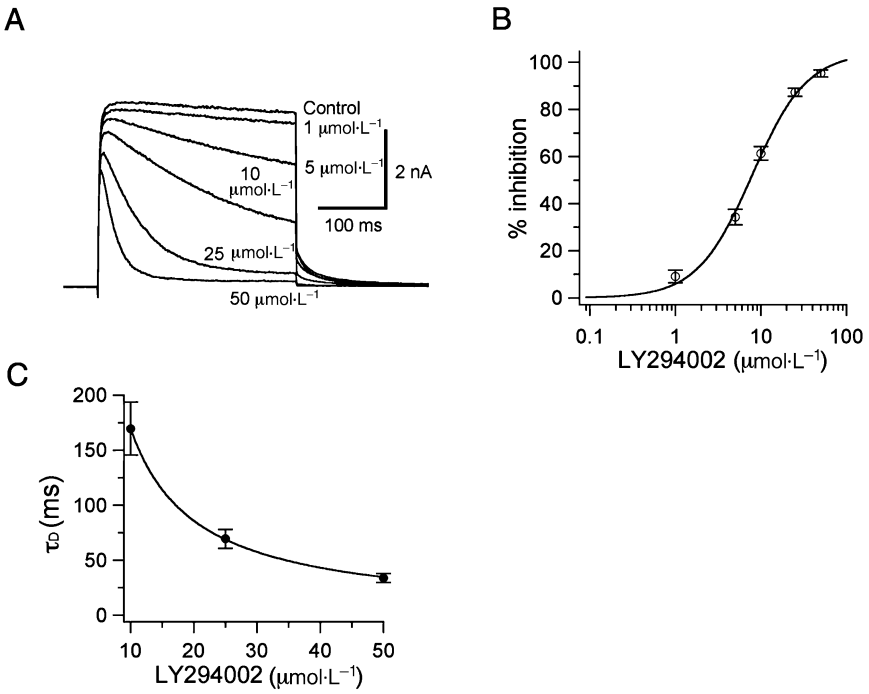

Figure 2 Concentration-dependent block of human Kv1.5 (hKv1.5) current by LY294002. (A) Superimposed hKv1.5 current traces evoked by 300 ms depolarizing step from a holding potential of –80 to +30 mV, followed by a 200 ms repolarization step to –40 mV before (Control) and during exposure to various concentrations (1, 5, 10, 25 and 50 μmol·L^{-1}) of LY294002. (B) Concentration–response relationship for the inhibition of hKv1.5 current by LY294002. Percentage inhibition (% inhibition) represents the fraction of hKv1.5 current reduced by each concentration of LY294002 with reference to the control amplitude, measured at the end of 300 ms depolarizing step to +30 mV. The smooth curve through the data points (mean ± s.e.mean of seven different cells) represents a least-squares fit of a Hill equation, yielding an IC_{50} of 7.9 ± 0.5 μmol·L^{-1} and a Hill coefficient of 1.3 ± 0.2 ($n = 7$). (C) Kinetics of LY294002 block of hKv1.5 current. LY294002-induced time constant at +30 mV was plotted as a function of the drug concentration (10, 25 and 50 μmol·L^{-1}). Note that control hKv1.5 current exhibited no appreciable decay during 300 ms depolarization to +30 mV. The solid line represents the fit of the data to the hyperbolic function (see text).

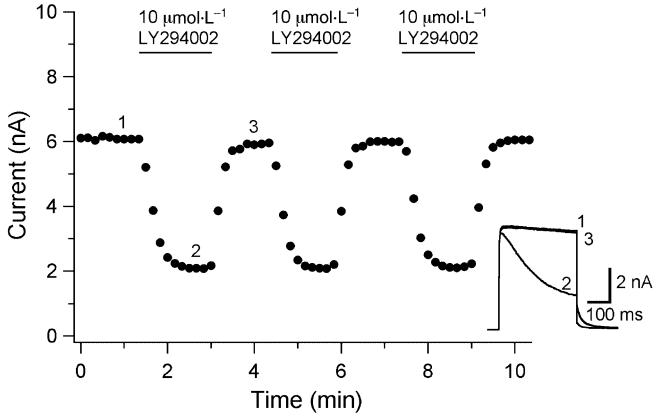

Figure 3 Reversible inhibition of human Kv1.5 (hKv1.5) current by LY294002. The hKv1.5 current was repetitively activated every 10 s by 300 ms depolarizing step to +30 mV from a holding potential of –80 mV, followed by a 200 ms repolarization step to –40 mV. The amplitude of hKv1.5 current measured at the end of the depolarizing step was plotted as a function of time for the entire experiment. LY294002 at a concentration of 10 μmol·L^{-1} was added to the bath during the period indicated by the horizontal bar. The inset illustrates the original current traces recorded at time points indicated by numerals on the graph.

