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SPECIAL REPORT

Molecular impact of MinK on the enantiospecific block of I_{Ks} by chromanols

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Slowly activating I_{Ks} (KCNQ1/MinK) channels were expressed in *Xenopous* oocytes and their sensitivity to chromanols was compared to homomeric KCNQ1 channels. To elucidate the contribution of the β-subunit MinK on chromanol block, a formerly described chromanol HMR 1556 and its enantiomer S5557 were tested for enantio-specificity in blocking I_{Ks} and KCNQ1 as shown for the single enantiomers of chromanol 293B. Both enantiomers blocked homomeric KCNQ1 channels to a lesser extent than heteromeric I_{Ks} channels. Furthermore, we expressed both WT and mutant MinK subunits to examine the involvement of particular MinK protein regions in channel block by chromanols. Through a broad variety of MinK deletion and point mutants, we could not identify amino acids or regions where sensitivity was abolished or strikingly diminished (>2.5 fold). This could indicate that MinK does not directly take part in chromanol binding but acts allosterically to facilitate drug binding to the principal subunit KCNQ1.
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Abbreviations: I_{Ks}, slow delayed rectifier current, comprised by KCNQ1 and MinK

Introduction Coassembly of KCNQ1 (KvLQT1, Wang *et al*., 1996) and the MinK accessory subunit (IsK, Takumi *et al*., 1988) underlies the slowly activating delayed rectifier I_{Ks} in the heart (Barhanin *et al*., 1996; Sanguinetti *et al*., 1996). The I_{Ks} conductance contributes to the repolarization of cardiac action potentials and mutations in both subunits, which impair channel function are known to prolong the QT interval in the ECG. Prolongation of the QT interval in such patients results in a congenital cardiac disorder described as long QT syndrome (LQTS) that can lead to ventricular arrhythmia and sudden death (Sanguinetti, 1999).

Blockers of I_{Ks} are discussed as promising new class III antiarrhythmic drugs because of an increased prominence of this current during β-adrenergic sympathetic stimulation (Sanguinetti *et al*., 1991). The overall effect of β-adrenergic stimulation on cardiac ion currents is a shortening of the ventricular action potential and it seems to be an important factor in the genesis of malignant ventricular tachyarrhythmias. Accordingly, Schreieck *et al*. (1997) showed that the effect of the specific I_{Ks} blocker chromanol 293B on action potential prolongation is increased after isoproterenol treatment.

Coexpression of MinK alters the biophysical channel features of KCNQ1 by shifting the voltage dependence, slowing activation kinetics, abolishing inactivation, and increasing the single channel conductance and the current amplitude. Additionally, the pharmacology of KCNQ1 is altered by the β-subunit and MinK is the molecular target for the regulation of I_{Ks} by PKC (Pusch *et al*., 1998; Suessbrich & Busch, 1999).

There are conflicting data concerning the discrepancy in block of homomeric KCNQ1 and heteromeric KCNQ1/MinK channels by the chromanol 293B (Suessbrich *et al*., 1996). Whereas Loussouarn *et al*. (1997) did not find any difference in blocking either potassium channel in COS-7 cells, Busch *et al*. (1997) and Seebohm *et al*. (1999) found significantly different IC_{50} values in *Xenopus* oocytes for both the racemate and the single enantiomers of the chromanol. Here, we also demonstrated in *Xenopus* oocytes that another chromanol, HMR 1556 (Gögelein *et al*., 2000), and its enantiomer S5557 had significantly different effects on I_{Ks} and KCNQ1, thus supporting our previous data on 293B and indicating an important contribution of MinK to the chromanol blockade.

To investigate this impact on a molecular level, we tested 293B sensitivity on different Mink mutant proteins. As we had already identified a potential binding region for chromanols within the H5/S6 region of KCNQ1 (Lerche *et al*., 2000b) this study was also designed to increase the knowledge about the molecular basis for the interplay of MinK and KCNQ1.

Methods Handling of *Xenopus* oocytes, synthesis of cRNA (mMESSAGE mMACHINE mRNA synthesis Kit, Ambion), two-electrode voltage-clamp experiments and the analysis thereof have been described in detail (Lerche *et al*., 2000a). Human and rat MinK mutants were published before (Takumi *et al*., 1991; Abitbol *et al*., 1999; Tai & Goldstein, 1998). In general, we injected 5 ng MinK cRNA and additionally 10 ng KCNQ1 cRNA for I_{Ks} expression and 10 ng KCNQ1 cRNA for KCNQ1 expression. Most of the cysteine point mutations (with the exception of: 62C, 63C, 65C, 66C, 69C, 70C, 71C, Tai & Goldstein, 1998) and the deletion mutants Δ10–39, 94–130 were co-expressed with the endogenous *Xenopus* KCNQ1 (Sanguinetti *et al*., 1996) resulting in currents sufficient for analysis (current amplitude at the end of test pulse >1 μA). Currents were analysed 2–5 days after injection. All cysteine point mutants bear an additional mutation C107A. For this single point mutation 293B sensitivity was not altered (Figure 3C). All experiments were verified in three different batches of oocytes.

