Investigating the structure and pharmacology of SK channels using a dual biological and structural strategy

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Introduction

- Small conductance calcium-activated potassium (**SK**) channels are selective for K+ ions and are gated by Ca2+ via calmodulin molecules[1]. Three isoforms exist and are expressed differentially within the central nervous system (CNS), SK1 & SK2 being mostly expressed in the cortex and hippocampus while SK3 expression is higher in the thalamus, the hypothalamus and the brainstem [2,3].
- SK channels underlie the medium duration component of the afterhyperpolarization (AHP, shown on *Figure 1*) and play an important role in modulating the firing rate/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.
- SK channels have been shown to be involved in the development of some mental illnesses such as schizophrenia[6] and mood disorders.

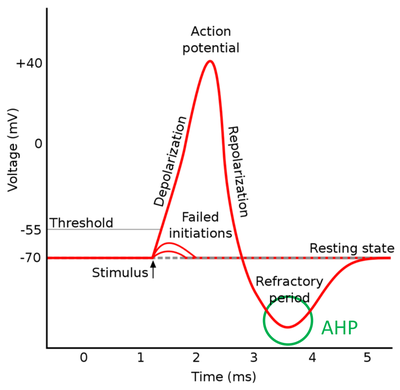

***Figure 1**. Excitation cycle of a neuron.*

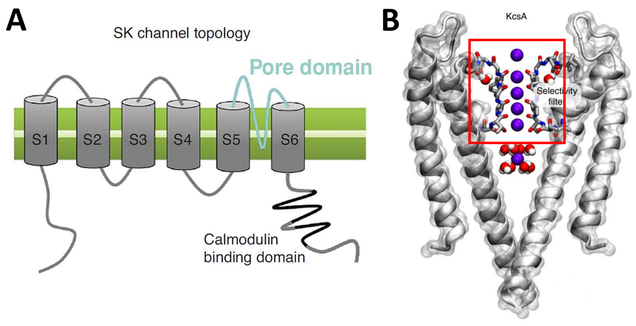

***Figure 2**. (**A**) General topology of SK proteins [7], (**B**) 3D representation of the S5-S6 domains with the selectivity filter in KcsA channel.*

- One monomer consists of 6 transmembrane domains, S1 to S6, with the N and C-terminal ends in the cytoplasm and the pore domain between the S5 and S6 helixes (*Figure 2A*)[7].
- The pore of the channel contains a region called the selectivity filter (circled in red on *Figure 2B*), essential for the regulation of the flux of K+ ions. Functional SK channels are tetramers.
- SK channels can be blocked by a whole series of inhibitors, including apamin, a neurotoxin found in bee venom[8]. By studying the structure and the pharmacology of SK channels, **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** 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

↓

4) Testing the **affinity** for apamin for each mutant channels by using **binding assay** with radiolabeled ^{125}I-apamin

5) Testing the **activity** of channels with ***in vitro*** **patch clamp** experiments

Analysis of the interactions between SK channels and blockers with **molecular docking**

AlphaFold Models

- The models obtained with AlphaFold highlight a particular conformation of the extracellular S3S4 loop (shown in red on *Figure 3A*) that is conserved in the three subtypes SK1-3 but different from the loop in SK4 *(Figure 3C)*. This loop contains a phenylalanine residue located just at the exit of the pore of the channel as shown in *Figure 3B-C*.
- As previous papers demonstrated the importance of amino acids next to this phenylalanine for the interaction with apamin[9], our hypothesis is that **the phenylalanine residue could also play a key role in the interaction and in the blocking mechanism by apamin** and potentially by some other blockers.

