Structure of HERG S5P extracellular linker

Structure of the HERG K\(^+\) channel S5P extracellular linker: Role of an amphipathic \(\alpha\)-helix in c-type inactivation

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ABSTRACT

The HERG K⁺ channel has very unusual kinetic behavior that includes slow activation but rapid inactivation. These features are critical for normal cardiac repolarization as well as in preventing lethal ventricular arrhythmias. Mutagenesis studies have shown that the extracellular peptide linker joining the fifth transmembrane domain to the pore-helix is critical for rapid inactivation of the HERG K⁺ channel. This peptide linker is also considerably longer in HERG K⁺ channels, 40 amino acids, than in most other voltage gated K⁺ channels. In this study we show that a synthetic 42-residue peptide, corresponding to this linker region of the HERG K⁺ channel, does not have defined structural elements in aqueous solution; however, it displays two well-defined helical regions when in the presence of SDS micelles. The helices correspond to W585-I593 and G604-Y611 of the channel. The W585-I593 helix has distinct hydrophilic and hydrophobic surfaces. The G604-Y611 helix corresponds to an N-terminal extension of the pore-helix. Electrophysiological studies of HERG currents following application of exogenous S5P peptides show that the amphipathic helix in the S5P linker interacts with the pore region of the channel in a voltage-dependent manner.
INTRODUCTION

The human ether-a-go-go related gene (HERG) encodes the alpha, pore-forming, subunit of the rapid delayed rectifier potassium channel, $I_{Kr}$ (1) The channel is an important contributor to repolarization of the cardiac action potential (1-3). Furthermore, mutations in HERG cause congenital long QT syndrome type 2 (4) that results in a markedly increased risk of ventricular arrhythmias and sudden cardiac death (5,6). A wide range of drugs that block the HERG K$^+$ channel also result in drug-induced long QT syndrome, the most common cause of serious drug-induced arrhythmia and death (7,8). Therefore, there is considerable interest in gaining a better understanding of the structure and structure-function relationships in the HERG K$^+$ channel.

HERG is a member of the family of voltage-gated K$^+$ channels (VGK) that contain six transmembrane domains, denoted by S1-S6 and a pore helix that is interposed between S5 and S6. The positively charged S4 acts as the voltage sensor for activation (9). Unlike other members of the VGK family, the HERG channel also undergoes very rapid voltage-dependent inactivation and recovery from inactivation (1,10-12). Consequently the HERG K$^+$ channel functions as an inward rectifier, i.e. it passes little current at depolarized potentials but large currents during the terminal repolarization phase of the cardiac action potential (13,14). This rapid inactivation is also critical for the role of the channel in suppressing arrhythmias initiated by ectopic electrical excitation (11,15).

Inactivation of the HERG K$^+$ channel results from conformational changes in the outer pore region of the channel (11,12,16-19) and involves so-called "collapse of the pore" (20). The pore region of HERG, including the pore-helix, selectivity filter and S6, is highly
homologous to that of other members of the VGK family (8,9) as well as to the bacterial K\(^+\) channel KcsA (21,22). This feature has enabled homology models of HERG K\(^+\) channels to be constructed, based on the KcsA structure (21). However, the extracellular loop connecting the pore helix to the top of S5 (S5P loop) in HERG is very different from that in other VGK family members. First, the S5P loop in HERG is about 40 amino acids long, compared to 10-15 in most other members of the VGK family (9,23) (also see Figure 1). Second, many mutations in the S5P loop disrupt the inactivation process in HERG (19,23,24). Third, toxins that bind to the S5P loop of other VGK channels, e.g., agitoxin and charybdotoxin (25) do not bind to HERG. Conversely, toxins that bind to this region of HERG (e.g., ErgToxin and BeKm-1) do not bind to other members of the VGK family (26,27). The S5P loop of HERG therefore appears to be a critical region of the protein but at present there is little specific information known about its three-dimensional structure.

Following the determination of the structure of a number of prokaryotic K\(^+\) channels (22, 28-30), great progress has been made in the understanding of the spatial arrangement of amino acid side chains in the pore region of K\(^+\) channels. Nevertheless, the crystallization and determination of the structure of ion channels remains a tremendously difficult task (31) and to date no structures of mammalian ion channels have been determined (32). For this reason, circular dichroism spectropolarimetry (CD; 33-34) and Nuclear Magnetic Resonance spectroscopy (NMR; 35) have been used to gather information on the structure of ion channels and/or domains. Although NMR has limitations in terms of the size of proteins whose structure can be determined, it has the advantage of permitting the determination of structures in a lipid environment (36), thereby eliminating the problems associated with crystallization of membrane proteins (31).
In this study we have used a combination of CD, two dimensional (2D) $^1$H NMR spectroscopy and electrophysiology to investigate the structure and function of the S5P linker of the HERG $K^+$ channel. Our findings show that the S5P linker contains an amphipathic $\alpha$-helix. Exogenous application of the S5P peptide fragment, or a peptide corresponding to the amphipathic $\alpha$-helix, results in altered ionic selectivity and disruption of inactivation of the HERG $K^+$ channel. These results suggest that the amphipathic $\alpha$-helix in S5P is critical for inactivation of HERG $K^+$ channels.

**Figure 1 near here (sequence)**
EXPERIMENTAL PROCEDURES

Peptide Preparation

Peptides were synthesized on a 0.50 mmol scale using HBTU activation of Boc-amino acids with in-situ neutralization chemistry, as previously described (37). The syntheses were performed on Boc-Tyr(2BrZ)-OCH2-Pam resin using standard amino-acid side-chain protection except that methionine residues at positions 5 and 10 were replaced by the isosteric norleucine residue to prevent adventitious oxidation of the peptide. This step is necessary to stabilize the synthetic peptide and is not expected to affect the peptide conformation (37). Each residue was reacted for 10 min and coupling efficiencies were determined by the quantitative ninhydrin reaction. Prior to a standard HF cleavage (10 mL of p-cresol:HF 1:9, 0°C, 60 min) and workup, the N-terminal Boc protecting group was removed (100% TFA), followed by formyl group removal (1.5 mL ethanolamine in 25 ml DMF/5% water, 2 times 30 min).

Three peptides were synthesised in this study: a 42 residue peptide corresponding to the S5P linker of HERG (residues A570-Y611), which we refer to as the S5P peptide, a 42 residue peptide in which the putative amphipathic helix corresponding to residues G584-K595 (23) was replaced with a GGGSGGGSGGGGS linker, which we refer to as the del-helix peptide and finally a 19 residue peptide corresponding to the putative amphipathic helix and four residues at each end (i.e., S581-S599 of wild type HERG), which we refer to as the helix peptide.

The NMR sample was prepared by dissolving 2.6 mg of the S5P peptide in approximately 400 µL of 90% H2O/10% D2O (v/v) containing 12 mg SDS-d25 (~100 mM;
the critical micellar concentration for SDS is 8 mM) in a 5-mm O.D. susceptibility-matched microcell (Shigemi, Tokyo, Japan). This resulted in a peptide concentration of 1.4 mM and pH 3.3.

**NMR spectroscopy**

NMR experiments were performed on a Bruker Avance-600 DRX spectrometer with a 5-mm 1H inverse probe with operating temperatures of 20°C, 30°C and 37°C. The homonuclear two-dimensional (2D) experiments that were performed included double-quantum filtered correlation spectroscopy (DQF-COSY, 38) with a phase cycle modified for fast recycle times (38); total correlation spectroscopy (TOCSY, 39) with MLEV spin-lock periods of 35 ms and 90 ms; and nuclear Overhauser enhancement spectroscopy (NOESY, 40) with mixing times of 200 and 300 ms. All 2D spectra were acquired using time-proportional phase detection (41). In DQF-COSY and NOESY experiments, water-signal suppression was achieved by low-power irradiation at the water resonance during the relaxation delay (1.3 s), and during the mixing period in NOESY experiments. In TOCSY experiments, the water signal was suppressed using the WATERGATE gradient module (42). All spectra were processed using XWIN-NMR software (Bruker, Karlsruhe, Germany).

