Uncovering Structural Determinants of SK2 and SK3 Channel Blockade for Selective Inhibitor Development

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Introduction

- Small conductance calcium-activated potassium (**SK**) **channels** are **selective for K^+** ions and are **gated by Ca^{2+}** via calmodulin (CaM) molecules[1]. **Three isoforms (SK1-3)** exist and are **expressed differentially within the central nervous system**[2,3].
- **SK channels** regulate the afterhyperpolarization (AHP) and **modulate the firing rate/pattern of different types of neurons**[4,5] (*Figure 1*).
- SK channels are involved in the development of mental illnesses such as **schizophrenia**[6] and **mood disorders**[7].
- Their activity can be regulated by using **blockers** like **apamin**[8], a neurotoxin found in bee venom, or **UCL1684**[9].

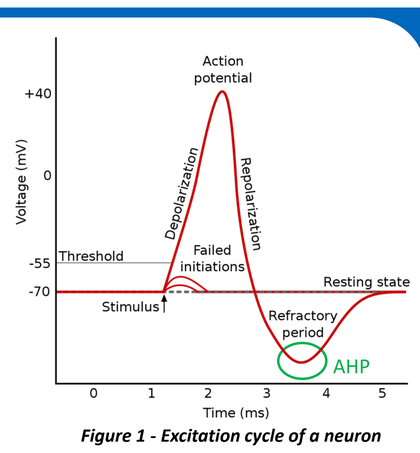

Figure 1 - Excitation cycle of a neuron

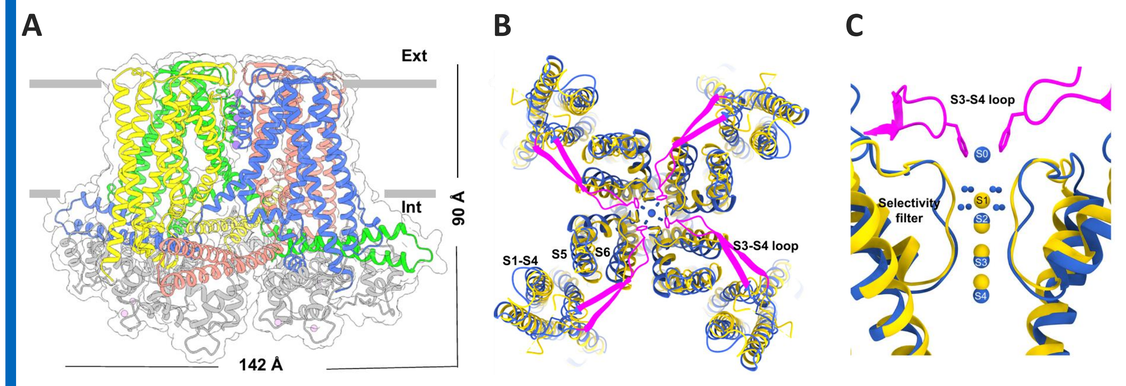

Figure 2 - SK2 structure *(Adapted from Nam YW, et al., 2025). (**A**) Cryo-EM density map with fitted model of SK2 viewed from the plane of the membrane. Four SK2 subunits are shown in blue, yellow, green and salmon, and CaM is shown in gray. (**B-C**) Superimposition of SK2 (blue) on SK4 (yellow). **(B)** Extracellular view - The four S3-S4 ß-hairpins (magenta) form a canopy over the outside of the pore. Phe243 residues at the tips of four S3-S4 ß-hairpins form an aromatic box with a central opening at the outer end of the pore. (**C**) Focus on the selectivity filter viewed from the plane of the membrane with two subunits shown.*

- Recently, a **cryo-EM structure of SK2** has been obtained (*Fig. 2A – PDB accession code : 8v2g*) [10]
- S3-S4 loops (β-hairpins) form a canopy over the outside of the pore (*Fig. 2B*).
- At the tip of the β -hairpin a Phe residue was shown to be essential for the proper folding of the loop and induces conformational changes that contribute to the low unitary conductance of SK channels.

