Structural and biological approaches to further understand the physiology and pharmacology of the SK channels

Mouchet A.[1], Vitello R.[2], Falinski S.[2], Robaye L.[3], Liégeois J.F.[2], Seutin V.[3], Brans A.[1], Kerff F.[1]

[1] Centre for Protein Engineering (CIP) - InBios ; [2] Laboratory of Medicinal Chemistry - C.I.R.M. ; [3] Laboratory of Pharmacology - GIGA-Neurosiences, University of Liège

Summary

- Small conductance calcium-activated potassium (SK) channels represent potential targets for numerous central and peripheral nervous system disorders such as schizophrenia or mood disorders. The development of new non-peptidic blockers combining high affinity and selectivity towards SK subtypes is crucial and requires a better knowledge of their structure and of their mechanism of action.

Objectives

- Generate mutants of SK channels and test their activity and their affinity for inhibitory peptides such as apamin *in vitro*.
- Study the three-dimensional structure of SK channels (wild-types and mutants) and identify the important features for their mechanism of action at the atomic level using cryo-EM.

Introduction

- SK channels are selective for K+ ions and are gated by Ca^{2+} via calmodulin molecules[1].
- Three subtypes of SK subunits have been cloned and shown to be expressed differentially within the central nervous system (CNS)[2,3]. SK1 and SK2 are mostly expressed in the cortex and hippocampus while SK3 expression is higher in the thalamus and hypothalamus.

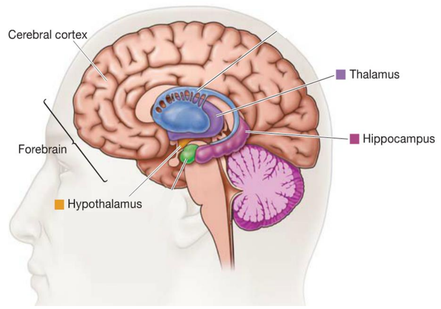

Figure 1. Scheme of the human brain featuring the cortex, hippocampus, thalamus and hypothalamus.

- SK channels underlie the medium duration afterhyperpolarization (mAHP) which plays an important role in modulating the firing rate and pattern of different types of neurons[4,5]. They slow down the return to the resting potential of the membrane and lower the frequency of the excitation peaks. The excitation cycle of a neuron is detailed in *Figure 2A*. The repolarization and refractory period are caused by the release of K+ ions du to the opening of K+ channels. *Figure 2B* highlights the different steps of the AHP period.

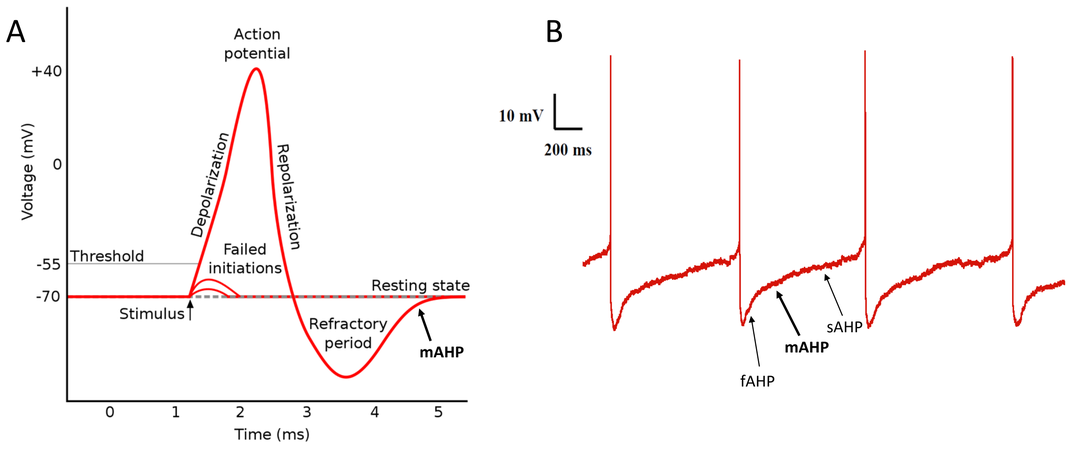

Figure 2. (A) Excitation cycle of a neuron with the different steps detailed, (B) experimental recording of the excitation peaks of a neuron. fAHP, mAHP and sAHP represent fast, medium and slow afterhyperpolarization respectively.

