

Intracellular Ca^{2+} Modulates PKA Compartmentalization and Dynamics in Human iPSC-Derived Cardiomyocytes

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Abstract

The automaticity of human-induced Pluripotent Stem Cell-derived cardiomyocytes (hiPSC-CMs) is governed by coupled Ca^{2+} and membrane clocks, coordinated through local Ca^{2+} releases (LCRs) and cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling. We investigated the role of PKA in hiPSC-CM energetics by measuring its dynamics in the cytosol and mitochondria, and its crosstalk with Ca^{2+} . We tested three hypotheses: (i) Ca^{2+} -activated PKA signaling regulates energy balance; (ii) adenylyl cyclase activity correlates with spontaneous beating; and (iii) PKA compartmentalization is Ca^{2+} -dependent. We also compared hiPSC-CMs with rabbit sinoatrial node cells (SANCs). The key findings are: (i) PKA inhibition (H-89), Ca^{2+} chelation (BAPTA), or mitochondrial Ca^{2+} blockade (Ru360) led to energy imbalance; (ii) H-89 induced compartmentalized PKA activity in the cytosol, mitochondrial matrix, and outer mitochondrial membrane; (iii) Ca^{2+} chelation with BAPTA reduced PKA activity globally; and (iv) PKA dynamics and Ca^{2+} -dependent regulation were similar in hiPSC-CMs and rabbit SANCs. In conclusion, intracellular Ca^{2+} -mediated PKA compartmentalization is present in hiPSC-CMs and rabbit SANCs.

Keywords: cyclic adenosine monophosphate (cAMP); protein kinase A (PKA); pacemaker; PKA compartmentalization; hiPSC-CMs; sinoatrial node cells (SANCs)

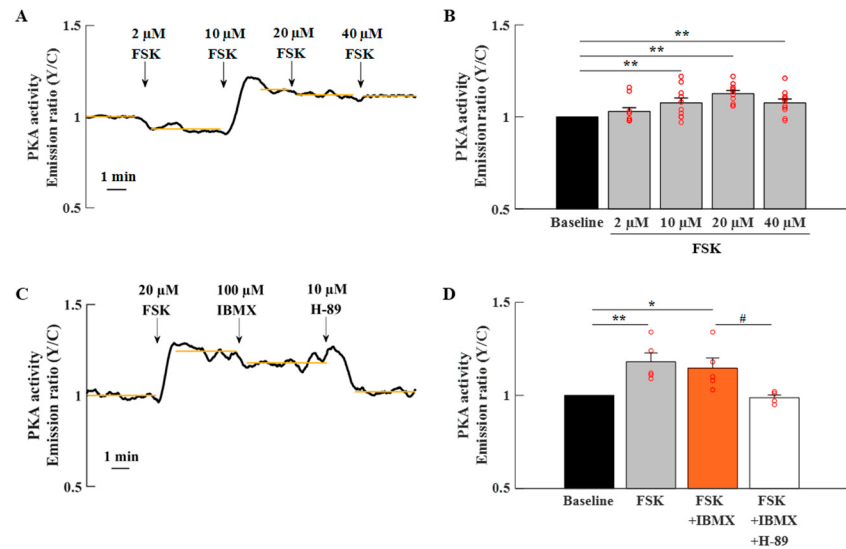


Figure S1. The effect of the adenylyl cyclase (AC) activator (forskolin) on PKA activity in the cytosol of spontaneously beating hiPSC-CMs. **(A)** A representative example of cytosolic PKA activity (AKAR4 emission Y/C) in a single hiPSC-CM in response to increasing concentrations of forskolin (FSK) (2, 10, 20, and 40 μ M). **(B)** The mean PKA activity in the cytosol in response to increasing concentrations of FSK ($n = 10$). **(C)** A representative example of cytosolic PKA activity in a single hiPSC-CM in response to 20 μ M FSK, followed by addition of the phosphodiesterase inhibitor IBMX (100 μ M) and 10 μ M H-89. **(D)** The mean PKA activity in the cytosol in response to FSK, IBMX, and H-89 ($n = 5$). The data are presented as the mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ FSK, IBMX, or H-89 compared with a value of 1.0 using a one-sample t -test. # $p < 0.05$ compared among FSK, IBMX, and H-89 using a one-way ANOVA with Dunn-Šidák multiple comparison.