Objective

- **Study of the conserved Phe residue at the tip of the S3-S4 loop and its role in the blocking by apamin and other compounds for the development of non-peptidic molecules with specificity for SK3.**

Methods

1) Mutation of the phenylalanine of interest in SK2 and SK3 through **sited directed mutagenesis**

↓

2) Expression of the proteins **in HEK293** cells

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

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

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Modeling of the mutants with **AlphaFold 3**

Phylogenetic analysis of species with a naturally occurring tyrosine variant

Affinity and activity tests

Mutants of hSK2 and hSK3 were generated by replacing the phenylalanine (F243) 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 Ca^{2+} in the pipette).

in vitro Binding Assays

A

Channels	Radioactive activity (dpm)		
	Total Binding	Non Specific	Specific Binding
SK2 WT	4305 ± 239	199 ± 35	4106 ± 270
SK2 F243Y	5854 ± 245	252 ± 29	5602 ± 224
SK2 F243A	298 ± 68	267 ± 45	31 ± 93
SK3 WT	4660 ± 199	340 ± 125	4321 ± 304
SK3 F392Y	254 ± 69	233 ± 40	21 ± 105
SK3 F392A	385 ± 246	238 ± 93	147 ± 155

B

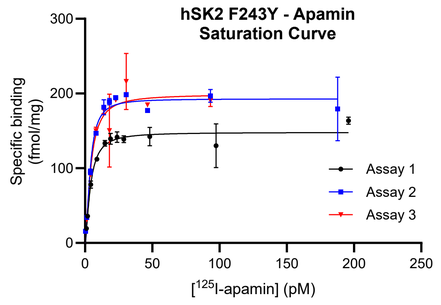


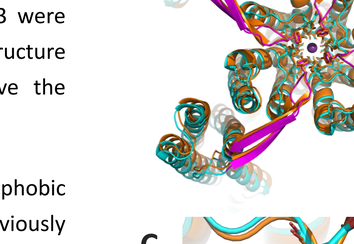
Figure 3 - Binding assay with radiolabeled apamin
*(**A**) Mutants screening. **(B)** Saturation assay with hSK2 F243Y.*

- Mutant screening revealed that only hSK2 F243Y retained apamin binding (*Figure 3A*). Saturation assays confirmed that this tyrosine substitution preserves high-affinity binding, with K_D values (3.7–4.7 pM) comparable to those of native SK channels (~5 pM) (Figure 3B).

in vitro Patch-clamp Assays

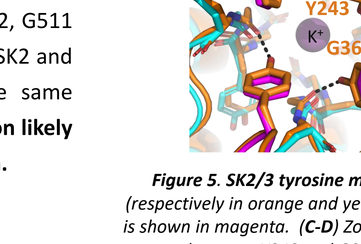
Tetraethylammonium (TEA)

A



IC_{50}
- hSK2 WT : 5,91 ± 1,72 mM
- hSK2 F243Y : 6,94 ± 1,46 mM
- hSK2 F243A : 5,86 ± 0,48 mM

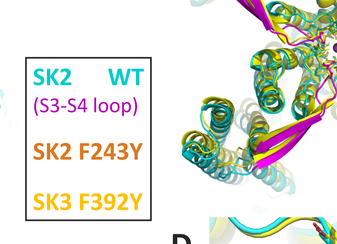
B



IC_{50}
- hSK3 WT : 7,83 ± 2,09 mM
- hSK3 F392Y : 9,89 ± 2,37 mM
- hSK3 F392A : 8,42 ± 1,67 mM

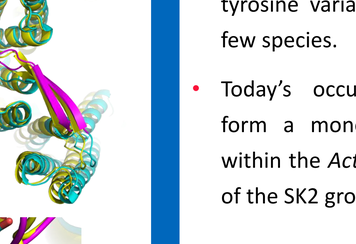
Apamin

C



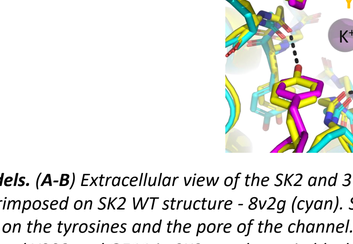
→ Apamin 30nM blocks > 75% of current at -80mV
→ Similar observation for hSK3 WT

D



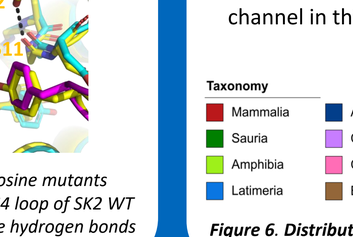
→ No current inhibition by apamin 1µM
→ Same observation for hSK3 F392A

E



IC_{50}
- hSK2 WT : 220,5 ± 41,3 pM
- hSK2 F243Y : 226 ± 37,7 pM

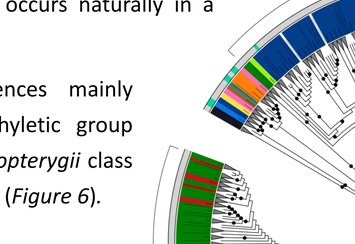
F



IC_{50}
- hSK3 WT : 377,6 ± 94,7 pM
- hSK3 F392Y : 2033 ± 245 pM

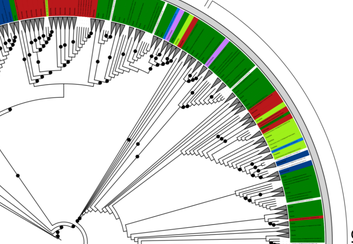
UCL1684

G



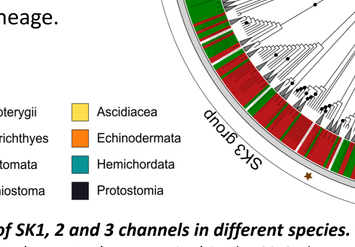
→ UCL1684 300nM blocks > 80% of current intensity