**Structure calculations**

Analyses of 2D spectra were carried-out using the XEASY program (43). Distance constraints were obtained from cross-peak volumes in the NOESY spectra recorded at 30°C with a mixing time of 200 ms. This yielded 416 non-redundant upper-distance constraints. An additional 14 distant constraints for H-bonding were obtained from a hydrogen-deuterium
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exchange experiment. No dihedral angle constraints were used in the structure calculations because backbone amide peaks were too broad, probably due to conformational averaging of the peptide structure when in the presence of SDS micelles. The simulated annealing protocol in the torsion-angle dynamics program DYANA (44) was used to obtain preliminary three-dimensional structures prior to refinement. Of the 1600 structures generated in DYANA, 40 of the "best" structures, with the lowest NOE violations, were chosen for refinement using the standard simulated annealing script in CNS (45). In this refinement process, the high-temperature dynamics and cooling cycle were performed in Cartesian space. Analysis of the ensemble of S5P structures was also carried out using PROMOTIF, a program that identifies structural motifs in proteins (46). The figures were generated using MOLMOL (47).

**Circular Dichroism Spectropolarimetry**

CD spectra were recorded on a Jasco J-720 spectropolarimeter equipped with a Neslab RTE-111 temperature controller. CD data were collected using a 1 mm cuvette, over the wavelength range 190-250 nm and with a resolution of 0.5 nm, a bandwidth of 1 nm and a response time of 1 s. Final spectra were the sum of three scans accumulated at a speed of 20 nm/min and were baseline corrected. Peptide concentration was 0.45 mg/ml for the S5P peptide or 0.15 mg/mL for the Del-helix and helix peptides, in 10 mM sodium phosphate, pH 3.0 and pH 7.0, with or without the addition of 100 mM SDS. Data are presented as molar ellipticity $[\theta]$, where $[\theta] = \theta/(10 \times c \times l)$ and $\theta$ is ellipticity (mdeg), $c$ is the molar concentration of the sample (mole/L) and $l$ is the pathlength in cm.
Electrophysiology

Chinese Hamster Ovary (CHO) cells stably transfected with HERG K\(^+\) channels were maintained in culture using DMEM F12 (Gibco) with 5% fetal bovine serum, as previously described (48). CHO cells were plated on 13 mm glass coverslips 48-72 hours prior to patch clamp analysis. The coverslips were then placed in a 0.5ml perfusion chamber mounted on the stage of a Nikon Eclipse TE200 inverted microscope. Cells were superfused with normal Tyrode solution that contained in mM (NaCl 130, KCl 4.8, KH\(_2\)PO\(_4\) 0.3, NaH\(_2\)PO\(_4\) 0.35, CaCl\(_2\) 1.8, MgCl\(_2\) 1.0, HEPES 10, pH adjusted to 7.4 with NaOH). In the Na\(^+\)-free external solution NaCl was replaced with N-methyl-D-glucamine (NMDG)-Cl and the NaH\(_2\)PO\(_4\) was omitted. Cells were patched using micropipettes fabricated from thin-walled borosilicate glass (Vitrex Microhematocrit tubes, Modulohm I/S, Denmark) with a horizontal pipette puller (Model P-87, Sutter Instrument Co., USA). The pipette solution contained in mM (K-Gluconate 120, KCl 20, MgATP 1.5, EGTA 5, HEPES 10, pH adjusted to 7.4 with KOH). The permeability ratio of K\(^+\)/Na\(^+\), \(a\), was calculated from the reversal potential measured in standard Tyrode solution using the constant field equation:

\[
E_{\text{rev}} = \frac{RT}{F} \ln\left(\frac{a[K^+]_o + [Na^+]_o}{a[K^+]_i + [Na^+]_i}\right)
\]  \hspace{1cm} (1)

and assuming that \([K^+]_i = 145\) mM and \([Na^+]_i = 5\) mM.

Conventional whole cell voltage clamp recordings were performed using an Axopatch 200B amplifier interfaced with a Digidata 1200 A/D converter operated using pClamp software (Axon Instruments, Foster City, CA, USA). All experiments were performed at room temperature. Whole-cell capacitance was determined from capacitative transient decay in current recordings following voltage steps \(\pm 10\) mV from the holding potential and at least 80% series resistance compensation was achieved in all reported experiments. Protocols used
in specific experiments are described in Figure legends. In all protocols a 20 ms duration 20 mV step from the holding potential of −80 mV to −100 mV was applied at the start of each sweep to enable offline leak-correction. We assumed that the leak was linear in the voltage range −120 to +40 mV. Data analysis was performed using the Clampfit module of the PClamp software. Data are expressed as mean ± standard error for n experiments and analysis of variance (ANOVA) was performed using Microsoft Excel. A p value <0.05 was considered significant.

For electrophysiology experiments peptides were prepared as 0.5 mM stock solutions in either normal Tyrode or Na⁺-free external solution and aliquots stored at −20°C. Once thawed aliquots were stored at 4°C for up to 2 weeks. Aliquots were diluted as required on the day of experiment. Peptides were applied to cells using a picospritzer II (Intracell, Cambridge UK) to ensure rapid application (typically less that 20 ms) and minimise the amount of peptide used in each experiment. A new coverslip was used for each experiment to ensure no residual contamination of cells with peptides.

**Analysis of peptide binding data**

Apparent on-rates, \( \lbrack \), and off-rates, \( k_\text{-}1 \), for peptide binding were obtained by fitting single exponential functions to the data for onset of current block and recovery of current following washout of the peptide. The on-rate, \( k_\text{+1} \), was calculated using the formula:

\[
\lbrack = [\text{peptide}] \cdot k_\text{+1} + k_\text{-1}
\]

(2)

To obtain the time constant for the apparent on-rate of peptide binding at negative potentials it was necessary to correct for channel deactivation (see Figure 9). We assumed that the rate of binding of the peptide to the channel was independent of the rate of deactivation and so:
$I_{\text{obs}} = A \exp(-t/\tau_{\text{obs}}) + C$

$= A \exp(-t/\tau_{\text{deact}}) \cdot \exp(-t/\tau_{\text{on}}) + C$ \hspace{1cm} (3)

where $A$ and $C$ are constants, $\tau_{\text{obs}}$ is the observed single exponential time constant measured from the rate of change in current following addition of the peptide, $\tau_{\text{deact}}$ is the time constant of deactivation (estimated from the single exponential fit to the current recorded in the absence of peptide) and $\tau_{\text{on}}$ is the apparent time constant for peptide binding, i.e. $\tau_{\text{on}} = 1/\beta$. 
RESULTS

Circular Dichroism Spectropolarimetry

Far-UV CD spectra of the S5P HERG peptide in 10 mM sodium phosphate buffer with and without 100 mM SDS at 20°C and pH 3.0 are shown in Figure 2. The large minimum between 195 nm and 200 nm and ellipticity close to zero at 222 nm for the S5P peptide in aqueous solution (thin line) indicates that the peptide does not have a well defined secondary structure under these conditions. This outcome prompted our investigation of the structural properties of the peptide under membrane-like conditions. A dramatic change in the CD spectral profile was observed upon addition of 100 mM SDS (Figure 2, thick line): positive ellipticity was observed between 190 and 195 nm, the position of the minimum shifted to 205 nm and a marked shoulder was present at 222 nm. This indicated that the S5P peptide contains helical elements in the micellar environment at pH 3.0. Similar CD profiles were obtained at pH 7.0 (also see Figure 10 below), and over the temperature range 20-30°C. NMR experiments were therefore performed at ~ pH 3 to facilitate data collection.

NMR spectroscopy

The 2D NOESY spectrum of the S5P peptide in aqueous solution at 25°C had only a few very weak cross-peaks suggesting that the peptide had a flexible, predominantly random coil structure (data not shown) that was consistent with the CD data (see Figure 2). The number and intensity of cross peaks in the 2D NOESY spectrum improved markedly upon
addition of 100 mM SDS. The appearance of amide-amide cross-peaks indicated that the peptide contained some helical structure when in the presence of SDS micelles (see Figure 3), in accordance with the CD results.

Assignments of proton resonances from the S5P peptide were made using standard methods (49) of analyzing 2D TOCSY and NOESY spectra. The cross-peaks were generally broader than those in aqueous solution, presumably due to the increased correlation time of the peptide, and possible conformational averaging and slow exchange between conformations. At 20°C, NOE cross-peaks were too broad to analyze and in several instances overlapped with other cross-peaks, especially in the 'fingerprint' region. Increasing the temperature brought about significant narrowing of the cross-peaks; however some NOE cross-peaks became less intense and a few disappeared. The NOESY spectrum obtained at 30°C was seen to be the best for resonance assignment and structural calculations, but, where possible, spectra obtained at 20°C or 37°C were used to resolve peaks that overlapped at 30°C (see Table S1, supplementary data).