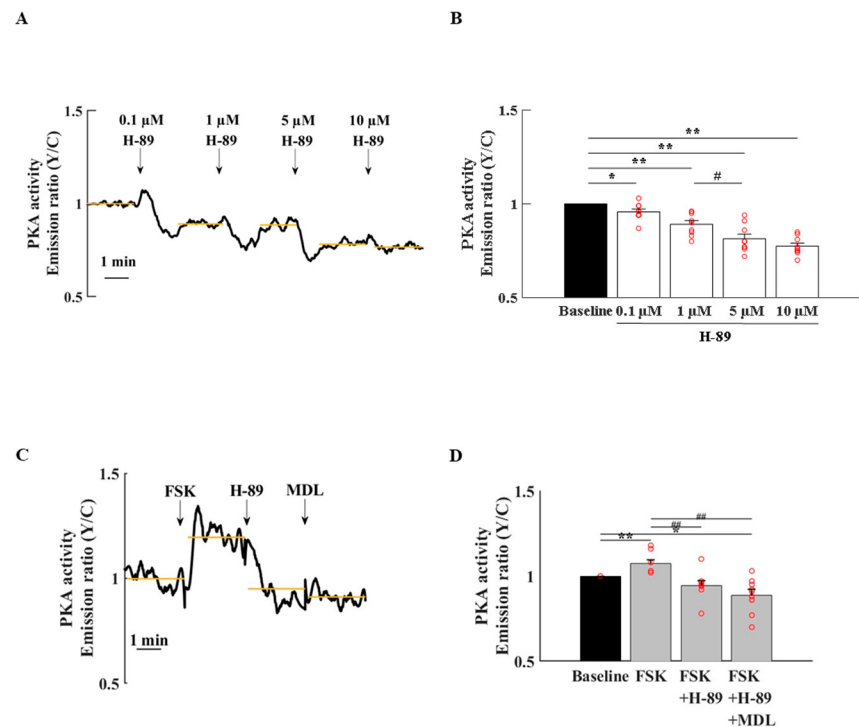


Figure S2. The effect of PKA inhibition (H-89) on PKA activity in the cytosol of spontaneously beating hiPSC-CMs. **(A)** A representative example of cytosolic PKA activity (AKAR4 emission Y/C) in a single hiPSC-CM in response to treatment with increasing concentrations of the PKA inhibitor H-89 (0.1, 1, 5, and 10 μ M). **(B)** The mean PKA activity in the cytosol in response to increasing concentrations of H-89 ($n = 9$). **(C)** A representative example of cytosolic PKA activity (AKAR4 emission Y/C) in a single hiPSC-CM in response to treatment with FSK, followed by the PKA inhibitor H-89 (10 μ M) and MDL (400 μ M). **(D)** The mean PKA activity in the cytosol in response to FSK, followed by the PKA inhibitor H-89 and MDL ($n = 9$). The data are presented as the mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ H-89 responses compared with a value of 1.0 using a one-sample t -test. ## $p < 0.05$ and ## $p < 0.01$ compared among H-89 responses using a one-way ANOVA with Dunn-Šidák multiple comparison.

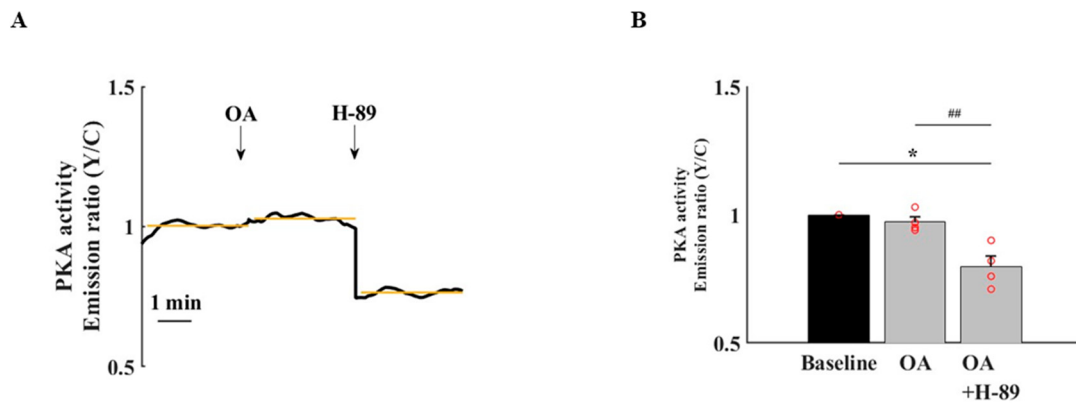


Figure S3. The effect of the PP2A inhibitor okadaic acid (OA) on PKA activity in the cytosol compartment of spontaneously beating hiPSC-CMs. **(A)** A representative example of cytosol compartment PKA activity in a single spontaneously beating hiPSC-CM in response to the PP2A inhibitor okadaic acid (OA) (100 μ M), followed by PKA inhibition with 10 μ M H-89. **(B)** The mean PKA activity in the cytosol compartment in a single spontaneously beating hiPSC-CM in response to the PP2A inhibitor okadaic acid (OA) (100 μ M), followed by PKA inhibition with 10 μ M H-89 ($n = 4$). The data are presented as the mean $\pm$ SEM. * $p < 0.05$ OA or H-89 compared with a value of 1.0 using a one-sample t -test. ## $p < 0.01$ OA versus H-89 using a paired Student's t -test.

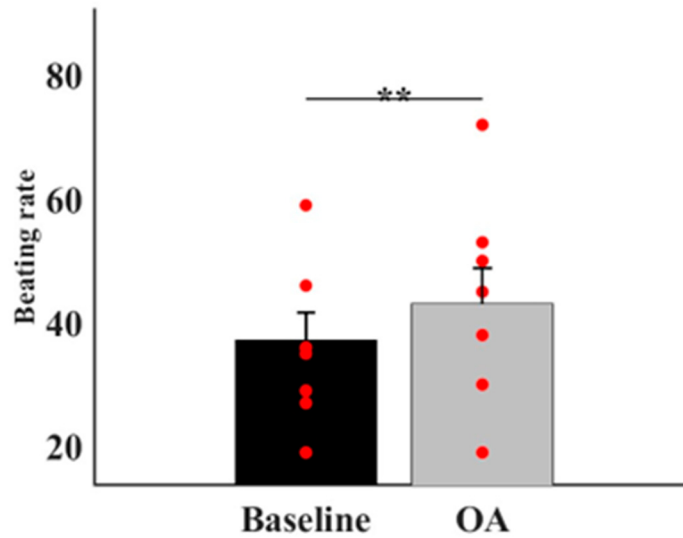


Figure S4. The effect of the PP2A inhibitor okadaic acid (OA) on the beating rate of spontaneously beating hiPSC-CMs. The mean beating rate of spontaneously beating hiPSC-CMs under baseline conditions and following treatment with the PP2A inhibitor okadaic acid (OA) (100 μ M) ($n = 4$). The data are presented as the mean $\pm$ SEM. The statistical analysis was performed using a one-sample *t*-test versus baseline values, followed by one-way ANOVA with Dunn–Šidák multiple comparison post hoc analysis. ** $p < 0.01$ versus the baseline.

