Specific block of cloned *Herg* channels by clofilium and its tertiary analog LY97241

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Abstract The class III antiarrhythmic drug clofilium is known to block diverse delayed rectifier K^+ channels at micromolar concentrations. In the present study we investigated the potency of clofilium and its tertiary analog LY97241 to inhibit K^+ channels, encoded by the human *ether-a-go-go* related gene (HERG). Clofilium blocked HERG channels in a voltage-dependent fashion with an IC_{50} of 250 nM and 150 nM at 0 and +40 mV, respectively. LY97241 was almost 10-fold more potent (IC_{50} of 19 nM at +40 mV). Other cloned K^+ channels which are also expressed in cardiac tissue, Kv1.1, Kv1.2, Kv1.4, Kv1.5, Kv4.2, Kir2.1, or I_{Ks}, were not affected by 100-fold higher concentrations. Block of HERG channels by LY97241 was voltage dependent and the rate of HERG inactivation was increased by LY97241. A rise of $[K^+]_0$ decreased both, rate of HERG inactivation and LY97241 affinity. The HERG S631A and S620T mutant channels which have a strongly reduced degree of inactivation were 7-fold and 33-fold less sensitive to LY97241 blockade, indicating that LY97241 binding is affected by HERG channel inactivation. In summary, the antiarrhythmic action of clofilium and its analog LY97241 appears to be caused by their potent, but distinct ability for blocking HERG channels.

Key words: Clofilium; HERG; K^+ channel; Torsades de pointes; Arrhythmia

1. Introduction

The quarternary ammonium derivative clofilium is well known for its antiarrhythmic efficacy [1]. However, clofilium does not appear to be a blocker of specific channels but has been demonstrated to block a variety of cation channels at micromolar concentrations. In guinea pig cardiac myocytes clofilium was demonstrated to block the voltage-activated K^+ current which is composed of the slowly and more rapidly activating conductances I_{Ks} and I_{Kr}, respectively, at concentrations as high as 100 μM [2]. However, it was not analyzed whether clofilium blocks both components with a similar potency. Interestingly, in the same study LY97241, a tertiary analog of clofilium, was shown to block inward rectifier K^+ currents in contrast to clofilium. For cloned cardiac K^+ channels expressed in *Xenopus* oocytes such as Kv1.2, Kv1.4, Kv1.5, and I_{Ks} channels clofilium was shown to have distinct IC_{50} values ranging from 1 to 100 μM [3–6]. The aim of the present study was 1) to test HERG channels for their sensitivity for the antiarrhythmic clofilium and 2) to compare its effects with its tertiary analog LY97241.

2. Materials and methods

Handling and injection of *Xenopus* oocytes and synthesis of cRNA has been described previously in detail [7]. The two-microelectrode voltage-clamp configuration was used to record currents from *Xenopus laevis* oocytes. In several sets of experiments, oocytes were individually injected with cRNA encoding for the K^+ channels HERG [8], HERG S631A [9], human I_{sK} [10], rat Kv1.1 [11], rat Kv1.2 [12], rat Kv1.4 [13], human Kv1.5 [14], rat Kv4.2 [15] or rat Kir2.1 [16]. The mutation S620T was introduced into HERG by PCR mutagenesis. The mutagenic oligonucleotide covered the unique *Bgl*II restriction site; the second oligonucleotide located upstream of a unique *Bst*EII site. The PCR product was cleaved with these two enzymes and subcloned into the HERG wild type plasmid. The sequence of the mutagenized fragment was confirmed by DNA sequencing. To increase the expression level of rat Kv4.2 we subcloned the gene into the expression vector pGEMHE [17]. The 2 kb fragment from *Bst*EII (endfilled) to *Eco*RI was introduced into the vector cleaved with *Sma*I and *Eco*RI.

Recordings were performed at 22°C using a Geneclamp amplifier (Axon Instruments, Foster City, USA) with MacLab D/A converter and software for data acquisition and analysis (ADInstruments, Castle Hill, Australia). Outward currents through HERG channels were in general evoked with 0.5-s depolarizing pulses from a holding potential of −80 mV to +40 mV at 3-s intervals (low-pass filtered at 0.5 kHz). Tail currents were analyzed at −85 mV. HERG-mediated currents were measured as the peak amplitude of the tail currents. Outward currents through Kv1.1 and Kv1.4 channels were evoked with 0.5-s and 0.3-s depolarizing pulses, respectively, to 0 mV every 3 s. K^+ outward currents through Kv1.2 and Kv1.5 were evoked every 3 s with 0.3-s depolarizing pulses to 0 mV and +40 mV, respectively; tail currents were analyzed at −30 or −80 mV. The holding potential for all Kv channels was −80 mV and the currents were low-pass filtered at 1 kHz. I_{Ks} was evoked with 15-s voltage steps to −10 mV from a holding potential of −80 mV (filtered at 10 Hz). Kir2.1 currents were evoked with 0.5-s hyperpolarizing pulses to −120 mV from a holding potential of −40 mV (filtered at 1 kHz). The amplitudes of the recorded currents were measured at the end of the test voltage steps; currents through inactivating Kv1.4 channels were measured at their maximum.