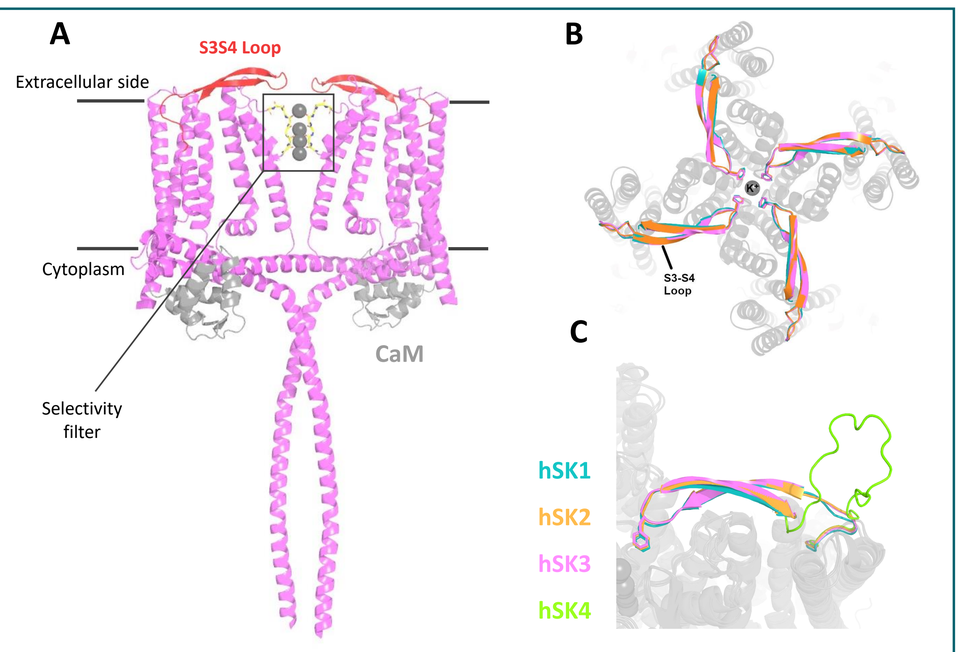


***Figure 3**. (**A**) Cartoon representation of the model obtained for human SK3 channel with AlphaFold. (**B**) Top view of SK channels highlighting the S3S4 loop. (**C**) Zoom in on the S3S4 loop in SK1 (blue), SK2 (orange), SK3 (pink) and SK4 (green).*

Molecular Docking

- In order to complement the patch clamp experiments shown below, molecular docking experiments were carried out with UCL1684, a chemical compound known to block SK channels currents. Simulation results show a similar position of UCL1684 when docking with the three SK channel subtypes and confirm the importance of the phenylalanine residue for the interaction with the compound.

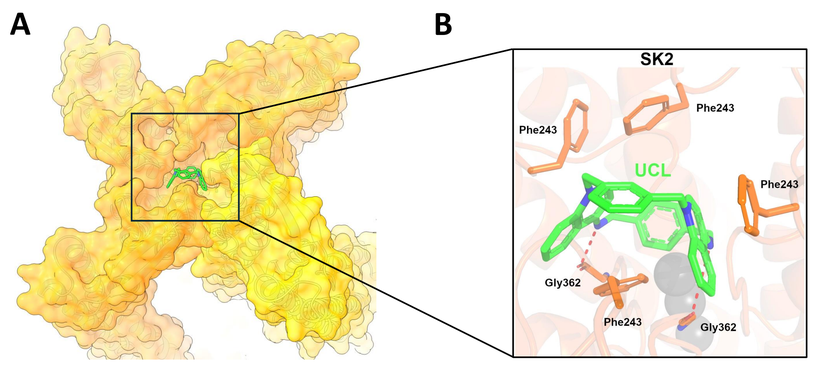


***Figure 4**. (A) Surface representation of hSK2 channel with UCL1684 in the binding pocket. (**B**) Zoom in on the binding pocket highlighting interactions between UCL1684 and hSK2 proteins.*

Affinity and activity tests

Mutants of hSK2 and hSK3 were generated by replacing the phenylalanine (F) of interest by either an alanine (A) or a tyrosine (Y). The affinity of mutant channels for apamin was screened through binding assays and their activity was tested with *in vitro* patch clamp experiments (whole-cell configuration, symmetrical K+ and 10 µM free Ca2+ in the pipette).