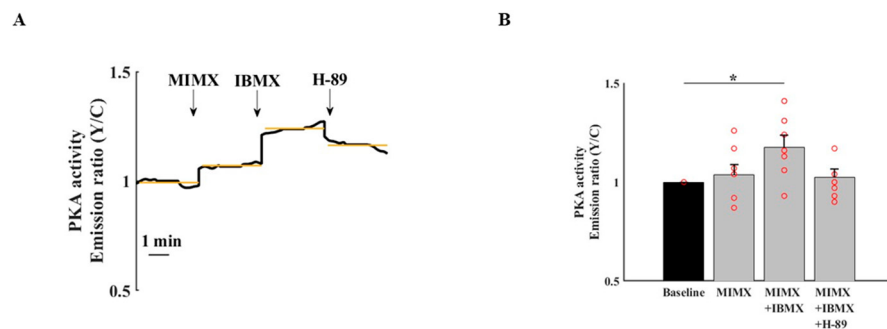


Figure S5. The effect of the PDE1 inhibitor MIMX on PKA activity in the cytosol compartment of spontaneously beating hiPSC-CMs. **(A)** A representative example of cytosol compartment PKA activity in a single spontaneously beating hiPSC-CM in response to the PDE1 inhibitor MIMX (50 μ M), followed by the PDE inhibitor (IBMX) at 100 μ M and PKA inhibition with 10 μ M H-89. **(B)** The mean PKA activity in the cytosol compartment in a single spontaneously beating hiPSC-CM in response to the PDE1 inhibitor MIMX (50 μ M), followed by the PDE inhibitor (IBMX) at 100 μ M and PKA inhibition with 10 μ M H-89 ($n = 7$). The data are presented as the mean $\pm$ SEM. * $p < 0.05$ MIMX, IBMX, or H-89 compared with a value of 1.0 using a one-sample *t*-test. Compared among MIMX, IBMX, or H-89 using a one-way ANOVA with Dunn–Šidák multiple comparison.

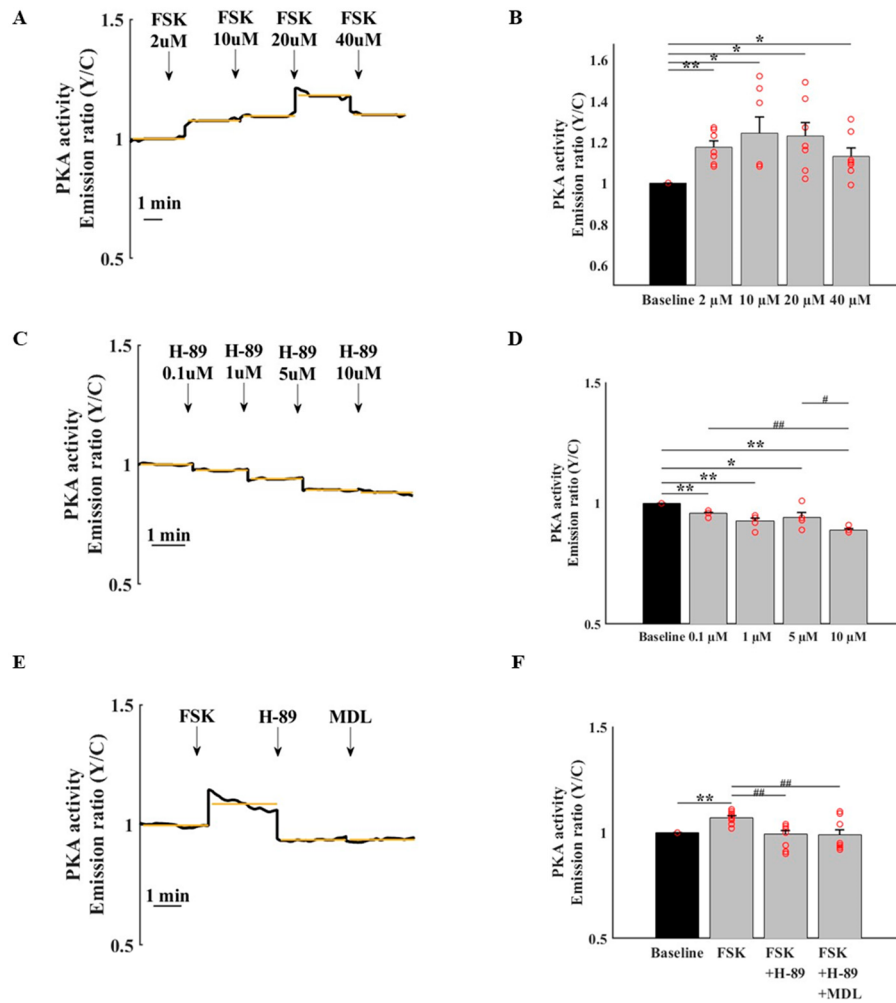


Figure S6. The effect of the adenylyl cyclase (AC) activator (forskolin) or inhibitor (H-89) on PKA activity in the mitochondrial matrix (MM) of spontaneously beating hiPSC-CMs. **(A)** A representative example of MM PKA activity (4Cox8-AKAR4 emission Y/C) in a single hiPSC-CM in response to increasing concentrations of forskolin (FSK) (2, 10, 20 and 40 μ M). **(B)** The mean PKA activity in the MM (4Cox8-AKAR4 emission Y/C) in a single hiPSC-CM to increasing concentrations of forskolin (FSK) (2, 10, 20, and 40 μ M ($n = 7$)). **(C)** A representative example of MM PKA activity (4Cox8-AKAR4 emission Y/C) in a single hiPSC-CM in response to treatment with increasing concentrations of the PKA inhibitor H-89 (0.1, 1, 5, and 10 μ M). **(D)** The mean PKA activity in the MM in response to increasing concentrations of H-89 ($n = 5$). **(E)** A representative example of MM PKA activity (4Cox8-AKAR4 emission Y/C) in a single hiPSC-CM in response to treatment with FSK and H-89 by the addition of the adenylyl cyclase inhibitor MDL (400 μ M). **(F)** The mean PKA activity in the MM (4Cox8-AKAR4 emission Y/C) in a single hiPSC-CM in response to 20 μ M FSK and 10 μ M H-89, followed by the addition of MDL ($n = 10$). The data are presented as the mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ FSK, H-89, and MDL responses compared with a value of 1.0 using a one-sample t -test. # $p < 0.05$ and ## $p < 0.01$ compared among H-89 responses/FSK/FSK + H89 using a one-way ANOVA with Dunn-Šidák multiple comparison.