the outward tail current at –40 mV (data not shown, see current traces in the inset of Figure 3) before and during exposure to LY294002, respectively, averaged 11.7 ± 0.82 and 10.7 ± 0.75 ms ($n = 7$, $P > 0.05$), indicating that the deactivation process was not appreciably influenced by this compound.

Frequency-dependent inhibition of hKv1.5 by LY294002

To evaluate whether LY294002 displayed any frequency-dependent effects on hKv1.5 current, trains of 20 depolarizing steps of 300 ms duration from –80 to +50 mV were applied at two different frequencies, 1 and 2 Hz, in the absence and presence of LY294002. The cell was equilibrated in LY294002 for 3 min at –80 mV to keep the channels in the closed state, so that the first sweep represents the first channel opening in the presence of the compound. For the comparison, the control cell was also held at –80 mV for 3 min before applying test pulses. Figure 4A shows superimposed current traces of hKv1.5 recorded during application of such pulse train at a frequency of 2 Hz in the absence (Control) and presence of 10 μmol·L^{-1} LY294002. Under control conditions, the peak current amplitude at the twentieth depolarizing step was reduced by 17.1 ± 1.4% ($n = 7$) at 1 Hz and 33.9 ± 1.6% ($n = 7$) at 2 Hz, compared with the first pulse (Figure 4B). Such a decline may reflect the accumulation of the slow inactiva-