The control solution (ND96) contained (mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5 (titrated with NaOH to pH 7.4). In some experiments the concentration of K^+ was 0 mM (0 K) or 10 mM (10 K). In the 0 K solution no K^+ was added, but additional 2 mM NaCl. Because of possible K^+ leak from the oocytes and an incomplete K^+ wash-out during the experiments we cannot exclude the local K^+ concentration at the K^+ channel pore to be up to 100 μM. 10 K (ND88) contained 88 mM NaCl, 8 mM KCl and the solution was titrated with KOH to pH 7.4. The microelectrodes were filled with 3 M KCl solution and had resistances between 0.5 to 0.9 MΩ. Clofilium and LY97241 were a generous gift from Eli Lilly, Indiana, USA. They were dissolved in DMSO as stock solutions and added to the control solutions before the experiments (maximal DMSO concentration < 0.1%). Data are presented as means with standard errors

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(S.E.M.) where n represents the number of experiments performed. Concentration-blockade relationships were calculated with the Hill equation: inhibition = 100*([inhibitor]^n/([inhibitor]+IC_{50}^n)), where n is the Hill coefficient. Student's t-test was used to test for statistical significance which was obtained for $p < 0.05$. In one batch of oocytes we detected HERG channels which were resistent to LY97241 block. These cells (about 20%) are indicative of a cellular regulation which was not studied in detail; these weakly responding cells were not considered for the statistics.

3. Results

Expression of HERG channels in *Xenopus* oocytes induced K^+ channels with characteristic properties, i.e. fast inactivation at positive potentials and large, slowly decaying tail currents upon repolarization [18]. The class III antiarrhythmic clofilium inhibited HERG channels in a concentration-dependent manner with an IC_{50} of 150 ± 9 nM ($n = 5$) at +40 mV (Fig. 1A, C). In addition, HERG channel block by clofilium was voltage dependent; at 0 mV an IC_{50} of 252 ± 62 nM was extrapolated ($n = 5$). A similar IC_{50} for clofilium has been reported for Kv1.5 channels, while other cardiac K^+ channels such as I_{Ks} channels appear to have a more than 100-fold lower affinity [5,6,19]. The clofilium block of HERG channels was almost irreversible; after a washout period of 2 hours approximately 40% of the control current could be recovered. This possibly indicates a channel block at an intracellular site.

LY97241 is an analog of clofilium with two chemical modifications, a p-substitution of the Cl^- residue by N_2O and the removal of the ethyl group at the amine function. Whereas the Cl^- substitution by N_2O is not expected to change the behaviour of the molecule, the removal of the ethyl residue is substantial, as it changes the quarternary, permanently positively charged clofilium into a tertiary amine. This tertiary analog of clofilium, LY97241, was 10-fold more potent (Fig. 1B, C) in inhibiting HERG channels. After depolarizations to +40 mV HERG tail currents were half-maximally blocked at a concentration of 19.0 ± 1.3 nM ($n = 5$), after depolarizations to 0 mV an IC_{50} of 39.3 ± 1.0 nM ($n = 5$) was determined. To test for the channel specificity of LY97241, Kv1.1, Kv1.2, Kv1.4, Kv1.5, Kv4.2, Kir2.1, and I_{Ks} channels, whose characteristics have been described elsewhere [15,20], were expressed in *Xenopus* oocytes. At 3 μM (100-fold higher concentrations than the IC_{50} for HERG channels) LY97241 inhibited all of these channels by less than 5% (data not shown).

We could recently describe a voltage-dependent HERG channel blockade by the histamine receptor antagonist astemizole and terfenadine [21] and the antipsychotic haloperidol [20]. Blockade of HERG channels by these compounds affected the rate of HERG inactivation. Similarly, LY97241 affected HERG inactivation in the present study (Fig. 2). At −15 mV, HERG channel inactivation could be fitted by a single exponential function with an inactivation time constant (τ_{inact}) of 14.5 ± 1.8 ms ($n = 5$). In the presence of 20 nM LY97241 τ_{inact} was decreased to 9.5 ± 0.6 ms ($n = 5$).