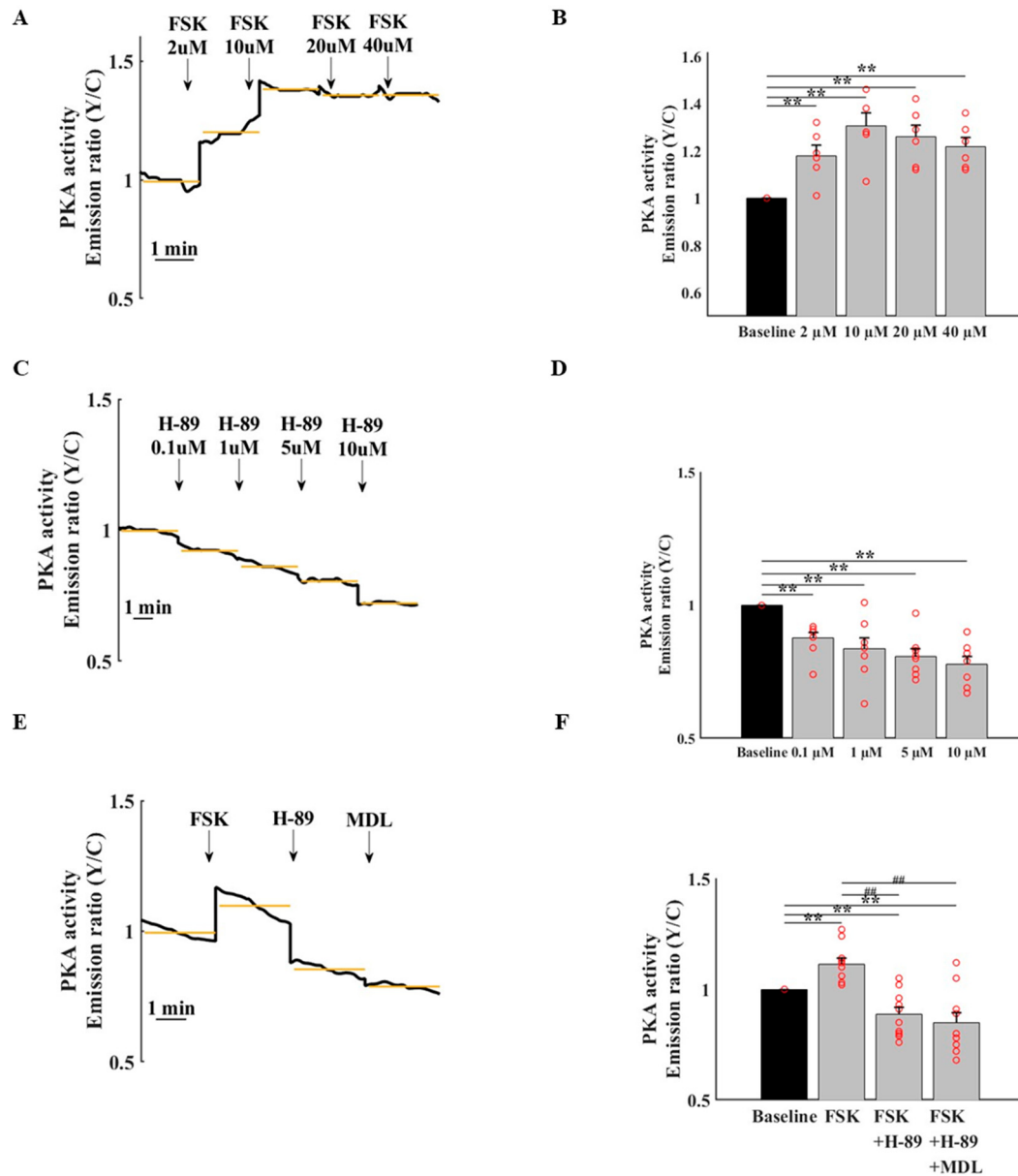


Figure S7. The effect of the adenylyl cyclase (AC) activator (forskolin) or inhibitor (H-89) on PKA activity in the OMM of spontaneously beating hiPSC-CMs. **(A)** A representative example of OMM PKA activity (yTom-AKAR4 emission Y/C) in a single hiPSC-CM in response to increasing concentrations of forskolin (FSK) (2, 10, 20, and 40 μ M). **(B)** The mean PKA activity in the OMM (yTom-AKAR4 emission Y/C) in a single hiPSC-CM in response to increasing concentrations of forskolin (FSK) (2, 10, 20, and 40 μ M) ($n = 6$). **(C)** A representative example of OMM PKA activity (yTom-AKAR4 emission Y/C) in a single hiPSC-CM in response to treatment with increasing concentrations of the PKA inhibitor H-89 (0.1, 1, 5, and 10 μ M). **(D)** The mean PKA activity in the OMM (yTom-AKAR4 emission Y/C) in a single hiPSC-CM in response to treatment with increasing concentrations of the PKA inhibitor H-89 ($n = 8$). **(E)** A representative example of MM PKA activity (yTom-AKAR4 emission Y/C) in a single hiPSC-CM in response to treatment with FSK and H-89 by addition of the adenylyl cyclase inhibitor MDL (400 μ M). **(F)** The mean PKA activity in the MM (yTom-AKAR4 emission Y/C) in a single hiPSC-CM in response to 20 μ M FSK and 10 μ M H-89, followed by

addition of MDL ($n = 10$). The data are presented as the mean $\pm$ SEM. ** $p < 0.01$, FSK, and H-89 responses compared with a value of 1.0 using a one-sample t -test. ## $p < 0.01$ compared among H-89 responses using a one-way ANOVA with Dunn-Šidák multiple comparison.