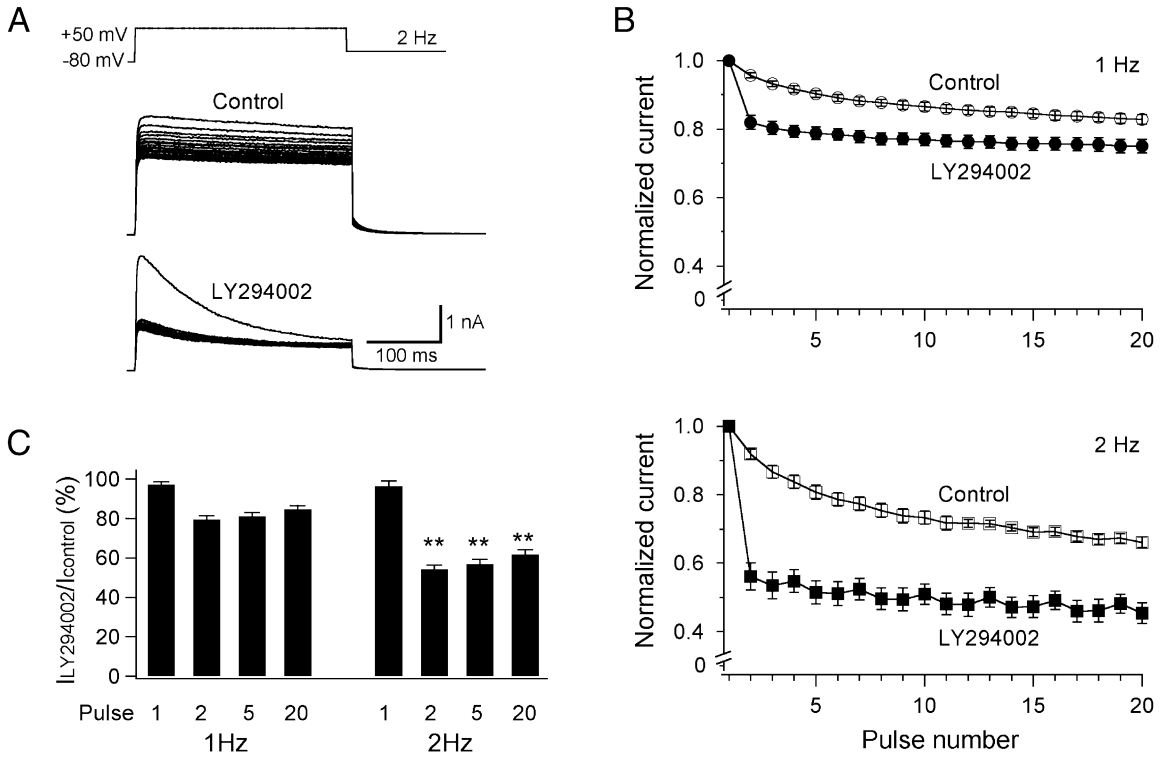

Figure 4 Frequency-dependent inhibition of human Kv1.5 current evoked by LY294002. Human Kv1.5 channels were subjected to 20 repetitive 300 ms depolarizing steps to +50 mV, from a holding potential of −80 mV at frequencies of 1 and 2 Hz, under control conditions and in the presence of 10 μmol·L^{-1} LY294002. (A) Original current traces recorded by application of a train of depolarizing steps at a frequency of 2 Hz under control conditions (upper panel) and after 3 min exposure to 10 μmol·L^{-1} LY294002 (lower panel). (B) Peak amplitudes of outward current at every pulse were normalized with reference to the peak amplitude of current obtained at the first pulse and then plotted against the pulse numbers. Data points represent mean ± s.e.mean ($n = 7$ for each frequency). (C) Normalized current amplitudes in the presence and absence of the drug measured for pulse numbers 1, 2, 5 and 20 at the two frequencies. $^{**}P < 0.01$ compared with that at 1 Hz.

tion of hKv1.5 channels. In the presence of 10 μmol·L^{-1} LY294002, the peak current amplitude at the first depolarizing step was not significantly modified (Figure 4A; control: 4.5 ± 0.8 nA; LY294002: 4.4 ± 0.7 nA, $n = 7$, $P = 0.19$), indicating the absence of tonic block. However, the peak amplitude of hKv1.5 current thereafter progressively decreased by 24.1 ± 1.8% ($n = 7$) and 54.6 ± 3.0% ($n = 7$) at twentieth depolarizing steps at 1 and 2 Hz respectively (Figure 4B). To determine the frequency-dependent effect of LY294002 without contamination of current decline observed under control conditions, the normalized current amplitudes ($I_{LY294002}/I_{control}$) were measured for pulse numbers 1, 2, 5 and 20 at the two frequencies (Figure 4C). These normalized current amplitudes were significantly less at 2 Hz (right hand graph) than the corresponding values at 1 Hz (left hand graph) for pulse numbers 2, 5 and 20. Thus, the degree of inhibition of hKv1.5 increased as the pulse frequency increased, showing that LY294002 blocks hKv1.5 channels in a frequency-dependent manner.

Effects of wortmannin and LY303511 on hKv1.5

PI3K is an important intracellular signalling enzyme that catalyses phosphorylation of phosphatidylinositols and affects a wide range of cellular functions, including growth, migration and survival (Knight *et al.*, 2004). It has also been shown that PI3K is involved in the up-regulation of Kv1 channels by insulin-like growth factor-1 (IGF-1) in HEK (human embryonic kidney) 293 cells (Gamper *et al.*, 2002). We tested whether the inhibition of hKv1.5 current by LY294002 is mediated through PI3K inhibition, using wortmannin, another pharmacological inhibitor of PI3K with a markedly distinct structure. Figure 5 shows the time-course of changes in amplitude of hKv1.5 current measured at the end of 300 ms depolarizing step to +30 mV during exposure to wortmannin at a concentration of 100 nmol·L^{-1}, more than 50-fold greater than the IC_{50} for inhibition of PI3K (Powis *et al.*, 1994). In a total of five cells, amplitude of hKv1.5 current was only slightly (by 11.2 ± 1.0%) decreased by 5–10 min exposure to 100 nmol·L^{-1} wortmannin. The addition of LY294002 (25 μmol·L^{-1}) caused a marked inhibition of hKv1.5 current that was almost insensitive to wortmannin (100 nmol·L^{-1}). These results indicate that inhibition of PI3K was not primarily involved in the LY294002-induced reduction of hKv1.5 current.

