State-Dependent cAMP Binding to Functioning HCN Channels Studied by Patch-Clamp Fluorometry

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Supporting Material

Supporting Figures:

Fig. S1. Negative control experiments confirmed that the fluorescence signal near the membrane patch corresponded to ligand binding.

A. Fluorescence anisotropy assays on the binding of cAMP to WT mHCN2 (red) and mHCN2-R591E mutant (purple) proteins. 10 nM 8-Fluo-cAMP was used. Kd: WT, 0.32 ± 0.04 μM; R591E, 350 ± 9 μM.

B. From top to bottom, different concentrations of mHCN2-R591E protein (Red, 10; green, 1; blue, 0.1, magenta, 0.01 μM) were mixed with 5 μM 8-NBD-cAMP. BSA was added accordingly to keep the total concentration of protein at 10 μM. Background (buffer only, no 8-NBD-cAMP) was subtracted from the final results. Notice that there is an over 25 folds reduction in the peak value of the emission spectrum of mutant protein compare to that of WT protein shown in Fig. 1C. Black dash traces show the spectra of the same concentration of 8-NBD-cAMP in buffer without protein. Fluorescence intensity unit is in 10^5 a.u.

C. No obvious fluorescence signal could be detected from the cell membrane expressing mHCN2-R591E mutant channels. Macroscopic current recordings from the corresponding patch membrane are shown in the left.

D. Negative results (neither current nor fluorescence) from uninjected oocytes. Grey, -150 mV; black, -180 mV.

Supporting Movies:

Movie 1: Steady-state.mpg

This movie shows images of a patch-clamp recording pipette with a cell membrane patch. Position of the cell membrane expressing mHCN2 channel is indicated. The sequence of fluorescence images correspond to a series of hyperpolarizing voltage steps from +20 mV to -160 mV. By correlating the fluorescence intensity and the channel activity, it is evident that cAMP binding increases as the channel being activated by the voltage steps more negative than -120 mV.

Movie 2: Dynamic.mpg

This movie shows bright field image and a sequence of 20 fluorescence images collected along a single voltage step from -40 mV to -150 mV. Notice that the increase in fluorescence intensity largely matches the kinetics of channel activation, but the fluorescence signal decays much slower than channel deactivates. This observation suggests that cAMP stays in the binding pocket of the deactivated channel longer than the channel closes.