**Figure 3 near here (NOESY spectrum)**

Before doing the structure calculations, the chemical shift values obtained for C\[^\alpha\]-protons were analyzed to provide information on possible secondary structure present in the peptide. For this we used the chemical shift index method of Wishart et al. (50). A prominent grouping of chemical shift deviations below -0.1 ppm for most residues between 16-22 implied a prominent helix in this part of the molecule (see Figure 4). It is also possible that a short helix is present in the region formed by residues 39-41 since they form a small cluster
of three with low chemical shift deviations. The absence of a group of residues with chemical shift deviations greater than +0.1 ppm indicated that no β-sheet structure is expected for the S5P HERG peptide. The rest of the S5P HERG peptide, from residues 1-15, and residues 23-38, is likely to be flexible or in random conformations.

**Figure 4 near here (NMR summary)**

In addition to the presence of significant amide-amide NOE connectivities, the 2D NOESY spectrum of S5P showed many medium-range connectivities between residues 14-24 and 38-42 (see Figure 4). This supported the proposal that these regions formed helices as indicated by the CD spectra (Figure 2) and chemical shift indices (Figure 4). No long range NOEs were detected, implying that the S5P peptide does not have a well-defined tertiary structure. A total of 416 distance (upper-limit) constraints (of which 74 were medium range) were obtained from the 200 ms NOESY spectrum (see Table S2, supplementary data); the constraints were used in structure calculations using DYANA and CNS (see Methods). The "best" 20 structures, namely those with the lowest penalty values, were considered to be representative of the structure and were therefore used in the figures shown here.

**Structural fold**

Comparison of the 20 best S5P structures showed that S5P did not have a distinct three-dimensional fold in SDS micelles. However, two regions in the peptide were well defined; this was indicated by their relatively low root mean square deviation (RMSD) values, with respect to the local mean structure. These were regions defined by residues 14-
24 and 35-42 that had mean backbone RMSDs of 0.31 Å and 0.23 Å, respectively (see Table S2, supplementary data). These two well-defined regions encompassed all residues displaying medium range NOE values and were predicted to be helical by the CSI analysis (Figure 4). Also, PROMOTIF analysis (46) of the ensemble of S5P structures predicts that the regions defined by residues 16-25 and 35-42 form β-helices in the presence of SDS micelles. Figure 5a shows the superpositions of 20 structures on to the backbone of the well-defined region defined by residues 14-24. The rest of the molecule, except for the region defined by residues 35-42, appears disordered (see Figure 5b). It should also be noted that as the region defined by residues 24-35 is disordered (represented by a dashed line in Figure 5b) it is not possible to define the spatial relationship between the two helical regions. The conclusion that the regions defined by residues 1-14 and 24-35 lacked defined structures in solution was based on the absence of medium and long-range NOEs.

**Figure 5 near here (ensemble / overall fold)**

In the KcsA structure (22), the ends of the extracellular loop that corresponds to S5P in HERG, (i.e., A50 and Y62 in KcsA) are close together, being separated by only ~11 Å. We attempted to constrain the ends of the S5P peptide by introducing a "dummy" distance constraint of 10.9 Å between the backbone Cα of A1 and that of Y42 in the NMR structure calculation. This could have provided useful insights into the three-dimensional fold of the peptide, and also information about the relative orientation of the amphipathic β-helix (see below) with respect to rest of the molecule. However, the calculation failed to indicate a distinct structural fold.
The amphipathic helix

Detailed analysis of the first well-defined region showed that it was an amphipathic helix, with the side-chains of W16, L17, L20, and I24 forming the hydrophobic face, and the side-chains of H18, N19, D22, and Q23 defining a hydrophilic edge. (See Figure 5). The side-chains of residues 16-20 were well defined, with RMSD values of 0.23 Å when their side chain heavy atoms were superimposed on each other (see Table S2, supplementary data). There were numerous medium-range NOEs between the W16 aromatic side-chains and those of I14, L17 and L20; this helped to define the conformation of the hydrophobic region of the molecule, placing W16 as the center of this site.

Role of amphipathic helix in inactivation

The unusual kinetics of HERG K⁺ channels, viz. slow activation / deactivation and rapid inactivation / recovery from inactivation are illustrated in Figure 6A. Depolarizations to +40 mV cause HERG channels to open slowly and then inactivate rapidly, resulting in a relatively small current flow. Subsequent repolarization to −120 mV results in rapid recovery from inactivation and hence a large increase in inward current followed by a slower decay in the current as the channels deactivate. To investigate whether exogenous application of the S5P peptide would have any effect on full length HERG channels we superfused cells with 1 µM S5P peptide and the membrane was depolarized from −80 mV to +40 mV for 0.5 s followed by a hyperpolarization to −120 mV for 1 s and the protocol repeated every 5 s. Application of the 42-residue S5P peptide caused a reversible suppression of HERG current
(see Figure 6B and C). Application of 1 µM S5P peptide to CHO cells transfected with HEAG or rELK2 channels resulted in no reduction in current (data not shown).

The apparent on-rates and off-rates for peptide binding to the channels were obtained by fitting single exponential functions to the data for onset of current block and recovery of current following washout of the peptide. In the example illustrated in Figure 6C the time constants for peptide binding and washoff were 17.8 s ($k = 0.056 \text{ s}^{-1}$) and 53.7 s ($k^{-1} = 0.019 \text{ s}^{-1}$) respectively. The mean values obtained from four separate cells were $k = 0.045 \pm 0.007 \text{ s}^{-1}$ and $k^{-1} = 0.024 \pm 0.005 \text{ s}^{-1}$ (n=4). Substituting values for $k$ and $k^{-1} = $ into Equation (2) gave an on rate, $k_{+1}$, of $2.1 \pm 0.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. And the dissociation constant for peptide binding ($K_A = k^{-1} / k_+$) was $1.1 \pm 0.4 \mu\text{M}$ (n=4).

To investigate further the binding of the S5P peptide to HERG channels we used a picospritzer to rapidly apply peptides and minimise the amount of peptide used in each experiment. Firstly we investigated the dose-response of the effect of S5P peptide by applying the peptide at doses ranging from 0.1 to 100 µM (see Figure 6D) using the same voltage protocol as used in Figure 6A. The IC$_{50}$ for inhibition of the tail current at -120 mV measured from the dose-response curve was 1.9 µM (Figure 6D). This value is similar to that measured from the on- and off-rates for 1 µM S5P, i.e. 1.1 µM.

**Figure 6 near here**

To investigate the voltage dependence of the effect of S5P peptide on HERG channels we used a voltage protocol where within each sweep channels were first activated by stepping to +40 mV for 500 ms then stepped to a test voltage in the range +30 to –110 mV
followed by a –120 mV step for 800 ms to fully deactivate the channels. The protocol was then repeated with peptide rapidly applied for 1 second during the test voltage step (see Figure 7A). Based on the on-rate for S5P peptide binding to HERG K⁺ channels calculated above, we used 100 µM S5P peptide in these experiments so as to achieve >90% binding within 1 s application.

The effect of rapid application of 100 µM S5P peptide was voltage dependent. For example, application of S5P peptide caused an increase in outward current at +30 mV (solid arrows, Figure 7A) but a significant decrease in the current at –50 mV (dotted arrows, Figure 7A). In the protocol illustrated in Figure 7A we waited 60s between sweeps to ensure that the effect of the peptide had washed out (also see Figure 7B). The traces in Figure 7B show the average (± SEM) current recorded during five successive sweeps at 60 s intervals where the test potential was -70 mV for all five sweeps. Both the control currents (thin line Figure 7B) and currents recorded during application of the S5P peptide (thick line, Figure 7B) were superimposable. This illustrates two important points. Firstly, during the 60 s inter sweep interval the effect of the peptide was completely washed out and secondly the effect of the peptide was very reproducible. Also of note from Figure 7B is that in the presence of the S5P peptide the current recorded at -70 mV reversed from an outward current to a small inward current, suggesting that application of the peptide altered the selectivity of the channel (see below).