We also checked the effect of LY303511, a structural analogue of LY294002, on hKv1.5 current. LY303511 contains a piperazine ring instead of the morpholine ring of LY294002 (Figure 6B) and has no effect on PI3K activity (Ding *et al.*, 1995; Kristof *et al.*, 2005). As shown in Figure 6A, LY303511 at 25 μmol·L^{-1} produced little effect on hKv1.5 current, which was in contrast with a marked inhibition evoked by subsequent application of LY294002 on the same cell. As judged from the structural differences between LY294002 and

LY303511 (Figure 6B), the morpholino oxygen in LY294002 may play a functional role in inhibiting the hKv1.5 currents.

Inhibitory effects of LY294002 on mutant hKv1.5 channels

Recent studies have found that several amino acid residues located in the pore (outer pore or pore helix) and in the S6 domain of hKv1.5 channels provide critical structural components for blockade of the channel by drugs (Decher *et al.*, 2004; 2006; Herrera *et al.*, 2005; Rezazadeh *et al.*, 2006). To investigate possible binding sites of LY294002 to the hKv1.5 channel, nine residues located in the pore and S6 domain were mutated by site-directed mutagenesis. These mutants are T462C and H463C located in the outer mouth of S5-pore linker, T480A located at the base of the pore helix, R487V located in the outer pore region and A501V, I502A, I508A, L510A and V516A located in the S6 domain. As has been reported (Decher *et al.*, 2006; Rezazadeh *et al.*, 2006), all these mutant channels can produce substantial outward currents during depolarization. Figure 7A demonstrates representative examples for the effect of LY294002 (25 μmol·L^{-1}) on WT and three mutant channels (H463C, R487V and I508A) activated during 300 ms depolarizing step to +30 mV from a holding potential of –80 mV. The kinetic properties of both the H463C and the R487V mutant channels appeared to be qualitatively similar to WT, whereas the I508A mutant channel exhibited slower channel kinetics compared with WT channels. Figure 7B summarizes the inhibitory effect of LY294002 (25 μmol·L^{-1}) on WT and various mutant channels, measured as percentage inhibition of late currents at the end of 300 ms depolarizing step (to +30 mV). Whereas the T462C, H463C and A501V channels were inhibited by LY294002 (25 μmol·L^{-1}) to an extent similar to WT, the inhibitory effect of LY294002 (25 μmol·L^{-1}) was significantly less in the T480A, R487V, I502A, I508A, L510A and V516A mutant channels. We also determined the concentration–response relationships for the inhibition of the R487V mutant channel by LY294002 (Figure 7C), yielding an IC_{50} of 16.5 ± 3.6 μmol·L^{-1} (n = 5) for the R487V mutant and 7.9 ± 0.5 μmol·L^{-1} for WT (n = 7, see also Figure 2B). These results indicate that Thr480 at the base of the pore helix, Arg487 in the outer pore region and Ile502, Ile508, Leu510 and Val516 in the S6 domain are important for the block by LY294002.

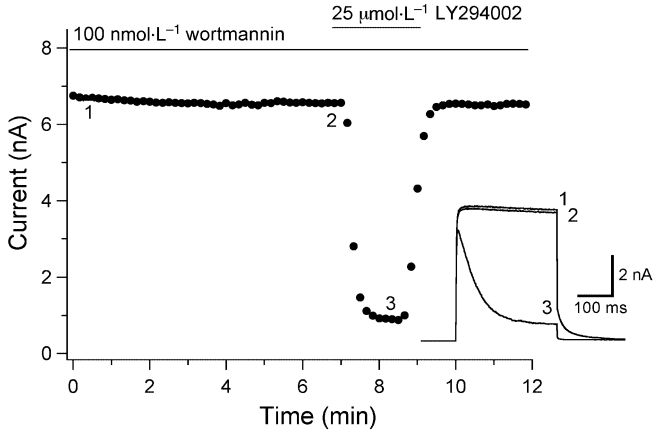

Figure 5 Effect of wortmannin on human Kv1.5 current. The human Kv1.5 current was repetitively (every 10 s) activated with 300 ms depolarizing step to +30 mV from a holding potential of –80 mV, followed by a 200 ms repolarization step to –40 mV and current amplitude measured at the end of depolarizing step was plotted. Inset shows the superimposed original current traces recorded at time points indicated by numerals.

