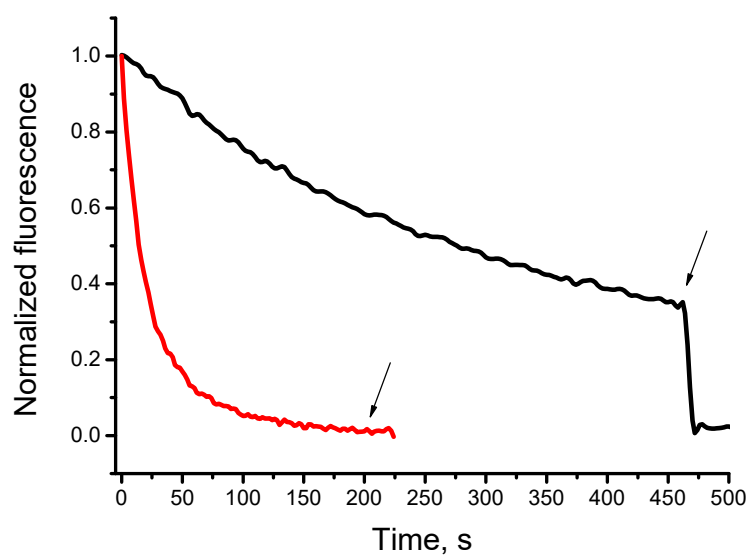


*Supplementary information*

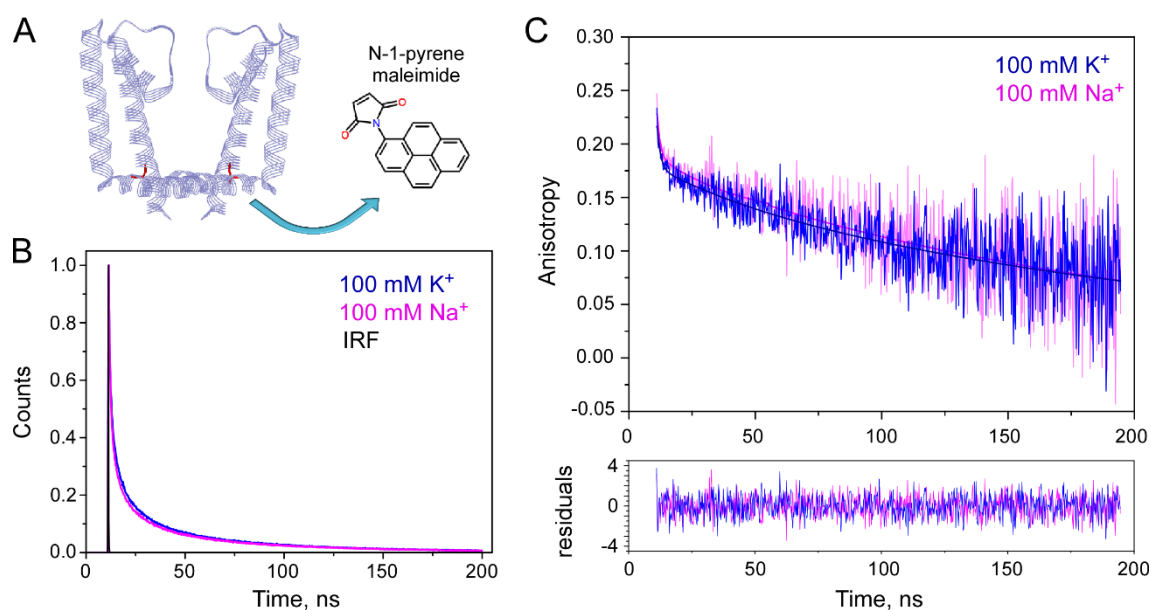


**Figure S1.** Normalized  $K^+$  flux of NaK (black) and NaK2K (red) channels in asolectin liposomes measured through the ACMA/CCCP fluorescence assay. The addition of CCP sets the time zero of the experiment. Valinomycin was added at the end of the recording (black arrow) to induce channel-independent  $K^+$  efflux.