H



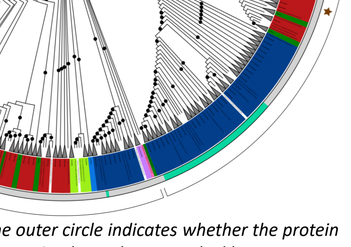
→ No current inhibition by UCL1684 1µM

I



IC_{50}
- hSK2 WT : 365,9 ± 55,9 pM
- hSK2 F243Y : 457,5 ± 81,6 pM

J



IC_{50}
- hSK3 WT : 413,6 ± 97,2 pM
- hSK3 F392Y : 380,4 ± 51,6 pM

Figure 4*. (**A-B**) Concentration-inhibition curves of TEA on SK2 and SK3 show similar sensitivity for wild-type channels and their Phe-Tyr and Phe-Ala mutants (n = 5 ; p > 0.05 ; Kruskal-Wallis test). (**C-D**) I–V relationships 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 show similar sensitivity to apamin for wild-type and tyrosine mutant in SK2 (n = 5 ; p > 0.05 ; Mann-Whitney test) but a differential sensitivity in SK3 (n = 5 ; p < 0.01). (**G-H**) I–V relationships 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 show similar sensitivity to UCL1684 for wild-type and tyrosine mutant in both SK2 and SK3 (n = 5 ; p > 0.05 ; Mann-Whitney test). All error bars correspond to SEM.*

SK2/3 tyrosine mutants models

- To further investigate the impact of the Phe -> Tyr mutation, structural models of tyrosine mutants in SK2 and SK3 were generated using AlphaFold3, with the SK2 wild-type structure (PDB ID – 8v2g) serving as a template to preserve the conformation of the selectivity filter (*Figure 5*).
- The mutation in tyrosine maintains the hydrophobic interactions of the phenylalanine it replaces and previously shown to induce conformational changes in the SK2 selectivity filter. In addition, tyrosine forms a supplementary hydrogen bond with the carbonyl group of a glycine (G362 in SK2, G511 in SK3). This hydrogen bond is 2.8 Å and 2.6 Å long in SK2 and SK3 respectively and is ideally oriented within the same monomer (*Figure 5 C and D*). **This additional interaction likely enhances the stability of the S3–S4 loop conformation.**

Figure 5. SK2/3 tyrosine mutant models. *(**A-B**) Extracellular view of the SK2 and 3 tyrosine mutants (respectively in orange and yellow) superimposed on SK2 WT structure - 8v2g (cyan). S3-S4 loop of SK2 WT is shown in magenta. (**C-D**) Zoom on the on the tyrosines and the pore of the channel. The hydrogen bonds between Y243 and G362 in SK2, and Y392 and G511 in SK3 are shown in black dotted lines.*

Phylogenetic analysis of tyrosine variant

- Phylogenetic analysis showed that the tyrosine variant occurs naturally in a few species.
- Today's occurrences mainly form a monophyletic group within the *Actinopterygii* class of the SK2 group (*Figure 6*).
- This suggests a potential functional adaptation of the channel in this lineage.

Figure 6. Distribution of SK1, 2 and 3 channels in different species. *The outer circle indicates whether the protein contains a Phe (grey) or a Tyr (aquamarine) in the S3-S4 loop. Human SK channels are marked by a star*

Conclusion

Confirmation of the critical role of the Phe residue in the S3–S4 loop of SK2/SK3 channels

- ***In vitro*** **binding and patch-clamp experiments demonstrated that substitution of Phe with Ala abolishes the sensitivity of SK2/SK3 channels to apamin and UCL1684, whereas substitution with Tyr preserves sensitivity to these compounds.**
- **AlphaFold models of Tyr mutants show that the Tyr would maintain the interactions observed for the Phe and allow an additional H-bond with a glycine from the selectivity filter, potentially enhancing the stability of the S3-S4 loop.**
- **The mainly monophyletic occurrence of Tyr variant in the *Actinopterygii* class of the SK2 group suggests a potential functional adaptation.**

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