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Protein Surface Recognition by Rational Design: Nanomolar Ligands for Potassium Channels

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Potassium channels are among the core features of life.[1] They have been identified in organisms as diverse as archaebacteria and humans,[2] occurring in virtually every eukaryotic and prokaryotic cell. Their ability to control the transmembrane-potential underpins elementary cellular functions such as excitability, proliferation, secretion, and volume regulation.

Recently, the three-dimensional structures of the prokaryotic potassium channels KcsA[3] and MthK,[4] as well as the voltage-gated channel K_vAP,[5] have been solved by X-ray crystallography. These crystal structures confirmed the remarkably conserved architecture of all potassium channels. As shown in Figure 1, four subunits assemble to form the central pore. Amino acid residues close to the pore line the so-called outer vestibule, a shallow depression on the extracellular surface of the channels.

Due to the detailed structural information that is now available, potassium channels provide a unique platform with which to explore the principles of protein surface recognition.[6] The interaction of ligands with channels can be determined in binding assays using radiolabeled peptide inhibitors[7] or in functional assays involving radioactive $^{86}Rb^+$ ions.[8] Electrophysiological methods can also be used to investigate the blocking characteristics of ligands, either at the whole cell (voltage clamp) or single channel (patch clamp) level.

A large number of natural peptide inhibitors that bind to the pore region of these channels have been isolated from scorpions, snakes, spiders, and other organisms.[9] In addition to this, a limited set of small-molecule blockers that bind in the same region of the channel, such as tetraethylammonium ion (TEA), have been identified. None of these inhibitors, however, make full use of the four-fold symmetry of a homotetrameric channel. Double-cycle mutant studies have identified residues in the channel's outer vestibule that are essential for high-affinity interaction of peptide inhibitors.[10] Some of these “hot spot” residues A-C are highlighted in a sequence alignment of KcsA with several potassium channels of the K_v1x class. They are mapped onto the crystal structure of KcsA in Figure 1. Note that the residues shown in blue (A) and green (C) are highly variable. By contrast, the residue highlighted in red (B), aspartate or glutamate, is conserved within the K_v1x family. The conserved GYG signature sequence of potassium channels is highlighted in yellow.

We now report the development of a new class of synthetic ligands for potassium channels. These four-fold symmetrical molecules are designed to mimic the peptide toxins and to interact

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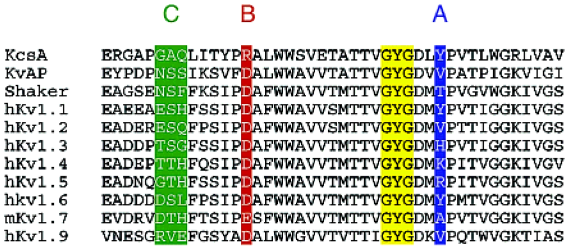
with all four channel subunits simultaneously, resulting in a strong polyvalency effect.[11] They bind to voltage-gated potassium channels of the K_v1x class, such as *Shaker* and $K_v1.3$, with nanomolar affinities, and partially block the conductance of the channels in a reversible fashion.



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KcsA      ERGAPGAQLITYPRALWWSVETATTVGYGDLYPVTLWGRLVAV
KvAP      EYPDPNSSIKSVFDALWWAVVTATTVGYGDVVPATPIGKVIGI
Shaker    EAGSENSFFKSIPDAFWWAVVTMTTVGYGDMTPVGVWGKIVGS
hKv1.1    EAEEAESHFSSIPDAFWWAVVSMTTVGYGDMYPVTIGGKIVGS
hKv1.2    EADERESQFPSIPDAFWWAVVSMTTVGYGDMVPTTIGGKIVGS
hKv1.3    EADDPTSGFSSIPDAFWWAVVTMTTVGYGDMHPVTIGGKIVGS
hKv1.4    EADEPTTHFQSIPDAFWWAVVTMTTVGYGDMKPITVGGKIVGV
hKv1.5    EADNQGTHFSSIPDAFWWAVVTMTTVGYGDMRPITVGGKIVGS
hkv1.6    EADDDDSLFPSIPDAFWWAVVTMTTVGYGDMYPMTVGGKIVGS
mKv1.7    EVDRVDTHFTSIPESFWWAVVTMTTVGYGDMAPVTVGGKIVGS
hKv1.9    VNESGRVEFGSYADALWWGVVTVTTIGYGDKVPQTWVGKTIAS
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Our ligands are water-soluble tetraphenylporphyrin derivatives whose structures are shown below.[12] The compounds were synthesized by coupling of tetraphenylporphyrin tetracarboxylic acid **1** or its acid chloride **2** with protected amino acids, peptides, or diamines, followed by deprotection (see Supporting Information). A similar molecular architecture has been used by Hamilton to recognize the surface of cytochrome