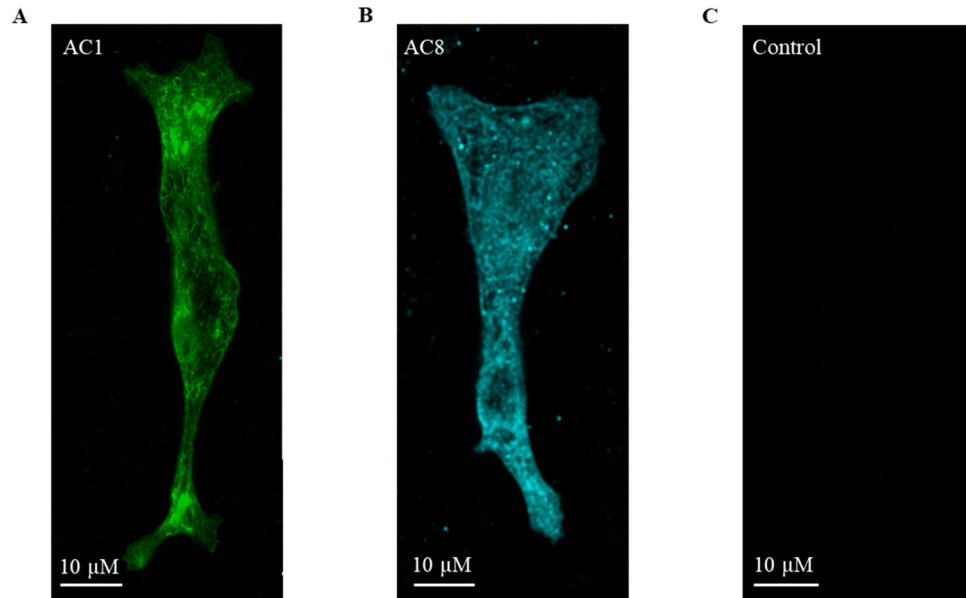


Figure S8. Immunofluorescence staining of Ca^{2+} -activated adenylyl cyclase (AC) in a single hiPSC-CM. Images of immunofluorescence staining of AC1 with an anti-AC1 antibody (A), AC8 with an anti-AC8 antibody in hiPSC-CMs, and (B) the control with a secondary antibody only (C).

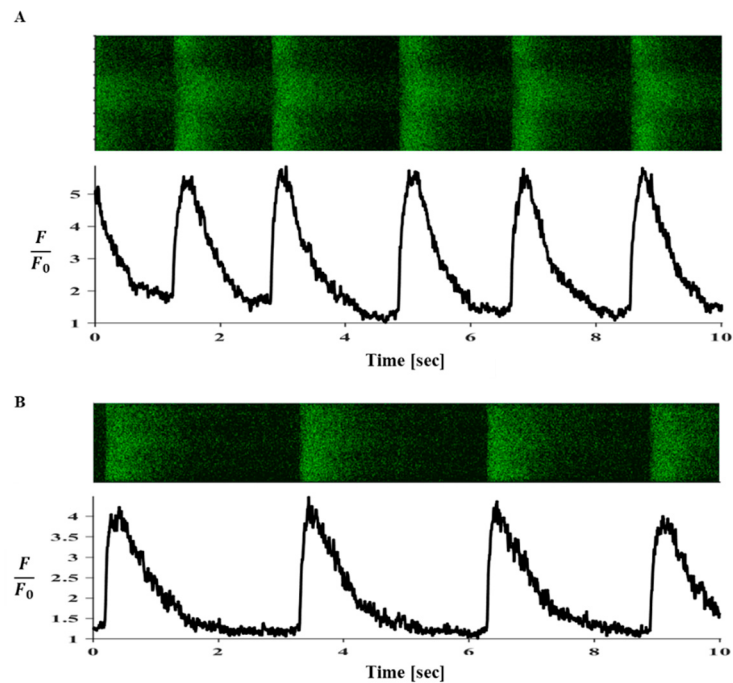


Figure S9. The effects of BAPTA on the beating rate of hiPSC-CMs. (A, B) Representative confocal line-scan images prior to (A) and following exposure to 25 μM BAPTA.