HERG channels have been described to be modulated by the extracellular K^+ concentration ($[K^+]_0$) [18]. Here we analyzed the effects of K^+_0 on HERG inactivation and LY97241 sensitivity. An increase in $[K^+]_0$ significantly decreased the rate of HERG inactivation (Fig. 3A). With nominally 0 mM, 2 mM, and 10 mM $[K^+]_0$ at −15 mV the inactivation time constants were 8.0 ± 0.4 ms, 14.4 ± 0.7 ms, and 19.5 ± 0.8 ms ($n = 7$), respectively. Steady-state inactivation was decreased from 90.1 ± 0.3% at 2 mM to 83.1 ± 1.4% ($n = 7$) at 10 mM $[K^+]_0$. As shown in Fig. 3B and C, an increase in $[K^+]_0$ to 10 mM also decreased the LY97241 sensitivity of HERG channels. The IC_{50} for LY97241 was more than threefold increased (66.8 ± 4.3 nM; $n = 5$) at 10 mM $[K^+]_0$.

The mutation S631A in the HERG channel pore region has previously been shown to decrease HERG inactivation [9]. This mutant also displays a reduced affinity to the histamine receptor antagonists astemizole and terfenadine (unpublished data) and the antipsychotic haloperidol [20]. Similarly to these drugs, the affinity of LY97241 was reduced 7-fold by the mutation S631A (Fig. 4B; IC_{50} at 0 mV was 279 ± 9 nM; $n = 5$). The pore mutation S620T which was reported to remove channel inactivation more completely than S631A [22] showed an even lower sensitivity to LY97241 (Fig. 4 A and B; IC_{50} at 0 mV was 1.35 ± 0.30 μM; $n = 6$).

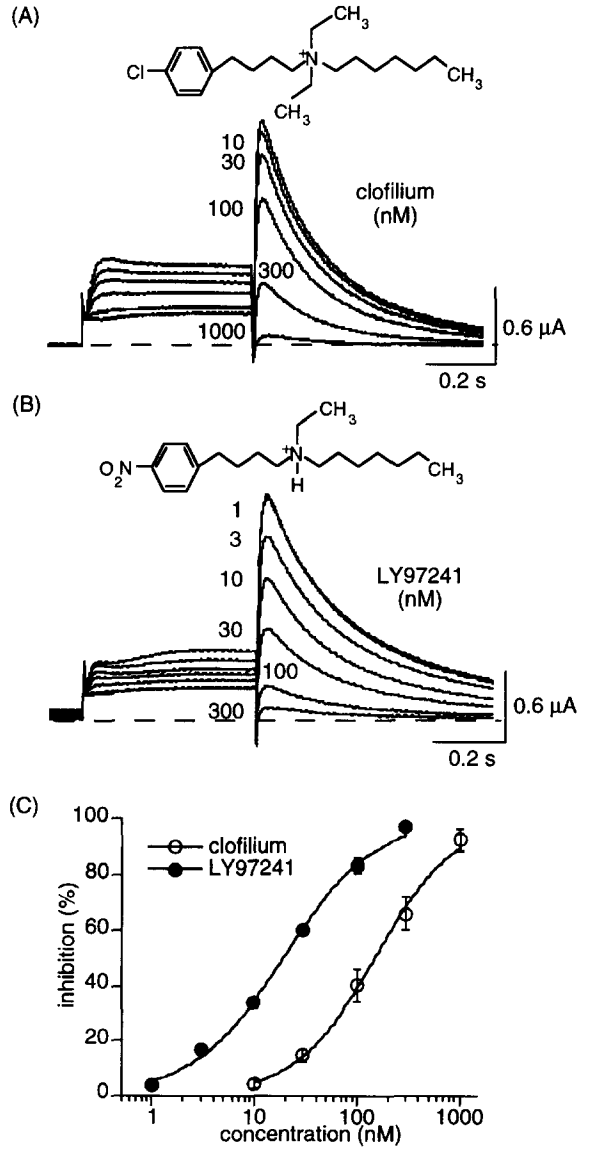

Fig. 1. Blockade of HERG channels by clofilium (A) and LY97241 (B). HERG channels were activated with depolarizations to +40 mV and the tail currents were recorded at −85 mV. The dashed line indicates the baseline. (C) Concentration-inhibition relation of HERG channel block by clofilium and LY97241 at +40 mV. The data represent means ± S.E.M. The continuous curves are data fits according to a Hill equation (see Section 2).