Discussion

The present study demonstrates that the specific PI3K blocker LY294002 potently and reversibly inhibits the hKv1.5 current in a concentration-, time- and frequency-dependent manner. LY294002 has been widely used to examine the physiological and pathophysiological roles of PI3K in the regulation of various cellular functions (Knight *et al.*, 2004). However, the present experiments strongly suggested that the inhibitory

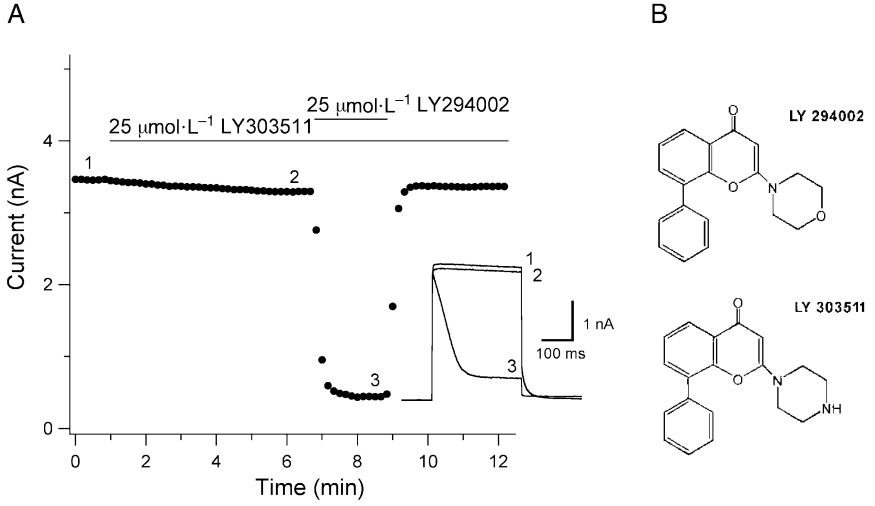

Figure 6 Effects of LY303511 on the human Kv1.5 current. (A) Human Kv1.5 current was activated by a protocol similar to that in Figure 5 in the presence of LY303511 and LY294002 (25 μmol·L^{-1}, each). (B) Chemical structures of LY294002 and LY303511. The morpholino oxygen in LY294002 is substituted by a nitrogen in LY303511, i.e., the morpholino ring becomes a piperazine ring.