**Figure 7 near here**
Examples of currents recorded at voltages in the range +30 to -110 mV (in 10 mV steps) using the voltage protocol illustrated in Figure 7A are illustrated in Figure 8. At positive potentials (panels a-c) application of the peptide resulted in an increase in current that was maintained for the duration of application of the peptide. At voltages in the range 0 to –20 mV (panels d-f) there was an initial increase in current followed by a slower decline in current during application of the peptide. In the voltage range -30 to -60 mV (panels g-j) there was a rapid decrease in current during application of the peptide. The most dramatic effects however occurred at –70 (panel k) and –80 mV (panel l) where the peptide caused a reversal in current flow. This suggests that the channel has lost its selectivity for K⁺ over Na⁺ (the only other cation present at significant concentrations in the extracellular solution). At progressively more negative potentials it became difficult to discern an effect of the peptide. From the data in Figure 6, however, we know that the peptide can inhibit the channel at -120 mV, therefore the lack of apparent effect at the most negative voltages in Figure 8 is presumably because the rate of channel deactivation is as fast if not faster than the rate of peptide binding to the channel at these voltages.

**Figure 8 near here**

To quantify the effect of the S5P peptide on HERG channels we analysed the rates of change in current magnitude following addition of the peptide (i.e., the apparent on-rates, see Figure 9A, B), the maximum increase (or decrease) in current during the 1 second application of the peptide (see Figure 9A, C) and the change in reversal potential caused by application
of the peptide (see Figure 9D). In a separate series of experiments we also measured the off-rate for peptide dissociation from the channels at +30 mV (see Figure 9E).

At potentials in the range −20 mV to +30 mV we fitted a single exponential to the increase in current to obtain apparent on-rates (see panels a and b in Figure 9A). At −20 mV there were two phases to the change in current during application of S5P peptide, i.e. an initial increase in current followed by a slower decrease in current. We fitted single exponential functions to both phases; in the example shown in Figure 9A (panel b) the time constant for the initial increase was 89 ms and the time constant for the subsequent decrease was 1.4 s. To obtain the time constant for the apparent on-rate at more negative potentials it was necessary to correct for channel deactivation (see Experimental Procedures). In the example illustrated in panel c) of Figure 9A the time constant of deactivation in the control trace (no peptide present) was 223 ms, and the time constant of current decline following application of the S5P peptide was 65 ms. Substituting these values into Equation (3) gives an apparent time constant for peptide binding of 92 ms, which is very similar to that obtained by dividing the two current traces (88 ms; as shown in the inset to panel c of Figure 9A). For voltages in the range +30 to -10 mV the apparent time constant for peptide binding, ~100 ms, did not vary with voltage (see Figure 9B). However, in the voltage range -20 to -80 mV the apparent time constant for peptide binding decreased significantly as the voltage became more negative (decreasing from 905 ± 59 ms at -30 mV to 47 ± 8 ms at -80 mV).

We measured the maximum increase (at potentials −20 mV) and decrease (at potentials −20 mV) in current during application of the S5P peptide from the single exponential curves fitted to the data after correcting for channel deactivation if appropriate (see inset to panel c of Figure 9A). The maximum change in current during 1 s application of
100 µM S5P peptide varied significantly with voltage. Note that the decrease at −70 mV (113 ± 8 %) was over 100% indicating that the current had reversed from an outward to an inward current.

To estimate the change in reversal potential we fitted single exponentials to the decaying phase of currents recorded in the voltage range -60 mV to -90 mV and extrapolated the fits back to the start of the test voltage step (see Figure 9D). In the example illustrated in Figure 9D the reversal potential changed from -80 mV to -64 mV. The mean change in reversal potential was from -83 ± 1.5 mV (normal Tyrode solution) to −68 ± 2.2 mV (in the presence of S5P peptide, n=6). From the change in reversal potential following application of S5P peptide we estimate, using Equation (1), that the permeability ratio for K⁺ over Na⁺, \( P_K / P_{Na} \), was reduced from 188 ± 38 to 27 ± 5 (n=6).

To estimate the off-rate for peptide dissociation from the channels at +30 mV we depolarized cells to +30 mV and after 1 s (when a steady-state current had been reached) we spritzed on the peptide for 1 s and then maintained the cell at +30 mV for a further 3 s (see Figure 9E). In the example illustrated in Figure 9E the time constant for the increase in current in the presence of S5P peptide was 123 ms and the time constant of the decrease in current following washoff of the peptide was 2.26 s. The time constant for washoff, 2.06 ± 0.56 s (n=4), was considerably shorter at +30 mV than was seen using the protocol in Figure 6 where the time constant for washoff was 42 ± 9 s (n=4), see above. This suggests that dissociation of the peptide from the channels is voltage-dependent. Substitution of the time constants measured at +30 mV into Equation (2) yielded an on-rate of \( 5.5 \times 10^4 \) M⁻¹s⁻¹ and a peptide affinity of 8.9 ± 2.2 µM (n=4).
The above results, most notably the altered ionic selectivity, suggest that the exogenously applied S5P peptide is interacting either directly with the selectivity filter of the channel or binding to the channel in such a way as to cause a change in the conformation of the selectivity filter. To determine which region of the S5P peptide was responsible for the interaction with the pore region of the HERG channel we synthesised two additional peptides. In the first peptide the amphipathic helix was deleted and replaced with a (GGGS)₃ linker (del-helix peptide) and in the second the N- and C-terminal regions were deleted, leaving just the amphipathic helix with four residues at either side (helix peptide). All three peptides, S5P-, del-helix and helix peptides, adopted a random coil conformation in aqueous solution at pH 7.0 (see Figure 10a). In the presence of SDS micelles the del-helix peptide remained in a random coil conformation, the S5P peptide contained some α-helical elements (the spectra obtained at pH 3.0, Figure 2, and pH 7.0, Figure 10a were indistinguishable) and the helix peptide adopted an almost pure alpha helix, as expected. Typical examples of HERG tail currents recorded at –80 mV during control sweeps or during application of the S5P, del-helix or helix peptides are shown in Figure 10b. Application of the del-helix peptide had no significant effect on HERG currents. The helix peptide, however, had a very similar effect to that observed with the full length S5P peptide (also see below).
Typical examples of the effects of the helix peptide on HERG tail currents recorded at 0 mV, -40 mV and -80 mV (using the same voltage protocol as shown in Figure 7A) are illustrated in Figure 11. Application of 100 µM helix peptide resulted in increased current at 0 mV, a decreased current at -40 mV and a clear reversal of current flow at -80 mV (Figure 11A). The apparent time constants for helix peptide binding to the HERG channel are plotted in Figure 11B and the maximal increase (at potentials > -20 mV) and decrease (at potentials < -20 mV) in current observed during 1 second application of 100 µM helix peptide are shown in Figure 11C. Qualitatively, the effects seen with application of the helix peptide are very similar to those seen with the S5P peptide (compare Figures 9 and 11). There were, however, some significant differences. The apparent time constants for peptide binding were faster for the helix peptide than for the S5P peptide (compare Figures 9B and 11B), the off-rate was also slightly faster for the helix (time constant for dissociation at +30 mV was 0.85 ± 0.15 s, n=5, for the helix peptide compared to 2.06 ± 0.56 s, n=4, for the S5P peptide) and the maximum increase in current seen at positive potentials was less for the helix peptide than for S5P peptide (see Table 1). However, the shift in the reversal potential (14 ± 2 mV, n=4) following addition of the helix peptide was similar to that seen with the S5P peptide (16 ± 2 mV, n=6). The del-helix peptide, 100 µM, had no effect on the reversal potential of HERG currents and caused no increase in current at positive potentials, rather it caused a very modest decrease in current at all potentials (see Table 1).
The shift in reversal potential following application of either the 42-residue S5P peptide or the 19-residue helix peptide was consistent with a decreased selectivity for $K^+$ over $Na^+$. To further investigate this possibility we looked at the effects of both peptides when all external $Na^+$ was replaced with NMDG$^+$. In the absence of external $Na^+$, acute application of either the S5P peptide or the helix peptide caused only modest decreases in current at all test potentials (see Figure 12A). Furthermore there was no current reversal at $-80$ mV following application of either peptide in NMDG$^+$ solutions. It should be noted, however, that both the S5P and helix peptides caused inhibition of current at $-120$ mV when applied for 1 minute in NMDG$^+$ external solutions (see Figure 12B). This confirms that the peptide was still active but its apparent affinity was either much reduced or the effect it has on the channel was substantially altered in the absence of external $Na^+$. 