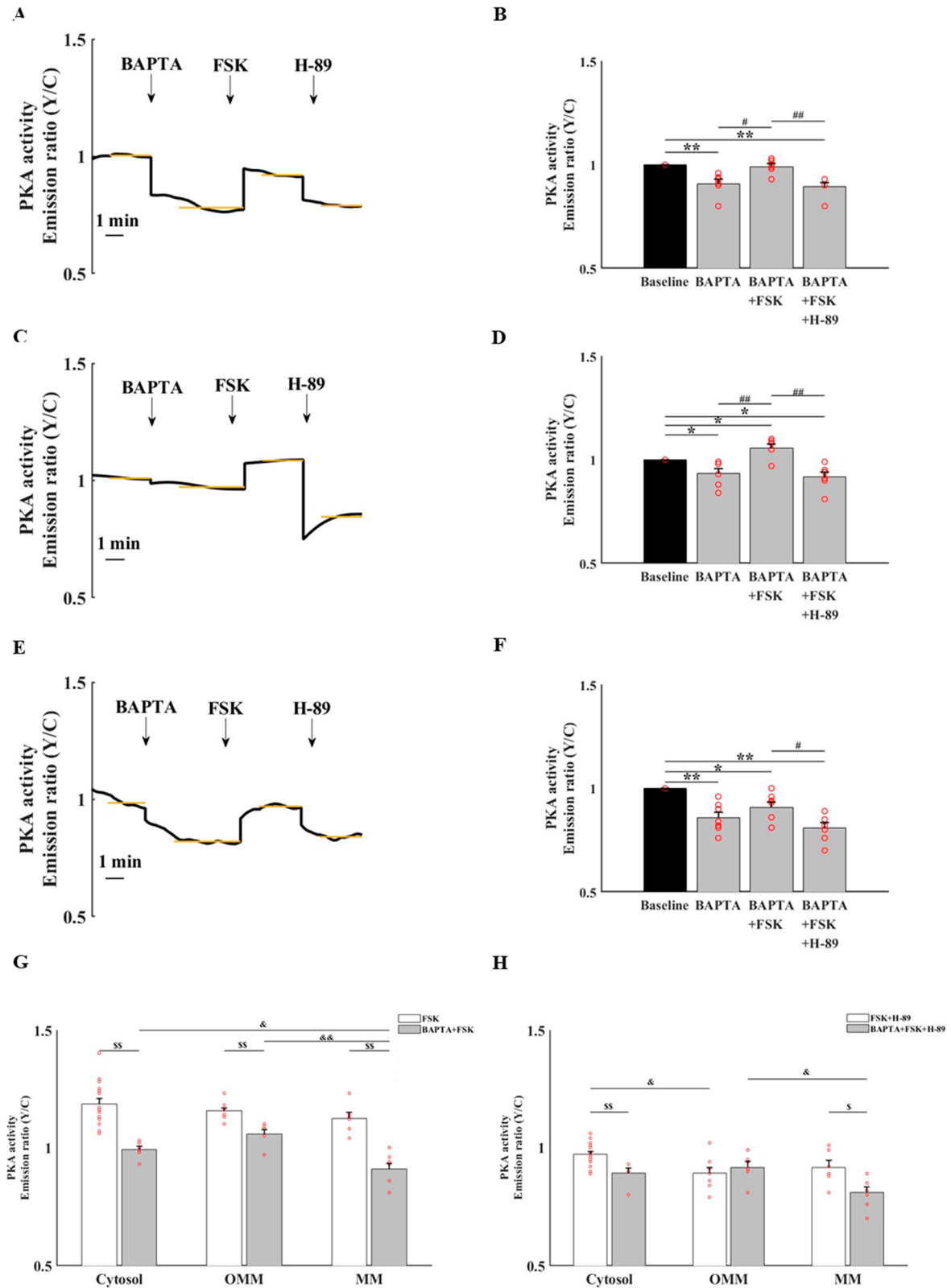


Figure S10. PKA activity in the cytosol and in mitochondrial compartments of spontaneously beating hiPSC-CMs. (A) A representative example of cytosolic PKA activity (AKAR4 emission Y/C) in a single hiPSC-CM in response to increasing concentrations of a Ca^{2+} chelator, BAPTA (25 μM), after 5 min recording, followed by adenylyl cyclase (AC) activation using 20 μM forskolin (FSK) and PKA inhibition using 10 μM H-89 after 3 min recording for each. (B)

The mean PKA activity in the cytosol in response to increasing concentrations of BAPTA, FSK, and H-89 ($n = 6$). (C) AS representative example of PKA activity in the outer mitochondrial membrane (OMM, γ Tom-AKAR4 emission Y/C) in a single hiPSC-CM in response to increasing concentrations of BAPTA (25 μ M), followed with 20 μ M FSK and 10 μ M H-89. (D) The mean PKA activity in the OMM in response to increasing concentrations of BAPTA, FSK, and H-89 ($n = 6$). (E) A representative example of mitochondrial matrix (MM) PKA activity (4Cox8-AKAR4 emission Y/C) in a single hiPSC-CM in response to increasing concentrations of BAPTA (25 μ M), followed with 20 μ M FSK and 10 μ M H-89. (F) The mean PKA activity in the MM in response to increasing concentrations of BAPTA, FSK, and H-89 ($n = 7$). (G) PKA activity in the cytosol, OMM, and MM of single hiPSC-CM following coadministration of BAPTA (25 μ M) and FSK (20 μ M). (H) PKA activity in the cytosol, OMM, and MM of single hiPSC-CM following coadministration of BAPTA (25 μ M) and H-89 (10 μ M). The data are presented as the mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ BAPTA, FSK, or H-89 compared with a value of 1.0 using a one-sample t -test. # $p < 0.05$ ## $p < 0.01$ compared among BAPTA, FSK, and H-89 using a one-way ANOVA with Dunn-Šidák multiple comparison. \$ $p < 0.05$ and \$\$ $p < 0.01$ FSK or H-89 versus the response in the presence of BAPTA in the same compartment using an unpaired Student's t -test. & $p < 0.05$, && $p < 0.01$ compared among H-89 responses in the cytosol, OMM, and MM using a one-way ANOVA with Dunn-Šidák multiple comparison.

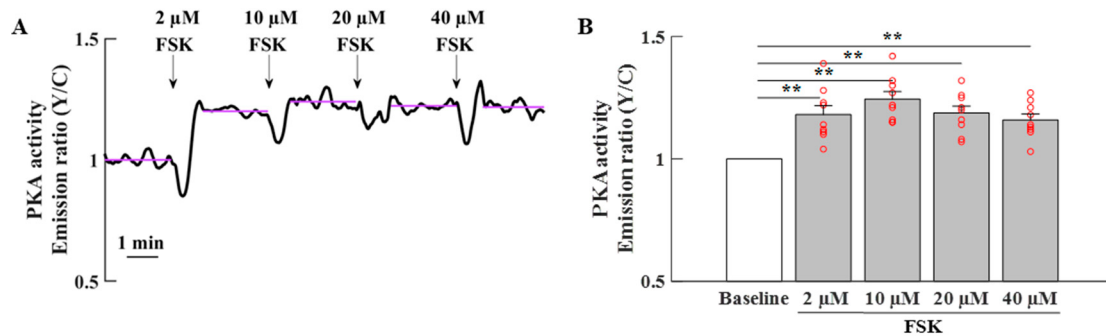


Figure S11. The effect of an AC activator (forskolin) on PKA activity in the cytosol of spontaneously beating SANCs. (A) A representative example of cytosolic PKA activity (AKAR4 emission Y/C) in a single pacemaker cell in response to increasing concentrations of forskolin (FSK) (2, 10, 20, and 40 μ M) ($n = 9$). (B) The mean PKA activity in the cytosol in response to increasing concentrations of FSK. The data are presented as the mean $\pm$ SEM. ** $p < 0.01$ FSK responses compared with a value of 1.0 using a one-sample t -test. # $p < 0.05$ compared among FSK responses using a one-way ANOVA with Dunn-Šidák multiple comparison.