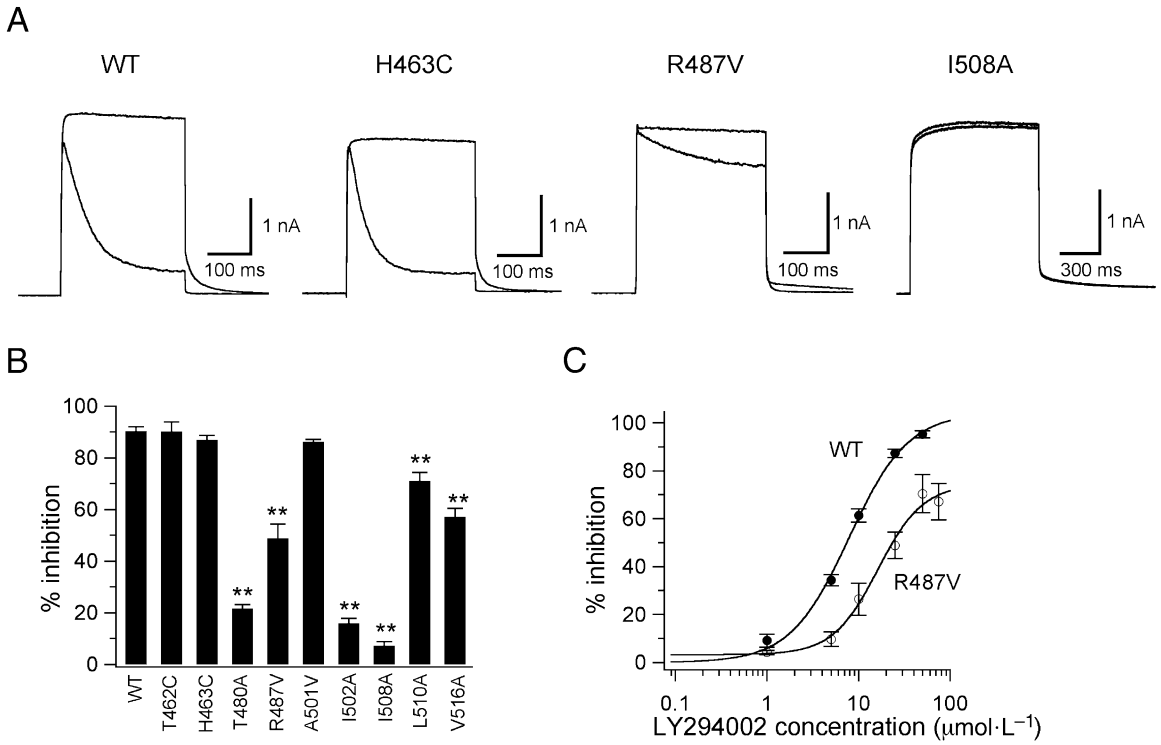

Figure 7 Inhibitory action of LY294002 on human Kv1.5 (hKv1.5) mutant channels. (A) Representative current traces recorded from wild-type (WT) and mutant channels (H463C and R487V) during 300 ms depolarizing step to +30 mV from a holding potential of –80 mV, followed by a 200 ms repolarization step to –40 mV before and during exposure to 25 μmol·L^{-1} LY294002. For I508A channels, because of slow kinetics, membrane potential was depolarized to +30 mV for 1 s, followed by a 1 s repolarization step to –40 mV. (B) Percentage inhibition of WT and various mutants of hKv1.5 channels by LY294002 (25 μmol·L^{-1}) was measured at the end of 300 ms depolarizing pulse. Numbers of cells are 6–16 in each channel. $^{**}P < 0.01$ compared with WT hKv1.5 channels. (C) Concentration – response relationship for LY294002 block of WT (●) and R487V (○) channels. The solid lines represent a least-squares fit of a Hill equation.

effect of LY294002 on hKv1.5 current was independent of PI3K signalling pathways for the following reasons. The block of hKv1.5 current takes place rapidly, within 10 s after addition of the compound, and reaches a steady-state inhibition within 1 min. This inhibitory effect was fully reversed in 1 min following removal of the compound (Figure 3). However, PI3K activity was completely abolished in 10 min by LY294002 (Vlahos *et al.*, 1994). Further, a structurally unrelated PI3K inhibitor, wortmannin, produced only a minimal effect on the hKv1.5 current (Figure 5).

Previous workers have shown that the 4′-oxygen heteroatom in the morpholine ring of LY294002 plays a critical role in determining its biological activities. Substitution at this position, with sulphur, hydroxymethyl, methylene or nitrogen, of the morpholine ring of LY294002, caused a marked reduction in the efficacy against PI3K activity (Vlahos *et al.*, 1994). Our present study clearly demonstrated that LY303511, which contains a 4′-nitrogen instead of the 4′-oxygen heteroatom in the morpholine ring of LY294002, i.e., a piperazine ring instead of the morpholine ring (see Figure 6B), had little inhibitory effect on the hKv1.5 current. It is therefore reasonable to assume that the 4′-heteroatom in the morpholine ring is also critical for determining the inhibitory action of this compound on the hKv1.5 current as well as on PI3K activity.