[redacted]

C.[13]

In aqueous solution near physiological pH, compounds **3-7** are, most likely, tetracations, compounds **8-10** would contain eight positively charged groups, whereas **11** and **12** are mostly neutral. Compound **13**, on the other hand, contains four negatively charged side chains.

The interaction of the porphyrin ligands with potassium channels was investigated in competitive binding assays with ^{125}I-hongotoxin$_1$-A19Y/Y37F (^{125}I-HgTX$_1$A19Y/Y37F) and in electrophysiological assays using the *Xenopus* oocyte system. Figure 2 shows the effect of our ligands on ^{125}I-HgTX$_1$A19Y/Y37F binding to membranes prepared from HEK293 cells stably transfected with the human $K_v1.3$ channel. The voltage-gated potassium channel $K_v1.3$ plays a crucial role in human T-lymphocyte activation.[14] ^{125}I-HgTX$_1$A19Y/Y37F binds to the outer vestibule of $K_v1.3$ channels with femtomolar affinity (K_d) 0.046 pM), in a bimolecular, reversible reaction.[15]

Ligands **3** and **5-12** inhibit ^{125}I-HgTX$_1$A19Y/Y37F, binding to $K_v1.3$ in a dose-dependent manner, whereas ligands **4** and **13** have no effect at concentrations up to 10 μM (Figure 2). The K_i values for inhibition of ^{125}I-HgTX$_1$A19Y/Y37F binding for these ligands are presented in Table 1.

Data from Figure 2 and Table 1 indicate that positively charged ligands with appropriate geometry, such as **3**, **9**, and **10**, strongly interact with the $K_v1.3$ channel. According to our binding model, their cationic side chains could form salt bridges to the conserved anionic aspartate residues in position B (D381). By contrast, tetracationic ligand **4**, which is a conformationally restricted and bulkier version of ligand **3**, does not compete for ^{125}I-HgTX$_1$A19Y/Y37F binding to $K_v1.3$ channels. Moreover, negatively charged porphyrins such as **1** and its glycine derivative **13** do not interfere with ^{125}I-HgTX$_1$A19Y/Y37F binding. Note, that the overall neutral ligands **11** and **12** bind with markedly different affinities.

Further evidence for the proposed interactions between the ligands and the pore of the potassium channel was obtained from electrophysiological experiments. Ionic currents were measured in *Xenopus* oocytes that express *Shaker*, the archetypical K_v1x channel.[16] The effect of two ligands on these currents is shown in Figure 3. Cationic porphyrin **3**, one of the ligands to bind with the highest affinity, significantly inhibited the *Shaker* current, in a reversible fashion, whereas anionic porphyrins, such as **13**, which lacked specific/high affinity binding, had virtually no effect. It is interesting to note that our ligands do not completely block ionic current through *Shaker* channels even at high concentrations. A similar observation concerning the lack of complete inhibition of currents has been observed for the peptide δ-dendrotoxin and the inward rectifier ROMK1 potassium channel.[17]