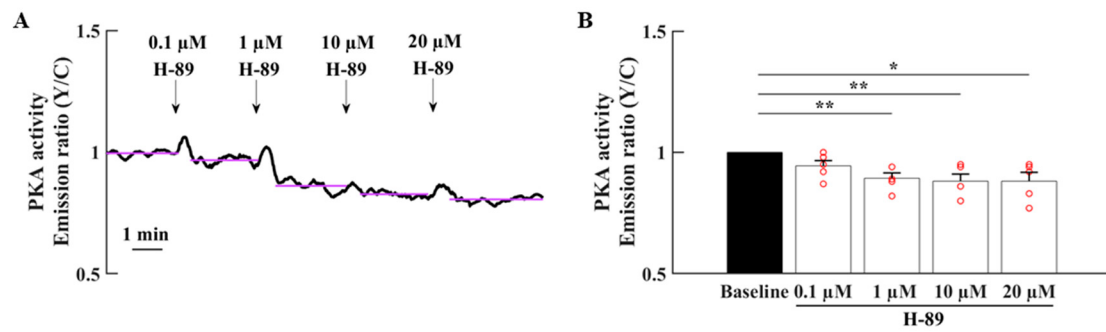


Figure S12. The effect of PKA inhibition (H-89) on PKA activity in the cytosol of spontaneously beating SANCs. **(A)** A representative example of cytosolic PKA activity (AKAR4 emission Y/C) in a single pacemaker cell in response to treatment with increasing concentrations of the PKA inhibitor H-89 (0.1, 1, 10, and 20 μM). **(B)** The mean PKA activity in the cytosol in response to increasing concentrations of H-89 ($n = 5$). The data are presented as the mean $\pm$ SEM. * $p < 0.05$. ** $p < 0.01$ H-89 responses compared with a value of 1.0 using a one-sample t -test.

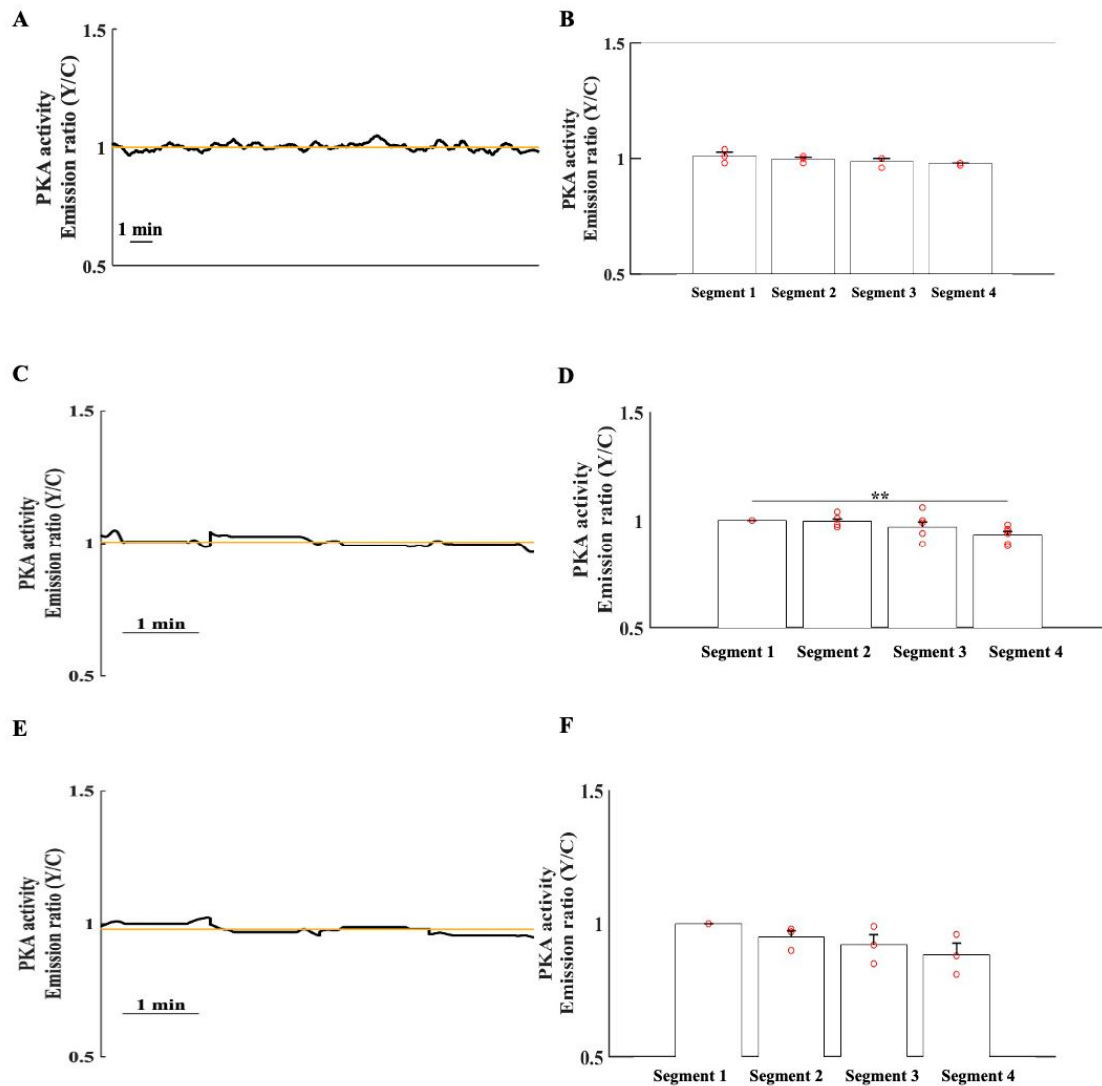


Figure S13. Time-control measurements of PKA activity in spontaneously beating hiPSC-CMs. **(A)** A representative example of cytosolic PKA activity (AKAR4