The action of LY294002 on the hKv1.5 currents can be best explained by block of the open channel, which is supported by the following experimental results. Firstly, LY294002 accelerated the hKv1.5 current decay during the depolarizing step in a concentration-dependent manner (Figure 2A,C). Next, LY294002 inhibition of hKv1.5 steeply increased at potentials between –30 and 0 mV, which corresponds to the voltage range of channel opening (Figure 1E). Then, LY294002 scarcely affected the activation time course of the channel and only modestly affected the peak amplitude at the onset of depolarization (Figs 1 and 2), suggesting that LY294002 exerted little, if any, effect on the channels in the closed state. These effects are similar to other drugs assumed to act as open-channel blockers of Kv1.5 channels, such as quinine, clofilium and tetrapentylammonium (Snyders and Yeola, 1995), zatebradine (Valenzuela *et al.*, 1996), bisindolylmaleimide (Choi *et al.*, 2000) and mibefradil (Perchenet and Clement-Chomienne, 2000). It should be noted, however, that LY294002 does not appreciably slow down the deactivation time course and fails to cause a 'crossover phenomenon' of tail current traces, which is in contrast with the properties of other open-channel blockers of Kv1.5 channels (Snyders and Yeola, 1995; Valenzuela *et al.*, 1996; Franqueza *et al.*, 1998; Choi *et al.*, 2000; Perchenet and Clement-Chomienne, 2000; Decher *et al.*, 2006). If the compound dissociates from the channel quickly upon repolarization and thereby does not interfere the time course for channel closure, nearly full amplitude of tail current (comparable to control conditions) is expected to appear. However, it is not the case for LY294002 (see

Figure 2A). Another possible explanation is that the functional dissociation of LY294002 from the channel that occurs upon repolarization may not interfere with the transition of the channel from an open to a closed state.

Previous studies have examined the molecular basis for high-affinity drug block of hKv1.5 channels (Yeola *et al.*, 1996; Decher *et al.*, 2004; 2006; Herrera *et al.*, 2005; Rezazadeh *et al.*, 2006). The anti-arrhythmic quinidine and the local anaesthetic benzocaine have been found to interact with Thr479 located near the pore helix and Thr507 and Val514 in the S6 domain of hKv1.5 channels (Snyders and Yeola, 1995; Yeola *et al.*, 1996; Caballero *et al.*, 2002). In recent years, Decher *et al.* (2004; 2006) and Eldstrom *et al.* (2007) have identified several amino acid residues (Thr479, Thr480, Val505, Ile508, Val512 and Val516) that could be essential for blocking action of S0100176 and AVE0118, using alanine scanning mutagenesis of the pore helix and S6 domain of the hKv1.5 channel. The present mutagenesis study has also found Thr480, Arg487, Ile502, Ile508, Leu510 and Val516 to be putative binding sites of LY294002. Of these six residues, Thr480, Ile508 and Val516 are believed to face towards the central cavity of the channel, whereas Ile502 and Leu510 are positioned away from the inner cavity (Decher *et al.*, 2004; Eldstrom *et al.*, 2007). It is therefore reasonable to propose that the reduced sensitivity of I502A and L510A mutants to LY294002 may be due to allosteric mechanisms, which alters the orientation of some amino acids towards the inner cavity of the channel.

A docking model has been used to compare the binding sites of novel atrial-selective, class III anti-arrhythmic compounds, such as S9947, MSD-D and ICAGEN-4 (Strutz-Seebohm *et al.*, 2007). This study suggests that hydrophobic interactions of the blocker molecules with Ile508 and Val512, as well as electrostatic interactions of the oxygen atoms of the inhibitor with the potassium ion in the selective filter Thr480 are important for the blocking action of these compounds. This binding complex formed by channel residues (selective filter), internal potassium ion and inhibitor oxygen was also proposed to be important for the binding of chromanol 293B to KCNQ1 (Kv7.1) channels (Lerche *et al.*, 2007). More recently, Andér *et al.* (2008) provided an improved model useful for further efforts to design ligands. These authors investigated binding of ortho,ortho-disubstituted bisaryl compounds to the open state of the hKv1.5 channels using a three-step procedure, including homology modelling, automated docking and binding free energy calculations, and suggested that, apart from the well-documented important residues Ile508, Val 512 and Val516 for ligand binding in the cavity, other residues, Ala509 and Pro513, also contribute to the non-polar binding interactions. Compared with the mutants I508A and T480A located deeper in the pore region, R487V at the outer mouth of the pore was found to partially attenuate LY294002 action (Figure 7 in the present study). We therefore propose that, as suggested for Ile502 and Leu510, an allosteric effect by the R487V mutation could be a possible explanation for the decreased potency of LY294002 on hKv1.5 channels.