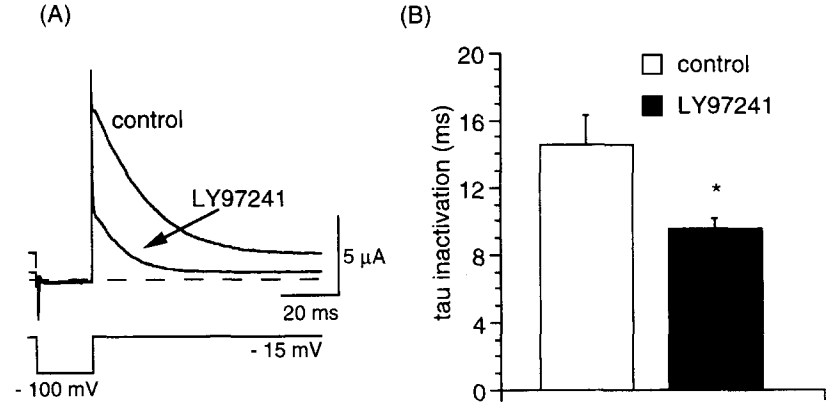

Fig. 2. Effects of LY97241 on HERG inactivation. (A) HERG channels were held at −15 mV where they are partially open but mostly inactivated. A brief repolarization to −100 mV causes a rapid relief of inactivation without significant deactivation. Subsequent depolarization back to −15 mV results in large, rapidly inactivating outward currents. Please note that the steady-state current before and after the repolarization is identical. (B) LY97241 (20 nM) significantly reduces the time constant for HERG channel inactivation. The data represent means ± S.E.M.

4. Discussion

The present paper describes the inhibitory effects of the class III antiarrhythmic clofilium and its analog LY97241 on HERG channels which underlie the cardiac K^+ conductance I_{Kr} [18]. The rather low IC_{50} of 150 nM for clofilium block of HERG channels compared to other cardiac K^+ channels (the IC_{50} for I_{Ks} channels which underlie the cardiac conductance I_{Ks} is close to 100 μM [6]) suggests that HERG channels are the primary targets for the antiarrhythmic action of clofilium.

Our data show a correlation between the sensitivity of HERG channels towards clofilium and LY97241 and the rate of channel inactivation, suggesting that changes in channel configuration during inactivation favour LY97241 binding. The inactivation of HERG channels is voltage dependent; the rate of inactivation increases about 3-fold between 0 and +40 mV [9]. We now observed a 2-fold higher sensitivity of HERG towards LY97241 at +40 mV compared with 0 mV. Consistent with this correlation, an increase of $[K^+]_0$ from 2 to 10 mM decreased both, the rate of channel inactivation (by about 40%) and the inhibitory potency of LY97241 (about 3-fold). Similarly, HERG S631A mutant channels, which display a strongly reduced inactivation, are also less sensitive to LY97241. In addition, LY97241 increases the rate of HERG channel inactivation, similarly as previously reported for the antipsychotic drug haloperidol [20]. Consequently, manipulations affecting HERG channel inactivation also affected their inhibition by LY97241. Although we cannot exclude an open-channel block as an additional component of HERG channel blockade, the present data suggest that conformational changes in the HERG channel protein leading to inactivation support binding of clofilium and LY97241.