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The enantiomers of chromanols 293B (trans-6-cyano-4-(N-ethylsulphonyl-N-methylamino)-3-hydroxy-2,2-dimethyl-chromane) and HMR 1556 (N-[3-hydroxy-2,2-dimethyl-6-(4,4,4-trifluorbutoxy)-chroman-4-yl]-N-methyl-methansulphonamid) were synthesized in-house. The drugs were added from respective 100 and 10 mM stock solutions in DMSO to the recording solution ND-96 (final DMSO concentration in the bath solution did not exceed 0.1%).

Concentration blockade relationship analysis was performed by Igor Pro software (Wavemetrix Inc.) and the Hill equation. Student's *t*-test was used to test for statistical significance, which was assumed if $P<0.05$.

Results and Discussion We expressed heteromeric KCNQ1/MinK and homomeric KCNQ1 potassium channels in *Xenopus* oocytes and tested their sensitivity to the enantiomers of two different chromanols. One is the lead compound 293B (Figure 1), for which we formerly characterized a specific and enantio-specific blockade of I_{Ks} and KCNQ1 (Suessbrich *et al*., 1996; Seebohm *et al*., 1999). The other is a recently described compound HMR 1556 and its enantiomer S5557 (Figure 1) both of which act more potently on I_{Ks} and KCNQ1 than the corresponding 293B enantiomers (respective IC_{50} values for 3R,4S 293B on I_{Ks} and KCNQ1 were (mean$\pm$s.e.m.) 2.6 ± 0.2 and 16.4 ± 0.7 μM; for 3S,4R 293B: 15.6 ± 0.8 and 30.1 ± 1.3 μM; for HMR 1556: 0.13 ± 0.01 and 0.42 ± 0.02 μM; for S5557: 0.56 ± 0.03 and 1.53 ± 0.03 μM, Figure 2C).

Thus, according to the results obtained for 293B, we discovered an enantio-specificity of HMR 1556 on I_{Ks} and KCNQ1 potassium channels in the way that 3R,4S HMR 1556 blocked either potassium channel significantly more potently than 3S,4R S5557. Additionally, we confirmed that co-expressing the β-subunit MinK increased the inhibitory potency of chromanols on the KCNQ1/MinK complex compared to homomeric KCNQ1 channels as shown for each enantiomer (Figure 2).

So far, it remains unknown, how MinK causes a shift in the concentration-blockade relation. Recently, we identified the inner pore region of KCNQ1 (Figure 3A) to be the molecular target of 293B (Lerche *et al*., 2000b). More specifically, we identified two residues which abolished or strongly decreased 293B sensitivity of mutant KCNQ1 as well as mutant KCNQ1/MinK potassium channels. These amino acids were located in the H5 pore loop, KCNQ1(V307L), and

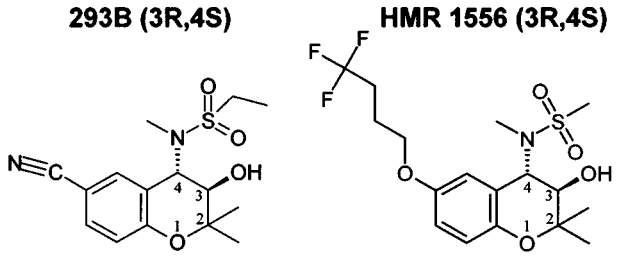

Figure 1 Molecular structures of chromanols 293B and HMR 1556. Both compounds carry two stereogenic centres at positions numbered 3 and 4 and have a *trans* configuration. The enantiomer of HMR 1566 is named S5557 (3S,4R).