Binding assay

A

Mutants	Radioactive activity (dpm)		
	Total binding	Non specific	Specific Binding
hSK2 WT	4456	181,5	**4274,5**
	4154	217	**3937**
hSK3 WT	4726,5	293,5	**4433**
	4594,5	386	**4208,5**
hSK2 F243A	275,5	305	-29,5
	321	230	91
hSK2 F243Y	5673	234,5	**5438,5**
	6035,5	270	**5765,5**
hSK3 F392A	499,5	288,5	211
	270,5	187	83,5
hSK3 F392Y	212,5	251,5	-39
	296	214,5	81,5

B

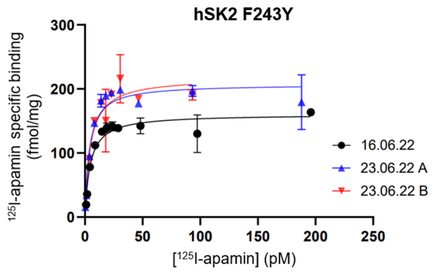
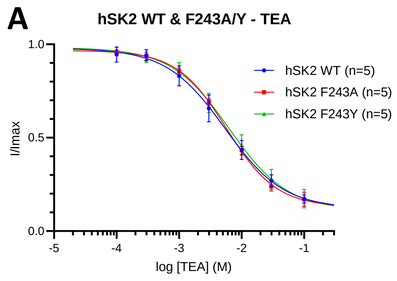


***Figure 5**. Binding assay with radiolabeled apamin. (**A**) Screening of the mutants. (B) Saturation assay with hSK2 F243Y.*

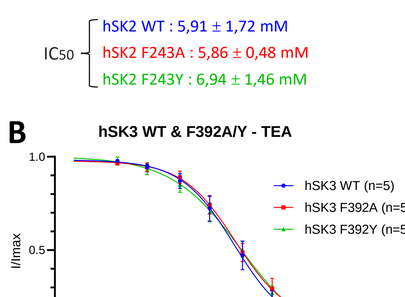
Screening of the mutants showed that only hSK2 F243Y bound to apamin (*Figure 5A*). Saturation assays with this tyrosine mutant (*Figure 5B*) showed that it has a K_D value similar to that of native SK channels (from 3.7 to 4.7 pM for the mutant, ~5pM for native channels).

in vitro Patch clamp

Tetraethylammonium (TEA)

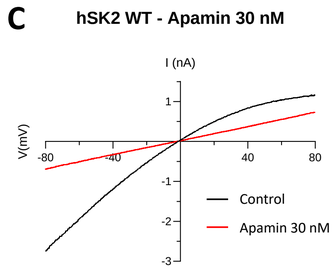


IC50 — hSK2 WT : 5,91 ± 1,72 mM; hSK2 F243A : 5,86 ± 0,48 mM; hSK2 F243Y : 6,94 ± 1,46 mM



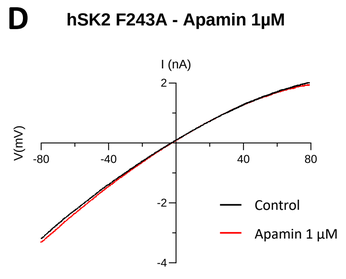
IC50 — hSK3 WT : 6,94 ± 2,09 mM; hSK3 F392A : 8,42 ± 1,67 mM; hSK3 F392Y : 9,89 ± 2,37 mM

Apamin



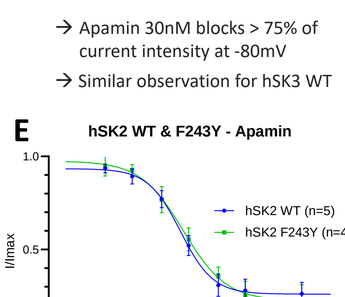
→ Apamin 30nM blocks > 75% of current intensity at -80mV