A similar dependence of drug binding on the inactivated

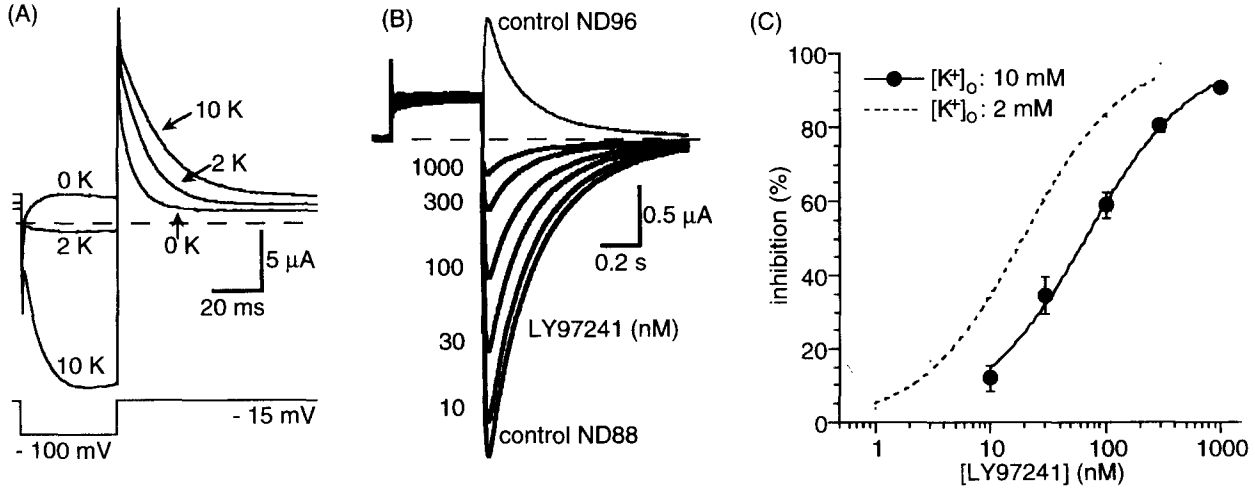

Fig. 3. Effects of $[K^+]_0$ on HERG channel inactivation and LY97241 block. (A) The same protocol as in Fig. 2 (A) was used at nominally 0, 2, and 10 mM $[K^+]_0$ as indicated. The currents flowing at −100 mV reflect the relief of HERG channel inactivation which is approximately complete at the end of the hyperpolarization. At 2 mM $[K^+]_0$ the reversal potential is close to −100 mV. (B) Concentration-dependence of HERG channel blockade in 10 mM $[K^+]_0$ (ND88). HERG channels were activated with depolarizing steps to +40 mV and inward tail currents were measured at −85 mV. The concentration of LY97241 is indicated in nM. As a control, a data trace recording in 2 mM $[K^+]_0$ (ND96) is included. (C) Dose-dependence of relative inhibition of HERG channels by LY97241. The data represent means ± S.E.M. The continuous curve represents a data fit according to a Hill equation (see Section 2). The dashed curve reflects the data in ND96 (2 mM $[K^+]_0$) as shown in Fig. 1C.

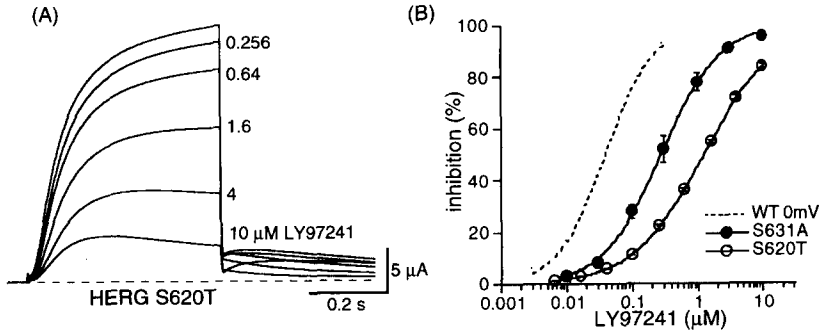

Fig. 4. Concentration-dependence of LY97241 block of HERG S631A and HERG S620T mutant channels. (A) HERG S620T channels were activated with depolarizing steps to 0 mV in ND96 solution. (B) Dose-dependence of relative inhibition of HERG S631A and HERG S620T mutant channels by LY97241 with a data fit according to the Hill equation. The data represent means ± S.E.M. For comparison, the corresponding result obtained from HERG wild-type channels (dashed curve) is indicated.

state of HERG channels has recently been described for the antiarrhythmic drug dofetilide [22]. In addition to the mutation S631A in the external mouth of the channel pore, these authors also analyzed a mutation at the inner vestibule of the pore (S620T) which had even stronger effects on drug sensitivity and channel inactivation [22]. It is shown here that this mutation is much less sensitive to LY97241 than the wild type and the mutant S631A. Thus, it is feasible that intracellular residues are important in forming a binding site for both, dofetilide and clofilium. In fact, clofilium has been shown to block Kv1.5 channels with a much higher potency in inside-out versus outside-out patches, suggesting a binding from the intracellular side [19]. The extremely slow reversal of HERG block by clofilium and LY97241 suggests a similar mechanism for these compounds. As the tertiary amine LY97241 is likely to penetrate the phospholipid membrane more easily than quaternary clofilium, it is conceivable that it has a better access to its binding site at the channel protein.

In summary, the relative specificity and potency of clofilium and LY97241 for blocking HERG channels versus other K^+ channels suggests that this block is the primary mechanism for their antiarrhythmic action. Furthermore, LY97241 is another valuable pharmacological tool for the characterization of HERG channels.

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