- One protein of SK channel consists of 6 transmembrane domains, S1 to S6, with the N and C-terminal parts located in the cytoplasm and a P-loop corresponding to the pore domain between S5 and S6 (*Figure 3A*)[6]. The sequence of this loop in SK1, 2 and 3 is shown in *Figure 3B*. A 4 amino acids motif forms the selectivity filter (circled in red in *Figure 3C*) which plays a major role in regulating the flux of potassium ions by reducing the space in the channel. One channel is formed by 4 SK subunits.

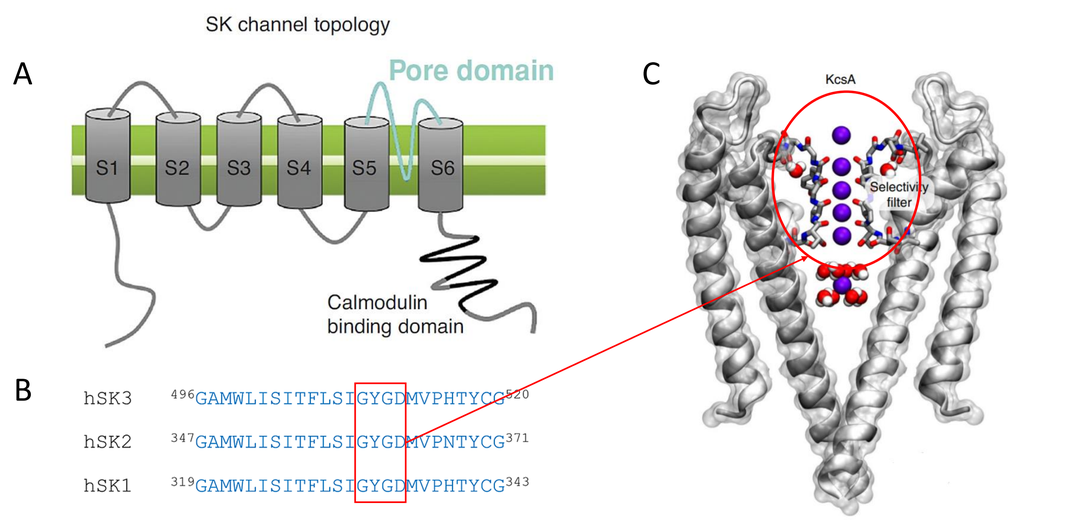

Figure 3. (A) General topology of SK proteins [6], (B) sequence alignment of the pore domain in SK1, 2 and 3, (C) 3D representation of the S5-S6 domains with the P-loop and the selectivity filter in KcsA channel.

- SK channels can be blocked by a whole series of inhibitors, including apamin, a neurotoxin found in bee venom[7]. By studying the structure of SK channels and their interactions with apamin, we aim to develop new non-peptidic blockers capable of acting specifically on the different subtypes of SK channels.

Methods

1) 3D modeling of SK channels by using the **AlphaFold** protein structure modeling software.

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2) Insertion of mutations in the genes coding for SK proteins by **sited directed mutagenesis**.

↓

3) Expression of the proteins **in HEK293** cells.

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4) Testing of **apamin affinity** to mutant channels by using **binding assay** with radiolabeled ^{125}I-apamin.

5) Testing of mutant activity with *in vitro* electrophysiological **patch clamp experiments**.

Analysis of the 3D structure of the channels by **cryo-electron microscopy**.

Preliminary results

- The models obtained (*Figure* 4) highlight a particular conformation of the extracellular L3 loop (blue) that has never been observed before. A phenylalanine residue (red) included in this loop seems to be located close to the pore of the channel, near the selectivity filter (pink). The proximity of the Phe residues of the four monomers in SK1, 2 and 3 could have an impact on the flux of K+ ions and make their transit more difficult. This could explain the lower currents displayed by those channels compared to SK4 channel in which this feature is not observed, and be involved in the blocking by apamin.