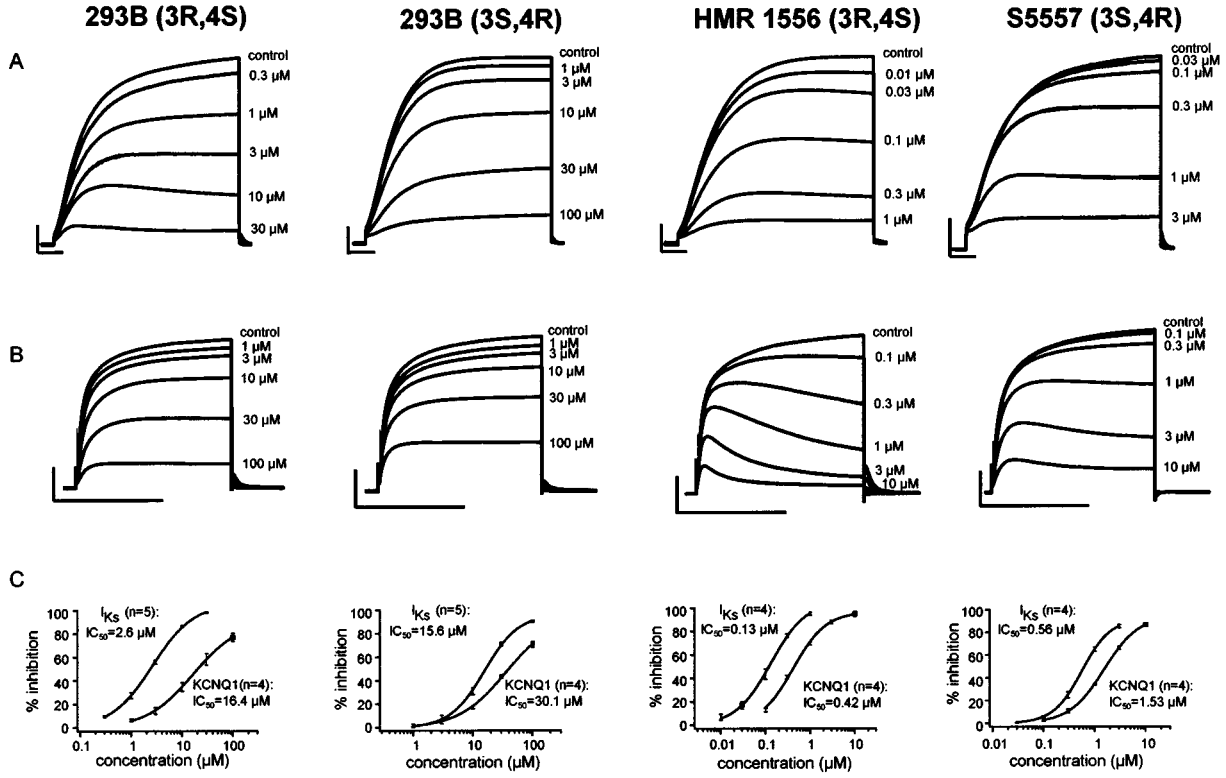

Figure 2 Effects of I_{Ks} blockers (as indicated on top) on heteromeric KCNQ1/MinK and homomeric KCNQ1 channels expressed in *Xenopus* oocytes. Each concentration was perfused for 5 min. For all blockers the inhibition effect was reversible with washing. Scale bars represent 1 μA (vertical) and 2 s (horizontal). (A) Representative current traces from an oocyte coinjected with cRNAs encoding KCNQ1 and MinK at different blocker concentrations while repetitively stepping the membrane potential from -80 to 0 mV for a 15 s test pulse every 60 s. (B) Representative current traces from an oocyte injected with cRNA encoding KCNQ1 at different blocker concentrations while repetitively stepping the membrane potential from -80 to 0 mV for a 3 s test pulse every 10 s. (C) Concentration dependence of inhibition of KCNQ1 and KCNQ1/MinK (I_{Ks}) by the respective blockers. Inhibition values were obtained at the end of the depolarizing step and normalized to the control. Error bars indicate s.e.mean.