Although the substitution of Arg (positively charged) by Val (neutral) at position 487 significantly reduced the inhibitory action of LY294002 (R487V, Figure 7B), we may exclude the possibility that this reduced sensitivity of the R487 mutant is related to some possible changes in electrostatic interaction between the residue and the compound, because the substitution of His (positively charged) by Cys (neutral) at position 463 (H463C) does not affect the sensitivity to LY294002 (Figure 7B). In addition, the substitution of neutral Ile by neutral Ala at positions 502 and 508 (I502A and I508A respectively) does markedly affect the LY294002 sensitivity. We also believe that the hydrophobicity and hydrophilicity of residues tested in the present study do not profoundly affect the sensitivity to inhibition by LY294002. For example, the replacement of His with Cys (hydropathy index changed from –3.2 to 2.5) at position 463 (Kyte and Doolittle, 1982) did not alter the sensitivity to LY294002, whereas the replacement of Arg with Val (hydropathy index changed from –4.5 to 4.2) at position 487 markedly reduced the sensitivity to LY294002 (Figure 7B).

Several hKv1.5 channel mutants (T480A, R487V and L510A) used for evaluating the blocking action have been shown to exhibit altered gating kinetics (Decher *et al.*, 2004; 2006; Rezazadeh *et al.*, 2006). However, there appears to be little, if any, clear evidence correlating the reduced sensitivity of these mutants to blockade, with possible kinetic changes. For example, T480A and V514A mutants exhibit similar slower changes in activation and deactivation rates (Decher *et al.*, 2005), but the responses to AVE0118 are very different between these two mutants (Decher *et al.*, 2006). In addition, the L510A mutant channel expressed in oocytes exhibits a pronounced inactivation, which is only slightly suppressed by AVE0118 (Decher *et al.*, 2006). In contrast, another novel channel blocker, S0100176, almost completely inhibits this mutant channel, as it does the WT channels (Decher *et al.*, 2004). These data suggest that the reduced affinity of LY294002 to hKv1.5 channel mutants is likely to be due to their changed binding sites, but probably not due to altered channel gating kinetics.

In the present study, we cannot completely rule out the possibility that inhibition of hKv1.5 current by LY294002 may be mediated, at least partly, through the inhibition of unidentified intracellular signalling pathways. However, an earlier study has confirmed that LY294002, which is a competitive inhibitor for the ATP binding site of PI3K, had no inhibitory effects on several other ATP-requiring, protein and lipid kinases, such as protein kinase A, protein kinase C, mitogen-activated protein (MAP) kinase, phosphatidylinositol 4-kinase and diacylglycerol kinase, when used at a concentration of 50 $\mu mol \cdot L^{-1}$ (Vlahos *et al.*, 1994). It is therefore reasonable to rule out the possible involvement of at least these ATP-requiring kinases in the inhibitory action of LY294002 on the hKv1.5 current.

LY303511 is an analogue of LY294002 that has no apparent effect on PI3K activity and thus provides a suitable control for evaluating cellular effects, unrelated to PI3K inhibition (Ding *et al.*, 1995). LY303511 has a marked inhibitory effect on cell proliferation and this effect is almost equal to that of LY294002 (Kristof *et al.*, 2005). It therefore seems likely that the substitution of an oxygen by nitrogen in LY303511 has little effects on its membrane permeability. In additional experiments, we have confirmed that the hKv1.5 current is not appreciably decreased, even by prolonging the exposure

time to 25 μmol·L^{-1} LY303511 to more than 10 min (data not shown). The present study clearly demonstrates that LY303511 has little effect on hKv1.5 current (Figure 6), which provides some structural basis to the suggestion that the morpholino oxygen is crucially involved in producing the inhibitory action of LY294002 on hKv1.5 currents. It is interesting to note that endogeneous Kv channels in MIN6 insulinoma cells and Kv2.1 channels heterologously expressed in tsA201 cells are substantially inhibited not only by LY294002, but also by LY303511 (El-Kholy *et al*., 2003). Such a difference in the effect of LY303511 on hKv1.5 and other Kv channels may also support some specific functional role of the morpholino oxygen for the inhibition of hKv1.5 channels. The present study may thus provide some important hints for future development of effective and specific blockers for hKv1.5 channels.

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Conflicts of interest

None.

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