Figure 12 near here
DISCUSSION

The S5P linker contains an amphipathic helix

In this work we have identified an amphipathic α-helix that is present in the extracellular linker connecting the outer ends of S5 and the pore-helix of the HERG K^+ channel. The 42-residue peptide spanning this region is unstructured in aqueous medium but contains α-helical elements when in the presence of SDS micelles. The major helical element extends from W585 to I593 (WLHNLGDQI; see Figure 5) and has a well-defined hydrophobic face. Interestingly, this helix contains a glycine, G590, which in the centre of a helix usually has a destabilising effect (51). It is possible that the presence of the glycine is the reason that the S5P peptide is unstructured in water. It appears that an interaction between the hydrophobic surface of the helix and the hydrocarbon chains of the SDS stabilises the helix in vitro. In vivo and in the native protein, the stabilising force, may be provided by the helix interacting with the membrane or with other parts of the protein.

The amphipathic α-helix we have identified (W585-I593) corresponds closely with the predictions originally made by Pardo-Lopez and colleagues, based on the effects of cysteine scanning mutagenesis on ErgToxin binding to the HERG K^+ channel (23). Therefore the amphipathic helix we have identified in the isolated peptide is almost certainly present in the intact protein as well.

Two pieces of evidence support the suggestion that the amphipathic α-helix interacts with another part of the HERG protein. First, in the ensemble of the 20 lowest energy structures the hydrophobic residues W585, L586 and L589 are contained within a region that is very tightly constrained (RMSD for the sidechain heavy atoms of residues WLHNL in the
peptide was 0.23 Å). Furthermore these hydrophobic residues are 100% conserved in all members of the ERG/EAG/ELK channel family (see Figure 1), whereas there is much less conservation amongst the hydrophilic residues. The conservation of sequence as well as the tight structural constraints argues in favour of the hydrophobic surface representing a region of specific protein-protein interaction. Second, application of exogenous S5P peptide to wild-type HERG K⁺ channels caused dramatic changes in channel properties (see below).

The amphipathic helix interacts with the pore region of the channel

Exogenous application of the S5P peptide had at least two effects on HERG currents. Firstly it suppressed the current (e.g. see Figures 6 and 12B). Secondly, it appeared to disrupt inactivation (e.g. it caused an increased current flow at positive potentials, see Figure 8) and reduced the selectivity for K⁺ over Na⁺ (e.g. see Figure 9D). We have classed the last two effects together as it is likely that they are related (see below). Furthermore, these effects were also seen following superfusion of CHO-HERG cells with the helix peptide that corresponded to residues S581-S599 but not with a peptide in which the amphipathic α-helix was replaced with a (GGGS)₃ linker. Thus it seems that the major site of interaction between the S5P peptide and the rest of the channel involves the amphipathic α-helix.

Current suppression

When cells were exposed for prolonged periods to S5P peptide current was suppressed in a dose dependent manner with an IC₅₀ of approximately 1.9 µM (see Figure 6D). This inhibitory effect of the S5P peptide was independent of external Na⁺ (see Figure 12B), however in the absence of external sodium the potency of the peptide appeared to be
Structure of HERG S5P extracellular linker

much reduced. For example 100 µM peptide only caused ~60% inhibition after 1 minute in cells superfused with Na\(^+\) free solution (e.g. Figure 12B) but ~93% inhibition after 10 s in cells superfused with Na\(^+\)-external solution (see Figure 6D).

*Disrupted inactivation and altered selectivity*

Addition of 100 µM S5P peptide to HERG K\(^+\) channels caused a depolarizing shift in the reversal potential by 16 ± 2 mV (see Figure 9D, Table 1). However, when all external Na\(^+\) was replaced by NMDG\(^+\) the S5P peptide no longer caused a shift in reversal potential (see Table 1). These results indicate that the channels had a decreased selectivity for K\(^+\) over Na\(^+\). If a decrease in selectivity for K\(^+\) relative to Na\(^+\) was the only effect of acute application of S5P peptide then one would not have expected an increase in current at positive voltages (see panels a-c, Figure 8) as the positive shift in reversal potential would result in a smaller driving force for outward current flow. The relatively small current flow through HERG K\(^+\) channels at positive potentials is due to rapid voltage-dependent inactivation (1,10-12). Thus one possibility is that in addition to altering the selectivity of the channels the peptide has altered inactivation. Such a hypothesis is also consistent with previous reports showing that many of the mutations in the S5P linker that affect inactivation also affect the selectivity for K\(^+\) over Na\(^+\) (19). The association between disruption in inactivation and changes in ionic selectivity is one of the strongest pieces of evidence supporting the “collapse of the pore” model of inactivation in HERG K\(^+\) channels (3). Thus we suggest that the exogenously applied S5P peptide is either itself directly binding to the pore region and thereby disrupting inactivation and K\(^+\):Na\(^+\) selectivity or it is binding to the
outer pore region in such a way that it induces a conformational change in the selectivity filter region.

The very significant reduction in the effect of acute application of S5P peptide when external Na\(^+\) was replaced by NMDG\(^+\) (compare Figures 9 and 12) suggests that the increase in current seen with application of S5P peptide at positive potentials is Na\(^+\)-dependent. Previous work from Balser and colleagues has shown that inactivation of HERG K\(^+\) channels is promoted by extracellular Na\(^+\) (52). Thus it may be that the S5P peptide is competing with external Na\(^+\) for binding to the site that when occupied by Na\(^+\) promotes inactivation (52).

**Is binding of S5P to the HERG K\(^+\) channel voltage dependent?**

The effect of the acute application of S5P peptide to HERG K\(^+\) channels was clearly voltage dependent (see Figures 8 and 9). The voltage-dependence of the time constants for change in current following addition of S5P peptide (see Figure 9B) also suggests that there are two components to peptide binding. Firstly, at positive potentials the time constant of current increase (open symbols, Figure 9B) is rapid and voltage-independent. Secondly, at potentials in the range –20 mV to –80 mV the time constant for the decrease in current (closed symbols, Figure 9B) is voltage-dependent. It should be noted that in our analysis of the apparent on-rates at negative voltages we assumed that the effect of peptide binding to the channels was independent of deactivation (see Experimental procedures and Figure 9A). This is the simplest model that is consistent with the data that we have, however we cannot assume that the model is necessarily correct. If our assumption re: independence of peptide binding and deactivation is incorrect then it is possible that part of the “voltage-dependence” of the time constants in the voltage range –20 mV to –80 mV seen in Figure 9B may reflect...
the voltage-dependence of the rates of deactivation in this voltage range rather than voltage-
dependence of binding per se. Conversely, the off rate measured at +30 mV (a voltage at
which no deactivation was observed) was considerably faster (0.48 s\(^{-1}\), see Table 1) than the
off-rate measured from Figure 6C (0.024 s\(^{-1}\)) when cells were held at –80 mV between
pulses. From these values the calculated on-rates were 5.5 x 10\(^4\) M\(^{-1}\)s\(^{-1}\) at +30 mV and 2.1 x
10\(^4\) M\(^{-1}\)s\(^{-1}\) at ~-80 mV. Thus we can conclude that there is a voltage-dependent component to
peptide binding, and that the off-rate appears to be more voltage-sensitive than the on-rate.
However, the precise influence of voltage on peptide binding in the voltage range –20 mV to
–80 mV remains to be determined.

The augmentation of current at positive potentials but inhibition at negative potentials
following application of the S5P peptide (see Figure 9C) is reminiscent of the effect of Ba\(^{2+}\)
on HERG K\(^{+}\) channels (53), i.e. 2 mM Ba\(^{2+}\) blocks HERG currents at negative voltages but
increases the current at positive voltages, an effect that has been attributed to a voltage-
dependent competition between Ba\(^{2+}\) and Na\(^{+}\) for binding to an outer pore binding site (52).
Although the voltage protocols used in the earlier Ba\(^{2+}\) studies (52,53) and this study are
different, it is possible that the S5P peptide, like Ba\(^{2+}\), may be competing with Na\(^{+}\) for
binding to an outer pore binding site and that this competition is voltage-dependent. The
significant change in the effect of the peptides on HERG channels when external Na\(^{+}\) was
replaced with NMDG\(^{+}\) also supports the hypothesis that the peptide is competing with
external Na\(^{+}\) for binding to an outer pore binding site.