These results, taken together, provide strong evidence that our ligands bind in the outer vestibule of potassium channels. Whether they simultaneously interact with all four subunits of the tetrameric channel remains to be determined. The modular composition of our ligands allows easy modifications and should provide a large set of synthetic probes that discriminate among different potassium channels. Future directions will concern the synthesis of metalloporphyrins and fluorescent porphyrins with extended side chains. Ideally, our compounds could surpass antibodies by discriminating between closely related channels, such as the K_v1x channel subtypes, or different functional states. Ultimately, the development of this class of compounds may lead to novel therapeutic agents against various ailments such as autoimmune disorders, diabetes, epilepsy, or cardiac diseases.[18]

Supporting Information Available

Synthetic procedures and spectroscopic data for compounds **3**-**13** as well as experimental details concerning the ^{125}I-HgTX$_1$A19Y/Y37F binding and electrophysiological assays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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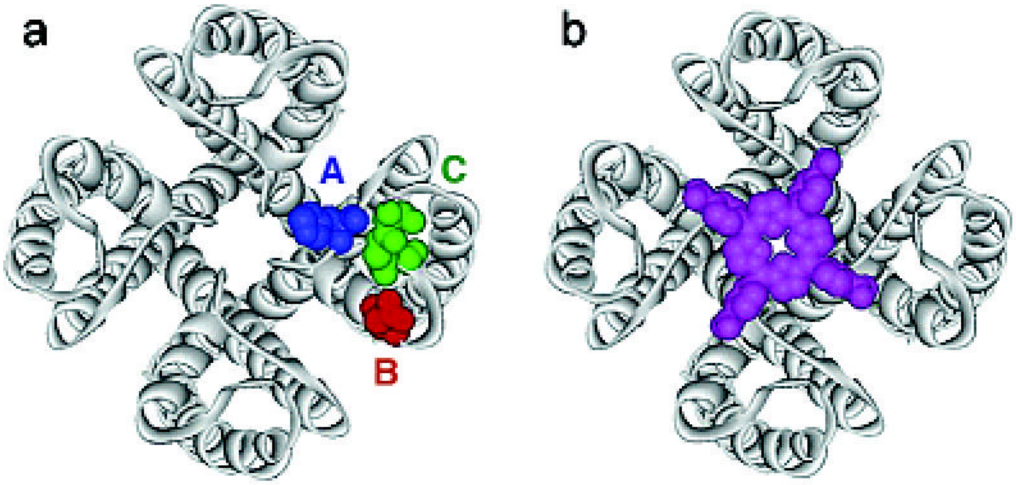

Figure 1.
(a) X-ray structure of KcsA. The approximate location of “hot spot” residues A-C on the surface of potassium channels is indicated. (b) Overlay of KcsA with tetraphenylporphyrin **1** (magenta).

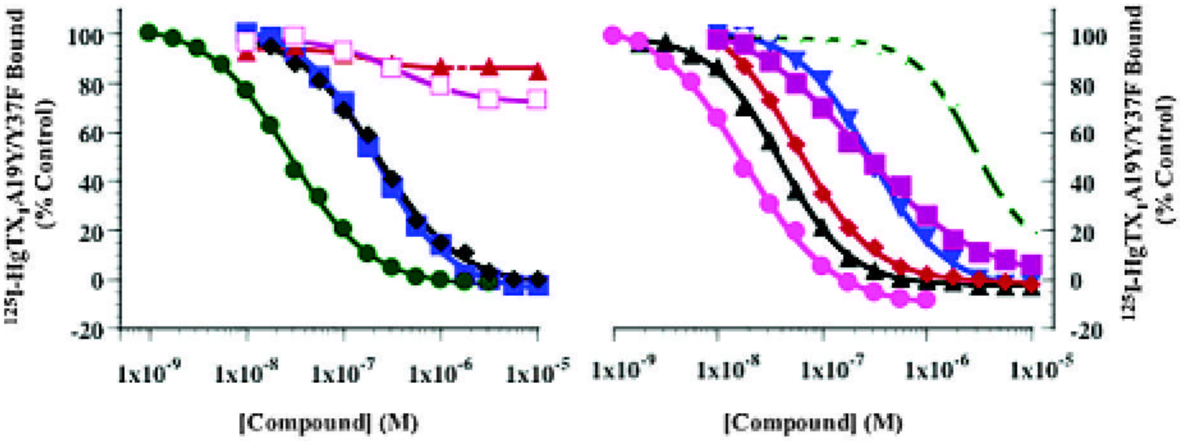

Figure 2.