→ Similar observation for hSK3 WT

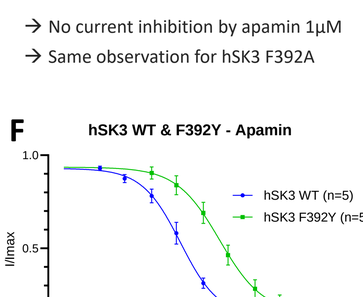


→ No current inhibition by apamin 1µM

→ Same observation for hSK3 F392A

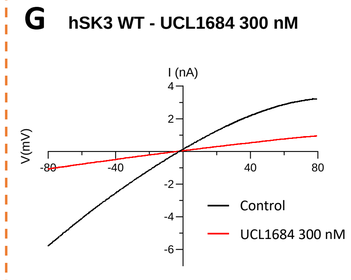


IC50 — hSK2 WT : 220,5 ± 41,3 pM; hSK2 F243Y : 247,3 ± 35,8 pM

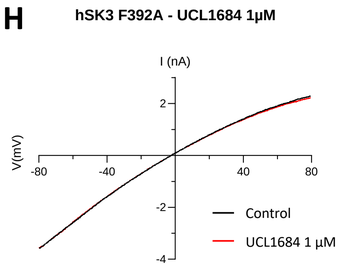


IC50 — hSK3 WT : 377,6 ± 94,7 pM; hSK3 F392Y : 2033 ± 245 pM; 2,03 ± 0,25 nM

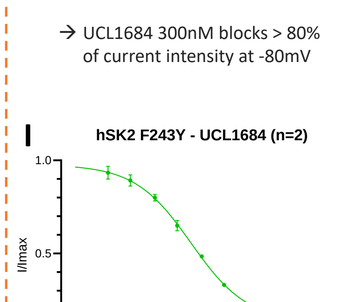
UCL1684



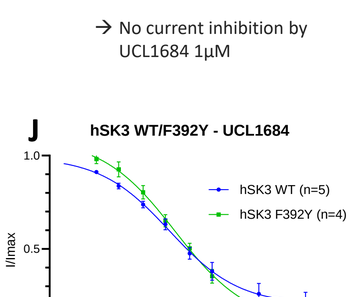
→ UCL1684 300nM blocks > 80% of current intensity at -80mV



→ No current inhibition by UCL1684 1µM



IC50: hSK2 F243Y : 605 ± 49 pM



IC50 — hSK3 WT : 413,6 ± 97,2 pM; hSK3 F392Y : 495,1 ± 72,7 pM

***Figure 6**. (**A-B**) Concentration-inhibition curves with TEA on wild type, alanine and tyrosine mutants in hSK2 and hSK3 channels respectively. (**C-D**) Current signal before (black) and after (red) adding high concentration of apamin on hSK2 WT and hSK2 F243A channels. Curves are obtained by averaging 5 experiments. (**E-F**) Concentration-inhibition curves with apamin on wild type and tyrosine mutants in hSK2 and hSK3 channels respectively. (**G-H**) Current signal before (black) and after (red) adding high concentration of UCL1684 on hSK3 WT and hSK3 F392A channels. Curves are obtained by averaging 5 experiments. (**I-J**) Concentration-inhibition curves with UCL1684 on wild type (hSK3) and tyrosine mutants (hSK2 & hSK3). All error bars correspond to SEM.*

Conclusion

- AlphaFold models of SK channels highlighted the particular conformation of the S3S4 loop and the presence of a conserved phenylalanine residue in hSK1, 2 and 3. Molecular docking experiments with UCL1684 supported the idea that this phenylalanine plays an important role in the interaction with the blocker. Binding assay and whole-cell patch clamp experiments on the alanine mutants in hSK2 and hSK3 proved that the phenylalanine residue is essential for the interaction and the subsequent blocking by apamin and UCL1684.

References

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