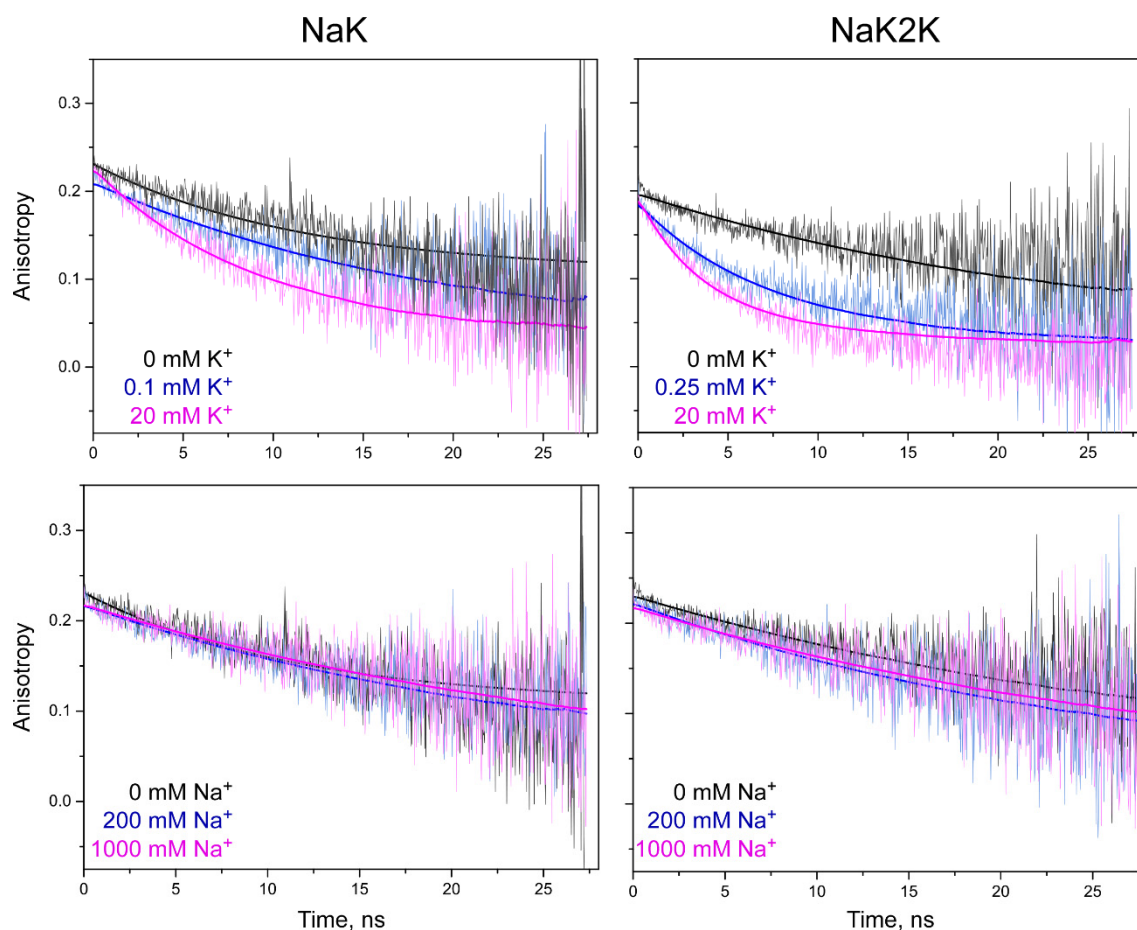
**Table S1.** Analysis of the fluorescence intensity decays obtained at room temperature for the NaK and NaK2K channels in 20 mM Hepes, 5 mM DDM, 5 mM NMG, pH 7 buffers ( $\lambda_{\text{ex}}$ = 300 nm;  $\lambda_{\text{em}}$ = 345 nm).