Comparison of the effects of the S5P peptide and the helix peptide
The effects of adding the S5P peptide to HERG channels could be reproduced, qualitatively, with a 19-residue peptide containing only the amphipathic helix and four flanking residues on either side. There were, however, some quantitative differences in the effects of the two peptides on HERG channels. Firstly, the amphipathic helix appears to bind more rapidly. For example the time constant for binding of 100 µM peptide at 0 mV was 145 ± 36 ms (n=5) for S5P peptide compared to 36 ± 7 ms (n=3) for the helix peptide (see Table 1). Secondly, the maximum increase in current seen during peptide application at positive potentials was greater for the S5P peptide than for the helix peptide. For example the maximum increase at +30 mV was 56 ± 22% (n=4) for the S5P peptide and 9 ± 3% (n=3) for the helix peptide (see Figures 9C and 11C and Table 1). These data suggest that a region of the S5P peptide not present in the helix peptide, for example, the charged motif at the C-terminal end, inhibits access of the amphipathic helix to a binding site on the channel. However, once it has bound the S5P peptide is more effective, than the helix peptide, at altering channel function. Whether such differences are of physiological significance however are debatable given that in the native channel movement of the amphipathic helix is likely to be constrained by its attachments to the S5 and pore helices.

How does the S5P peptide interact with the rest of the HERG channel?

Given that the S5P linker is critical for inactivation of HERG K⁺ channels and inactivation in HERG K⁺ channels is voltage dependent (1, 10-12) it is possible that voltage-dependent binding of the S5P linker to the outer pore of the channel could contribute to the voltage-dependent inactivation of HERG K⁺ channels. There are a number of charged residues in the S5P linker including an aspartate (D591) in the amphipathic α-helix (see
Figure 1) that in theory could contribute to voltage dependent binding of the S5P linker to the rest of the channel. The observation that mutation of D591 to a cysteine disrupts inactivation of HERG K^+ channels (19) suggests that this residue may be important for binding of the amphipathic helix to the rest of the channel but whether it could contribute to voltage-dependent binding of the amphipathic helix to the pore region of the channel has not been tested.

We were not able to define a unique three-dimensional fold for the S5P loop of the HERG K^+ channel despite constraining the ends of the peptide to the homologous residues in the KcsA structure (22). This result is consistent with the NMR data though, showing that significant stretches of the linker, A571-G584 and G594-G603, may be highly flexible. Flexibility in this region could be advantageous in that it may allow significant movement of the amphipathic \(-\)-helix; which it has been suggested may occur during inactivation (19). To further address this issue will require additional experimental data to help constrain the possible orientations of the S5P linker and to identify the specific site(s) of interaction between the S5P linker and the rest of the channel. Such data could be provided by mutant cycle analysis experiments analogous to those used to identify the sites of interaction between the scorpion toxin BeKm-1 and the outer vestibule of the HERG K^+ channel (54).

In summary, we have provided the first structural information on the outer pore region of the HERG K^+ channel, a region that is critical for the rapid inactivation of the channel (19,24). This unique feature of the HERG K^+ channel is essential for its function in normal cardiac repolarization. We have also shown that the S5P peptide is able to interact with the rest of the HERG channel resulting in suppression of current, an altered selectivity
for $K^+$ over $Na^+$ and partial disruption of inactivation. Furthermore, we have shown that it is the amphipathic $\alpha$-helix region that is critical for the effects on ion-selectivity and inactivation because when the amphipathic $\alpha$-helix region of the S5P peptide is replaced with a random coil linker the peptide no longer affects ion-selectivity or inactivation (see Figure 10b and Table 1) whereas a peptide containing only the helix region has very similar effects on the HERG channels as the full length peptide (compare Figures 9 and 11). Interestingly, the S5P peptide and the helix-peptide are able to affect HERG channels despite being applied in normal Tyrode solution where in isolation they would be expected to have a random coil conformation. Presumably, however, the peptides are able to form helices in the context of the HERG $K^+$ channels and the associated membrane environment.
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FOOTNOTES

1. Abbreviations used:
HERG: human ether-a-go-go related gene, VGK: voltage-gated K\(^+\) channel, NMDG: N-methyl-D-glucamine, NMR: nuclear magnetic resonance spectroscopy, CD: circular dichroism spectropolarimetry, DQF-COSY: double-quantum filtered correlation spectroscopy, TOCSY: total correlation spectroscopy, NOESY: nuclear Overhauser enhancement spectroscopy, SDS: sodium dodecyl sulfate, CHO: Chinese Hamster Ovary, RMSD: root mean square deviation

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FIGURE LEGENDS

Figure 1.

A. Alignment of the S5, pore (P)-helix and selectivity filter regions of Shaker, KcsA and HERG. Grey shadow indicates S5 (Shaker and HERG) or outer helix (KcsA) and the P-helices. Asterisks indicate methionine residues that were replaced with norleucine in the synthesised peptide to simplify structure assignment (see text for details). Residues in HERG shown in bold type indicate the peptide that was synthesised in this study. The inset shows a cartoon of a HERG $K^+$ channel subunit, with the region of interest highlighted by the grey shading and bold lines.

B. Alignment of the region surrounding the amphipathic helix (shaded region) of HERG with other members of the ERG/EAG/ELK family of $K^+$ channels. Residues in white with black shading indicate hydrophobic residues conserved in all 8 members of the ERG/EAG/ELK family.

Figure 2.

Comparison of the farUV CD spectra of the HERG S5P loop peptide in 10 mM phosphate, pH 3.0 (thin line) and in 10 mM phosphate, 100 mM SDS, pH 3.0 (thick line). In aqueous solution, the peptide displays a signal indicative of an unstructured protein, with a large minimum between 195 nm and 200 nm. In the membrane-mimic environment, the peptide assumes some helical structure, displaying the characteristic signals of positive ellipticity between 190 and 195 nm and negative minima at approximately 205 nm and 222 nm.
Figure 3.
Amide region of 200 ms NOESY spectrum of S5P peptide in 100 mM SDS at pH 3.3 and 30°C. Presence of non-diagonal NOESY cross-peaks in this region suggests turn-like or helical conformation in the molecule. The intense NOESY cross-peaks in the spectrum are labelled with two residue numbers corresponding to the two amide protons that are close in space.

Figure 4.
Summary of NMR data for secondary structure prediction in S5P peptide in 100 mM SDS. Two residues denoted by X are norleucines, which replaced methionines. Slowly exchanging backbone amide protons are indicated by circles; NOE connectivities are represented by shaded horizontal bars whose thickness are proportional to the observed NOE intensities; the chemical shift index (i.e. $\delta_{H^\alpha}$ chemical shift deviations from random coil values (50)) are indicated by vertical bars.

Figure 5.
NMR structure of S5P peptide in 100 mM SDS micelles. A. Region defined by residues 14-25 of ensemble of 20 structures superimposed over the backbone atoms of residues 14-24 of the local mean structure. B. The whole S5P peptide showing the helices at the center and the N-terminal end of the molecule. The dashed lines (residues 1-14 and 25-34) indicate that these regions did not have a defined structure. Diagrams were generated using the program MOLMOL (47).
Figure 6.

A. Typical example of ion currents recorded from a CHO-HERG cell during a step depolarization to +40 mV for 0.5 s followed by a step to –120 mV. At the holding potential of –80 mV channels are in the closed state (C). During the step to +40 mV channels open (O) slowly but inactivate (I) rapidly resulting in little current flow (thin line). During the step to –120 mV the channels rapidly recover from inactivation (on the timescale of ms) resulting in a large increase in current (thick line) and then deactivate slowly (on the timescale of 10s – 100s of ms) resulting in a decay in current (dashed line) back to the zero current level (indicated by dotted horizontal line at the left of the trace). The dotted box on the voltage protocol shows the region of the traces depicted in panel B.

B. Typical example of currents recorded from a CHO-HERG cell using the voltage protocol shown in A, (i) before, (ii) after 1 minute superfusion with 1 µM S5P peptide and (iii) six minutes after washout of the peptide. Horizontal dotted line shows the zero current level. The dotted vertical arrow indicates the point at which tail currents at –120 mV were measured (see panel C).