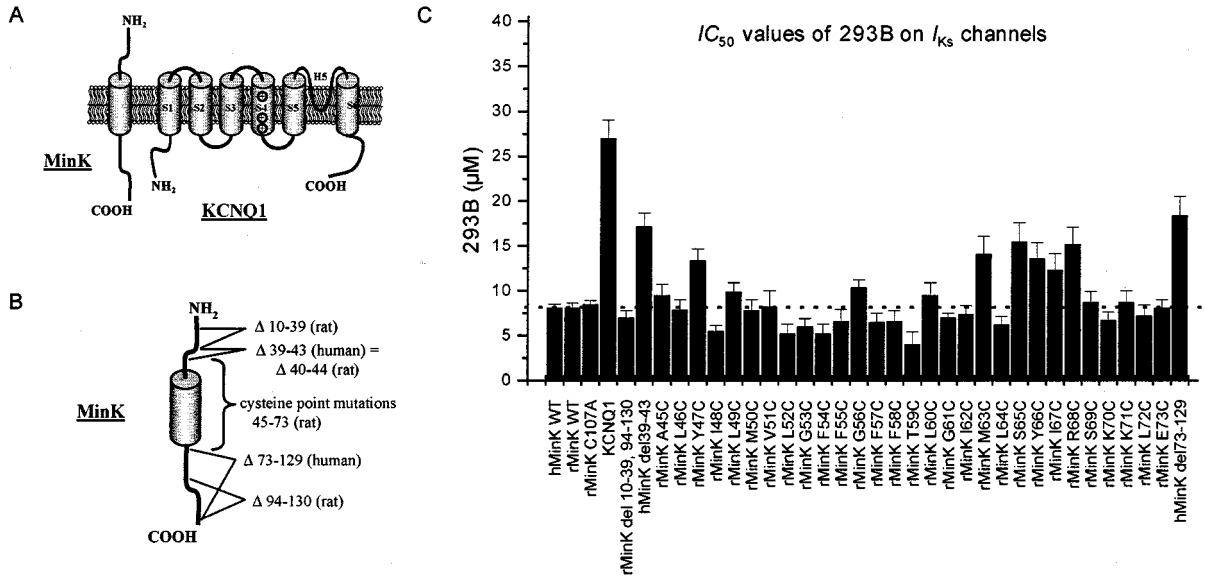

Figure 3 Effects of 293B racemate on WT and mutant I_{Ks}-channels expressed in *Xenopus* oocytes. (A) Topological model of KCNQ1 and MinK with six and one single transmembrane domain, respectively. MinK bears an extracellular N-terminus and an intracellular C-terminus, whereas in KCNQ1 both termini are located intracellularly. Throughout all potassium channels there is a conserved region between S5 and S6 forming the selective ion pathway (H5). (B) Draft of MinK illustrating the nearly complete coverage of the MinK protein by the mutants used. (C) Bar graphs plotting mean IC_{50} values for each WT and mutant potassium channel. Test pulses as described in Figure 2. Dotted line indicates IC_{50} of WT I_{Ks}. Error bars indicate s.e.mean. Numbers of experiments were 3–5 for each construct.

in the S6 transmembrane segment, KCNQ1(I337V). Moreover, there is evidence that MinK directly lines the pore of KCNQ1/MinK channels. This was postulated by Tai & Goldstein (1998), showing a decreasing ion flow through the channel when expressing distinct transmembranal MinK cysteine mutants and exposing them to internal and external Cd^{2+}.

To test this hypothesis and the arising question, whether MinK is directly involved on a molecular basis in chromanol block, we expressed mutant MinK proteins (Figure 3B) in *Xenopus* oocytes. All mutant MinK proteins were described before and most of them did not exhibit different kinetics compared to WT MinK. Deletion mutants Δ39–43 and Δ73–129 exhibit different activation kinetics with no sigmoidal delay (Abitbol *et al*., 1999), but they exert the characteristic slowly activating I_{Ks}-current. Rat and human MinK deletions and point mutations were chosen covering almost the entire protein, excluding only extracellular amino acids 1–9, which were shown to be crucial for functional expression (Takumi *et al*., 1991).

Figure 3C illustrates the influence on the IC_{50}-values of racemic 293B on KCNQ1/MinK channels by each mutation compared to WT KCNQ1 and WT KCNQ1/hMinK. Rat I_{Ks}-channels exhibited identical inhibition by 293B to the human channel. Summarizing this panel, most of the expressed mutants were inhibited to a similar degree as WT I_{Ks}. None of these mutants showed more than a 2.5 fold change in the sensitivity to 293B compared to WT I_{Ks}. All of the mutants were significantly more inhibited compared to homomeric KCNQ1 channels.

The latter result is most important regarding a potential direct involvement of MinK subunits in the inhibition of I_{Ks} channels. If the MinK protein takes part in forming the binding pocket of the drug, it would be expected that molecular changes would affect the inhibition more dramatically as e.g. recently shown for dofetilide on HERG potassium channels (Lees-Miller *et al*., 2000). It is likely, that at least the shift in the inhibition caused by MinK co-expression is abolished by a mutation that deconstructs the binding pocket or it would be rather possible that the inhibition ability of 293B is totally destroyed. Of course, we cannot exclude that conservative cysteine substitution at potential chromanol binding positions only slightly altered 293B sensitivity.

However, it seems conclusive that the MinK protein does not affect inhibition by chromanols directly, because alterations caused by the expressed mutations are only minor. Following our data it is more likely that MinK causes an allosteric change of the binding pocket conformation of KCNQ1.

Moreover, MinK sites immediately downstream of the proposed internal boundary of a selectivity barrier at rat MinK position 57 (Tai & Goldstein, 1998) did not display different chromanol inhibition when changed to cysteine (Figure 3C), whereas we assume the binding region of chromanols to the inner pore of KCNQ1. Thus, this study does not support MinK lining this part of the KCNQ1/MinK channel pore but argues for an allosteric modulation of KCNQ1 subunits by the β-subunit MinK. This study delivers important hints for the molecular pharmacology and the structure of a potassium channel which is only poorly understood until now.

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