Binding of ^{125}I-HgTX$_1$A19Y/Y37F to K_v1.3 channels. Membranes prepared from HEK293 cells stably transfected with the K_v1.3 channel were incubated with ~0.13 pM ^{125}I-HgTX$_1$A19Y/Y37F, in the absence or presence of increasing concentrations of (left panel) compound **3** (solid green circles), compound **4** (solid red triangles), compound **6** (solid black tilted squares), compound **7** (solid blue squares), compound **13** (open magenta squares); (right panel) compound **5** (solid blue triangles), compound **8** (solid black triangles), compound **9** (solid pink circles), compound **10** (solid red tilted squares), compound **11** (open green circles), compound **12** (solid magenta squares), for 20 h at room temperature. Inhibition of binding was assessed relative to an untreated control. Specific binding data can be fit to a single-site inhibition model, as described in the Supporting Information.

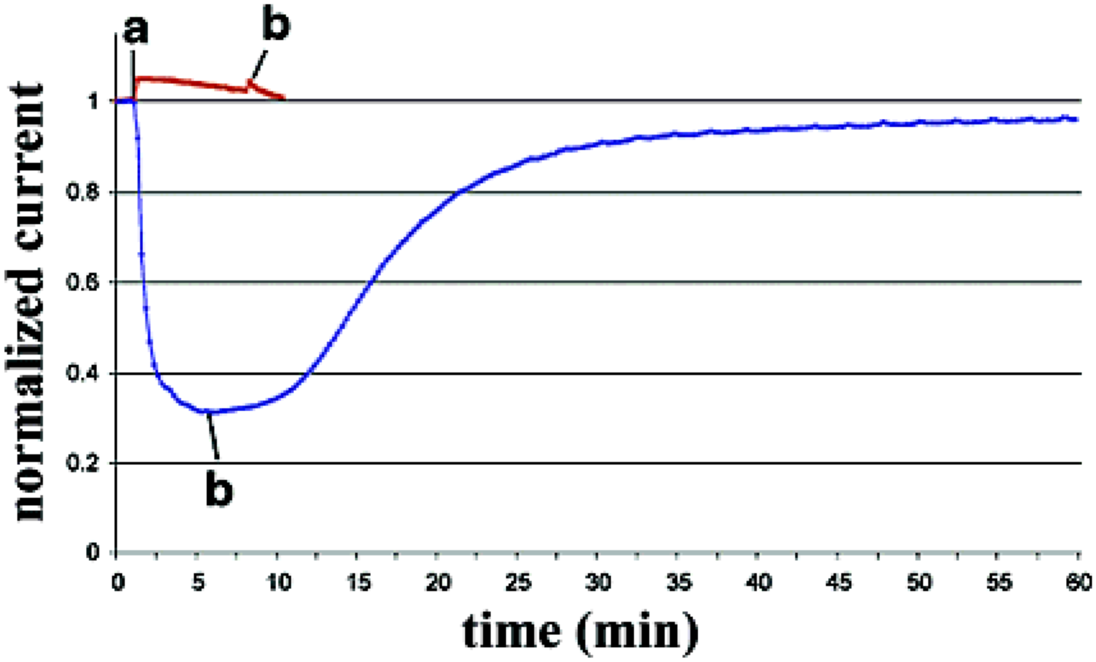

Figure 3.

Effect of 2.5 μM ligand **3** (blue) and 100 μM ligand **13** (red) on *Xenopus* oocytes expressing *Shaker* channels. Ligand was applied at a. Perfusion with control solution began at b.

Table 1.

K_i's for Ligands Based on Displacement Assays

ligand	**K_i[μM]**	**ligand**	**K_i[μM]**	**ligand**	**K_i[μM]**
3	0.020	7	0.144	10	0.038
5	0.138	8	0.026	11	1.918
6	0.157	9	0.013	12	0.156