C. Changes in peak tail current recorded at –120 mV prior to, during superfusion and following washout of 1 µM S5P peptide. The peptide was applied for 1 minute (shown by the solid bar at the top of the graph). The labels (i), (ii), and (iii) indicate the time points for which traces are shown in B. The thin lines show single exponential fits to the data for the onset of the effect of S5P peptide ($t_1 = 17.8$ s, $k_1 = 0.056$ s$^{-1}$) and the wash off of the S5P peptide effect ($t_2 = 53.7$ s, $k_2 = 0.019$ s$^{-1}$).
D. Dose dependence of effects of S5P peptide on currents recorded at -120 mV (n=3-4 at each dose), using the protocol shown in A. The IC\textsubscript{50} was obtained by fitting a Hill function to the mean data.

Figure 7

A. A typical example of a family of currents recorded during a double pulse protocol to examine the voltage dependence of S5P peptide effects on HERG channels. The cell was depolarized from a resting membrane potential of -80 mV to +40 mV for 500 ms then stepped to potentials in the range +30 to -110 mV for 2 s. The channels were deactivated by hyperpolarizing the cell to -120 mV for 800 ms and the protocol repeated except that during the second test pulse 100 µM S5P peptide was applied for 1 s (as indicated by the solid bar). The cell membrane potential was then held at -80 mV for 53 s between each sweep. The thick lines show the current traces recorded for the +30 mV test pulse (solid arrows) and –50 mV test pulse (dotted arrows). Addition of peptide caused a significant increase in current at +30 mV but a marked suppression of current at –50 mV. Horizontal dotted line indicates zero current.

B. A typical example of the mean ± SEM of the current recorded during five successive sweeps at 60s intervals where the test step was -70 mV for all five sweeps. The voltage protocol is illustrated above the current trace and the dotted boxes (thin, control; thick, + S5P peptide) indicate the regions that are plotted. 100 µM S5P peptide was applied for 1 s as indicated by the thick line in the voltage protocol. The effect of the peptide was reproducible (as indicated by the small error bars) and the effect of the peptide washed off within 60 s. The horizontal dotted line indicates the zero current level. It is worth noting that the current
recorded at -70 mV in the presence of the peptide reversed from an outward current to an inward current.

**Figure 8.**

Typical examples of the effect of 100 µM S5P peptide applied for 1 s on HERG currents recorded during test potential steps in the range +30 to –110 mV. The dotted boxes (thin, control; thick, + S5P peptide) on the voltage protocol indicate which regions are shown in the current traces. Application of S5P peptide resulted in increased current at depolarized potentials ( –20 mV), a more rapid decline in current at intermediate potentials (-30 to -60 mV) and a reversal of current from outward to inward at -70 and -80 mV. At the most negative voltages the peptide had little observable effect on the current.

**Figure 9**

A. Typical examples of current traces (control: thin trace; + S5P peptide: thick trace) obtained for test pulses of a) +30 mV, b) -20 mV and c) -70 mV (taken from the same cell as used in Figure 8). The effect of S5P peptide on HERG currents was quantified by fitting single exponential functions (thin lines superimposed on current traces) to the ascending (denoted by open boxes) or descending (denoted by closed box) portions of the currents recorded during application of S5P peptide. In panel a) the onset of the current increase in the presence of S5P peptide had a time constant of 123 ms and in panel b) the time constant was 89 ms. At -20 mV the current subsequently decreased in the continued presence of peptide with a time constant of 1.4 s. At -70 mV the exponential fit to the decrease in current had a time constant of 65 ms. This time constant represents a composite of the time constants of
deactivation (223 ms, measured in the corresponding control current trace) and peptide binding. The apparent peptide binding time constant was obtained by dividing the current recorded in the presence of peptide (I_{S5P}) by the control current (I_{con}) see inset (also see text for details of method used to deconvolve time constants). The inset also highlights how we calculated the maximum decrease in current for test potentials in the range –30 mV to –70 mV. In this example the maximum decrease was 140% indicating that the current had reversed from outward to inward.

**B.** The mean ± SEM for the time constants measured from the ascending phase (open symbols, n=4) and descending phase (after correction for deactivation, closed symbols, n=5) of currents following addition of S5P peptide. There were no significant differences between the time constants measured at positive potentials (ANOVA). Time constants at voltages in the range –70 to –30 mV were significantly different from each other.

**C.** Summary of the maximum change (increase at positive potentials, open symbols, or decrease at potentials <= -20 mV, closed symbols) in current following application of 100 µM S5P peptide (mean ± SEM, n=4-5). Note there are two points for -20 mV reflecting the fact that the current initially increased but subsequently decreased in the presence of the peptide at this voltage.

**D.** Typical examples of current traces recorded at -70, -80 and -90 mV during control conditions and -60, -70 and -80 mV in the presence of S5P peptide. The reversal potential was calculated from the extrapolated current at the start of the test pulse. Extrapolations were obtained by fitting a single exponential to the slow decaying phase of the current. In this example the reversal potential was shifted from 80 mV to 64 mV during addition of the S5P peptide.
Structure of HERG S5P extracellular linker

**E.** Typical examples of currents recorded at +30 mV during application and washoff of 100 µM S5P peptide (indicated by thick bar above voltage protocol). Thin lines show single exponential fits used to obtain time constants for the apparent binding (123 ms) and washoff (2.26 s) of the peptide.

**Figure 10.**

**A.** Far UV CD spectra for S5P peptides. All three peptides display a predominantly random coil conformation in aqueous environment (thin lines) with little evidence for secondary structure. However, in the presence of SDS micelles (thick lines) both the S5P peptide and the helix peptide display elements of helical structure whereas the del-helix remains unstructured. Calculation of the mean residue molar ellipticity, which takes into account the number of peptide bonds in the peptide, indicates that the helix peptide is almost completely helical in SDS micelles, with very little of the sequence in alternative conformations whereas the S5P contains significant random coil content in addition to the helical elements. This is consistent with the experimentally determined structure of the S5P peptide and what would be expected from secondary structure prediction algorithms.

**B.** Typical examples of HERG currents recorded at –80 mV test potentials under control conditions (thin traces) and following addition of peptides (thick traces). S5P peptide causes a shift to inward current, the del-helix peptide has minimal effect on the current and the helix peptide has a similar effect to the full-length peptide. The dotted line in each trace indicates the zero current level.

**Figure 11**
A. Typical examples of the effect of 100 µM helix peptide applied for 1 s (denoted by solid bar on voltage protocol) on HERG tail currents recorded during voltage steps to 0 mV, –40 mV and –80 mV. The control traces are shown using thin lines and the peptide traces shown using thick lines. Horizontal dotted lines indicate the zero current level.

B. Summary of the time constants for increases in current (open symbols) and decreases in current (closed symbols) following application of the peptide (mean ± SEM, n=4) analysed as described in the legend to Figure 9B.

C. Maximal percentage change in current (open symbols, increase in current for voltage range +30 to -20 mV; closed symbols, decrease in current for voltage range -20 to -70 mV) following addition of 100 µM helix peptide (mean ± SEM, n=4). Analysed as described in legend to Figure 9C. The error bars are in general smaller than the size of the symbols.

Figure 12

A. Typical examples of HERG tail currents recorded at 0, -40 and -80 mV in Na⁺ free (replaced with NMDG⁺) solution under control conditions (thin traces) or following addition of 100 µM S5P peptide (thick traces, left hand panel) or helix peptide (thick traces, right hand panel). The peptides caused a small decrease in current at all potentials. Neither peptide caused any reversal of current flow at –80 mV. Horizontal dotted lines indicate zero current level. Dotted box on the voltage protocol indicates region of the trace shown in each panel.