Channel	Salt	$\alpha_i$	$\tau_i$ (ns)	$\alpha_2$	$\tau_2$ (ns)	$\alpha_3$	$\tau_3$ (ns)	$\langle\tau\rangle_1$ (ns)	$\langle\tau\rangle_2$ (ns)
NaK	Na <sup>+</sup> *	0.41 [0.18-0.64]	0.69 [0.45- 0.92]	0.29 [0.15- 0.42]	3.0 [1.9- 4.1]	0.30 [0.18- 0.42]	6.7 [5.8-7.5]	3.1 [2.8-3.5]	5.1 [4.9-5.4]
		0.34 [0.20-0.47]	0.64 [0.55- 0.73]	0.23 [0.19- 0.27]	2.4 [1.8- 2.9]	0.43 [0.32- 0.55]	6.1 [5.9-6.3]	3.4 [2.8-4.0]	5.1 [4.9-5.3]
NaK2K	Na <sup>+</sup> **	0.669 [0.59-0.75]	0.72 [0.70- 0.75]	0.20 [0.15- 0.25]	2.7 [2.4- 3.0]	0.13 [0.09- 0.17]	7.3 [6.7-8.0]	2.0 [1.7-2.3]	4.4 [3.9-4.9]
		0.36 [0.20-0.52]	0.61 [0.46- 0.75]	0.21 [0.20- 0.23]	2.4 [1.7- 3.1]	0.4 [0.3-0.6]	6.1 [5.5- 6.6]	3.3 [2.6- 3.9]	5.1 [4.8- 5.3]

$\alpha_i$  and  $\tau_i$  are the normalized amplitude and the lifetime of the  $i^{\text{th}}$  decay component. The amplitude-weighted average fluorescence lifetime,  $\langle\tau\rangle_1$  and the average lifetime  $\langle\tau\rangle_2$ , was calculated as in [1]. \*The concentration of salt used was 100 mM, \*\*The concentration of NaCl used was 200 mM, The calculated  $\langle\tau\rangle_1$  and  $\langle\tau\rangle_2$  represent the mean of at least three independent experiments. Values in brackets correspond to the 95% confidence intervals.



**Figure S2.** Determination of the global rotational time of the NaK-DDM complex in 100 mM K<sup>+</sup> (blue lines) and 100 mM Na<sup>+</sup> (magenta lines). **Panel A:** schematic representation (side view) of two of the four monomers of the full-length wild-type NaK channel (PDB: 2AHZ) and the location of the native C15 position (red sticks). These residues were covalently attached to a N-1-pyrene dye following the maleimide chemistry. **Panels B and C** illustrate representative time-resolved fluorescence and anisotropy decays, respectively ( $\lambda_{\text{ex}} = 335 \text{ nm}$ ;  $\lambda_{\text{em}} = 400 \text{ nm}$ ). No significant changes were observed when K<sup>+</sup> or Na<sup>+</sup> were present in the buffer (20 mM Hepes, 1 mM DDM, pH 7.0). The solid lines in panel C represent the best fit of Eq. 3 to the anisotropy decays. The intensity-weighted average lifetime was  $\sim 50 \text{ ns}$  and the global rotational correlation time,  $\phi_g$ ,  $40 \pm 3 \text{ ns}$ .



**Figure S3.** Representative time-resolved anisotropy decays of NaK (left column) and NaK2K (right column) channels in the presence of increasing amount of  $K^+$  or  $Na^+$ . The straight lines correspond to the best fit of Equation (3) to the data.

## References

1. Renart, M.L.; Giudici, A.M.; Poveda, J.A.; Fedorov, A.; Berberan-Santos, M.N.; Prieto, M.; Díaz-García, C.; González-Ros, J.M.; Coutinho, A. Conformational Plasticity in the KcsA Potassium Channel Pore Helix Revealed by Homo-FRET Studies. *Sci. Rep.* **2019**, *9*, 6215–6228. <https://doi.org/10.1038/s41598-019-42405-5>.