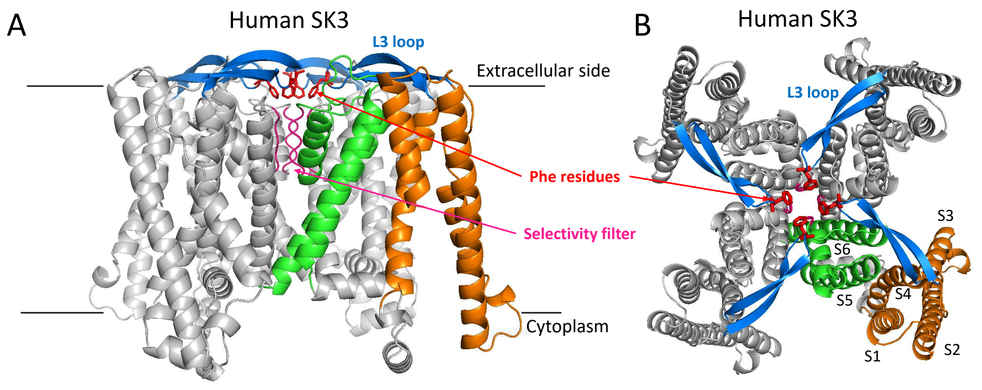

Figure 4. Cartoon representations of the transmembrane domains of the SK3 channel model using AlphaFold. (A) Side view (B) Extracellular side view.

- We generated mutants of SK2 and SK3 by replacing the Phe residue either by alanine (A), serine (S), tryptophan (W) or tyrosine (Y).
- Competition binding assays showed that only hSK2 F243Y mutant has a high affinity for apamin (*Figure 5A*). Saturation assays with this tyrosine mutant showed that it has a K_d value similar to that of native SK channels (from 3.712 to 4.719 pM for the mutant, ~5pM for native channels).

A

Mutants	Radioactive activity in dpm (duplicate)	
hSK2 F234A	-29,5	91
hSK2 F234S	25	17
hSK2 F234W	40,5	15
hSK2 F234Y	**5438,5**	**5765,5**
hSK3 F392A	211	83,5
hSK3 F392S	-36	32,5
hSK3 F392W	49	223,5
hSK3 F392Y	-39	81,5

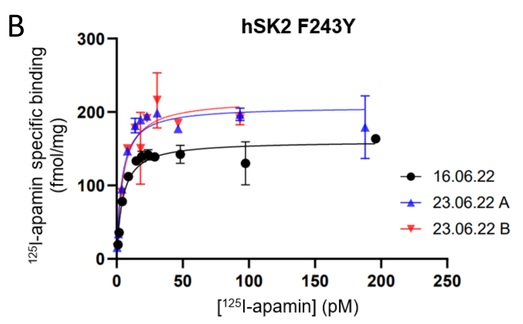

Figure 5. Binding assay with radiolabeled apamin. (A) Results (in dpm) of the competition assay performed on each mutant. (B) Saturation assay with the SK2 F243Y mutant (n=3).

- In line with the binding results, *in vitro* patch clamp experiments showed that the Tyr mutant is sensitive to apamin as can be seen from the reduction in current generated (Figure 6A). In contrast, the alanine mutant appears to be insensitive to apamin (Figure 6B). More tests are needed to confirm these results.

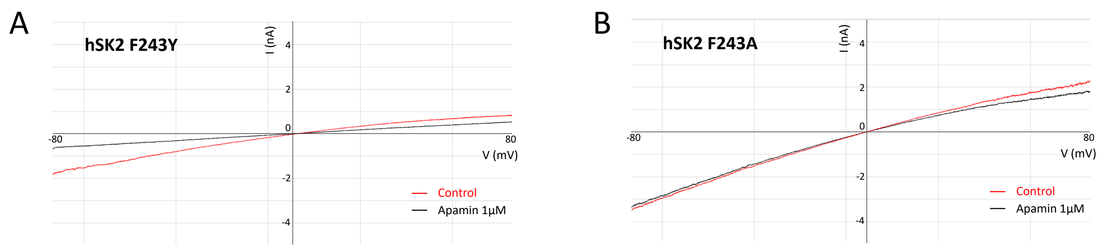

Figure 6. I-V relationships measured from whole-cell recordings obtained from HEK293 cells expressing (A) hSK2 F243Y mutant, (B) hSK2 F243A mutant. In red: control curves (without apamin), in black: recordings in presence of apamin 1µM.

References

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