B. Typical examples of current traces recorded before (thin trace) and after 1 minute superfusion with 100 µM S5P or helix peptide (thick traces) in Na⁺ free (replaced with NMDG⁺) solution. The dotted boxes on the voltage protocol indicate regions of the trace
shown in each panel. Both peptides, after 1 minute application, caused ~60% decrease in current at –120 mV. Horizontal dotted lines indicate zero current level.
Table 1: Summary of effects of S5P-, helix- and del helix-peptides on HERG current

|                  | S5P peptide | Helix peptide | Del-helix peptide |
|------------------|-------------|---------------|-------------------|
| $E_{\text{rev}}$ (mV) | 16 ± 2      | 14 ± 2        | 0.7 ± 0.3†        |
| $t_{\text{on}}$ (30 mV) (ms) | 168 ± 35    | 27 ± 6*       | 1630 ± 200†       |
| $t_{\text{off}}$ (30 mV) (s) | 2.06 ± 0.6  | 0.85 ± 0.15*  | Nd                |
| $I_{(30 \text{ mV})}$ (%)   | 56 ± 22     | 9 ± 3*        | -9 ± 4†           |
| $t_{\text{on}}$ (0 mV) (ms) | 145 ± 32    | 36 ± 7*       | 890 ± 140†        |
| $I_{(0 \text{ mV})}$ (%)    | 40 ± 8      | 33 ± 3        | -11 ± 3†          |
| $t_{\text{on}}$ (-60 mV) (ms)| 99 ± 9      | 71 ± 17*      | 2550 ± 440†       |
| $I_{(-60 \text{ mV})}$ (%)  | -71 ± 9     | -70 ± 10      | -21 ± 5†          |

* significantly different between S5P and helix peptides
† significantly different between del-helix peptide and S5P and helix peptides
Nd not determined. For the S5P- and Helix-peptides measurements are from n=4-6 cells and for the Del-helix peptide n=3.
Structure of HERG S5P extracellular linker
Structure of HERG S5P extracellular linker

Figure 2
Structure of HERG S5P extracellular linker

Figure 3
Structure of HERG S5P extracellular linker

Figure 4
Structure of HERG S5P extracellular linker

Figure 5
Structure of HERG S5P extracellular linker

Figure 6
Figure 7
Figure 8
Structure of HERG S5P extracellular linker

Figure 9
Figure 10
Structure of HERG S5P extracellular linker

Figure 11
Structure of HERG S5P extracellular linker

Figure 12
### Supplementary data – Structural statistics for the NMR data

#### Table S1 Proton chemical shifts of S5-P in SDS at pH 3.3 and 303K\(^1\)

| Residue | NH  | C\(\alpha\)H | C\(\beta\)H | C\(\gamma\)H | Others          |
|---------|-----|--------------|-------------|-------------|----------------|
| Ala1    | -   | 4.24         | 1.57        |             |                 |
| Ile2    | 8.39 | 4.13         | 1.91        | C\(\gamma\)H 0.97 | C\(\beta\)H 0.92 |
|         |      |              |             | C\(\gamma\)H 1.25,1.57 |                 |
| Gly3    | 8.27 | 3.93         |             |             |                 |
| Asn4    | 8.16 | 4.76         | 2.76,2.90   | N\(\alpha\)H 6.82,7.53 |                 |
| Nle5    | 7.97 | 4.21         | 1.82        | 1.41        | C\(\gamma\)H 1.34 C\(\beta\)H 0.90 |
| Glu6    | 7.96 | 4.39         | 1.98,2.12   | 2.46        |                 |
| Gln7    | 8.06 | 4.45         | 1.97,2.03   | 2.32        | N\(\alpha\)H 6.72,7.46 |
| Pro8    | -   | 4.46         | 2.02,2.28   | 1.87,1.96   | C\(\gamma\)H 3.66,3.78 |
| His9    | 8.37 | 4.77         | 3.24,3.30   | C(2)H 8.62; C(4)H 7.37 |
| Nle10   | 8.11 | 4.17         | 1.81        | 1.34        | C\(\gamma\)H 0.89 |
| Asp11   | 8.44 | 4.59         | 2.93        |             |                 |
| Ser12   | 8.07 | 4.45         | 3.94        |             |                 |
| Arg13   | 8.10 | 4.41         | 1.93        | 1.7         | C\(\gamma\)H 3.07,3.12; N\(\alpha\)H 7.12 |
| Ile14   | 7.76 | 4.05         | 1.89        | C\(\gamma\)H 0.86 | C\(\beta\)H 0.81 |
|         |      |              |             | C\(\gamma\)H 1.22,1.49 |                 |
| Gly15   | 8.34 | 3.93,4.04    |             |             |                 |
| Trp16   | 7.77 | 4.41         | 3.38,3.43   | C(2)H 7.37; N(1)H 9.96 |
|         |      |              |             | C(7)H 7.41; C(6)H 7.06 | C(5)H 6.96; C(4)H 7.42 |
| Leu17   | 7.76 | 3.90         | 1.49,1.54   | 1.58        | C\(\gamma\)H 0.81,0.89 |
| His18   | 8.04 | 4.40         | 3.29,3.34   | C(2)H 8.64; C(4)H 7.35 |
| Asn19   | 8.06 | 4.57         | 2.77,2.88   | N\(\alpha\)H 6.86,7.46 |
| Leu20   | 7.81 | 4.12         | 1.65        | 1.60        | C\(\gamma\)H 0.84 |
| Residue | δ  | J  | Chemical Shifts          |
|---------|----|----|--------------------------|
| Gly21   | 8.35 | 3.78,3.94 | N\H_2 6.72,7.31 |
| Asp22   | 7.93 | 4.60 | 2.96  |
| Gln23   | 8.05 | 4.33 | 2.12,2.23 | 2.41 |
| Ile24   | 7.68 | 4.16 | 1.97  | C[H]_3 0.98 | C[H]_3 0.89 | C[H]_2 1.26,1.59 |
| Gly25   | 8.02 | 3.90,3.94 | |
| Lys26   | 7.83 | 4.54 | 1.72,1.74 | 1.44,1.46 | N\H_3^+ 7.43 |
| Pro27   | 4.44 | 1.86,2.24 | 1.96  | C[H]_2 3.63,3.78 |
| Tyr28   | 7.83 | 4.53 | 3.02  | C(3,5)H 6.81; C(2,6)H 7.08 |
| Asn29   | 8.12 | 4.70 | 2.72,2.84 | N\H_2 6.85,7.54 |
| Ser30   | 8.12 | 4.41 | 3.87,4.00 | |
| Ser31   | 8.27 | 4.44 | 3.95  | |
| Gly32   | 8.20 | 3.95,4.03 | |
| Leu33   | 8.00 | 4.36 | 1.64,1.71 | 1.60  | C[H]_3 0.89,0.94 |
| Gly34   | 8.20 | 3.98 |   | |
| Gly35   | 8.06 | 4.09 |   | |
| Pro36   | -   | 4.46 | 2.32  | 1.96,2.04 | C[H]_2 3.64,3.69 |
| Ser37   | 8.17 | 4.48 | 3.92,3.97 | |
| Ile38   | 8.08 | 4.04 | 2.03  | C[H]_3 1.00 | C[H]_3 0.95 |
|         |     |     |     | C[H]_2 1.31,1.63 |
| Lys39   | 8.01 | 4.09 | 1.80,1.85 | 1.45,1.51 | C[H]_2 1.72; C[H]_2 3.01 |
|         |     |     |     |     | N\H_3^+ 7.43 |
| Asp40   | 7.88 | 4.62 | 2.93  | |
| Lys41   | 7.76 | 4.16 | 1.61,1.70 | 1.12,1.26 | C[H]_2 1.62; C[H]_2 2.95 |
|         |     |     |     |     | N\H_3^+ 7.41 |
| Tyr42   | 7.67 | 4.68 | 2.84,3.16 | |

1 Proton chemical shifts
Table S2 Structural statistics for the ensemble of 20 S5-P structures in presence of SDS micelles

| Distance restraints                      |       |
|------------------------------------------|-------|
| Intraresidue (i–j = 0)                   | 189   |
| Sequential (li–jl = 1)                   | 153   |
| medium-range (li–jl ≥ 5)                 | 74    |
| long-range (li–jl > 5)                   | 0     |
| hydrogen-bonds                           | 14    |
| Total                                    | 430   |

Atomic rms differences (Å)

|                              | versus mean | pairwise    |
|------------------------------|-------------|-------------|
| backbone atoms (14-24)       | 0.31 ± 0.12 | 0.44 ± 0.17 |
| heavy atoms (14-24)          | 0.73 ± 0.13 | 1.02 ± 0.22 |
| backbone atoms (35-42)       | 0.23 ± 0.07 | 0.31 ± 0.13 |
| heavy atoms (35-42)          | 0.68 ± 0.16 | 0.95 ± 0.23 |

1Atomic differences given as average rms difference against mean coordinate structure (mean) and average rms difference of all pairwise structures (pairwise). Values given as mean ± SD.
Structure of the HERG K+ channel S5P extracellular linker: Role of an amphipathic α-helix in c-type inactivation

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