emission Y/C) in a single hiPSC-CM measured for 20 min in the presence of DMSO. (B) The mean PKA activity measurements in the cytosol (AKAR4 emission Y/C) were divided into 5 min segments for comparison and assessment of decay ($n = 3$). No significant decay in fluorescence was observed. (C) A representative example of mitochondrial matrix (MM) PKA activity (4Cox8-AKAR4 emission Y/C) in a single hiPSC-CM measured for 20 min. (D) The mean PKA activity measurements in the MM (4Cox8-AKAR4 emission Y/C) were divided into 5 min segments for comparison and assessment of decay ($n = 6$). No significant decay in fluorescence was observed. (E) A representative example of outer mitochondrial membrane (OMM) PKA activity (yTom-AKAR4 emission Y/C) in a single hiPSC-CM measured for 20 min. (F) The mean PKA activity measurements in the OMM (yTom-AKAR4 emission Y/C) were divided into 5 min segments for comparison and assessment of decay ($n = 3$). No significant decay in fluorescence was observed. ** $p < 0.01$ using one-way ANOVA between adjacent segments (one after the other).

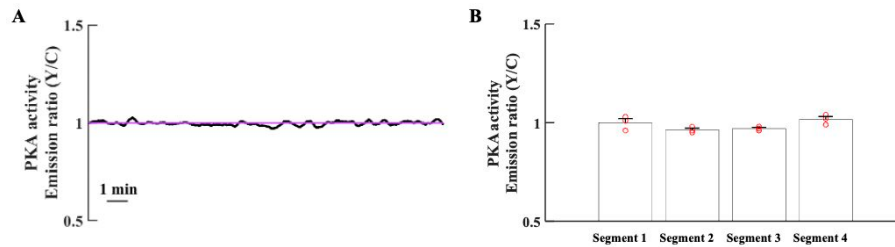


Figure S14. Time-control measurements of PKA activity in spontaneously beating SANs in the presence of DMSO. (A) A representative example of cytosolic PKA activity (AKAR4 emission Y/C) in a single pacemaker cell measured for 20 min. (B) The mean PKA activity measurements in the cytosol were divided into 5 min segments for comparison and assessment of decay ($n = 3$). No significant decay in fluorescence was observed.

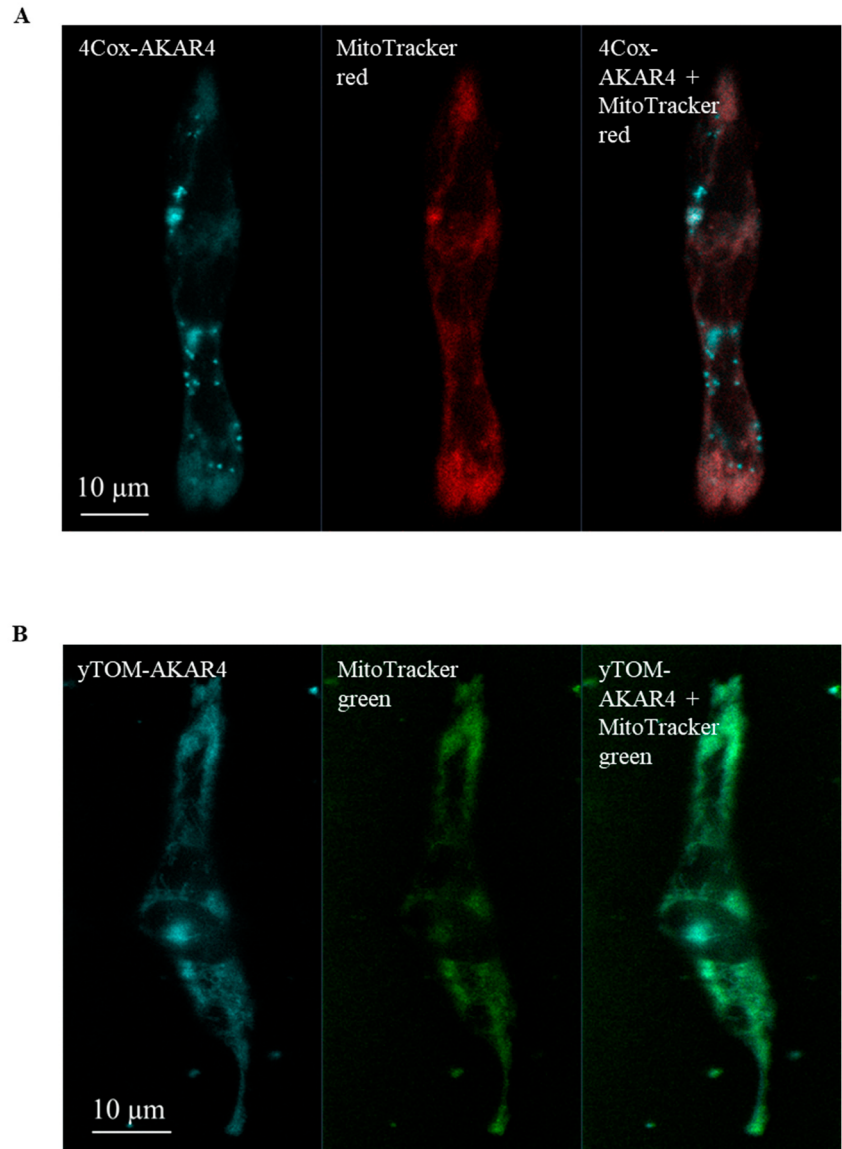


Figure S15. Localization of 4Cox8-AKAR4 in the mitochondrial matrix (MM) and of yTom-AKAR4 in the outer mitochondrial membrane (OMM) of spontaneously beating hiPSC-CMs. **(A)** A confocal image of a single hiPSC-CM expressing 4Cox8-AKAR4 and loaded with MitoTracker red as the MM indicator, excited alone and together. **(B)** A confocal image of a single hiPSC-CM expressing yTom-AKAR4 and loaded with MitoTracker green as the OMM indicator, excited alone and together.

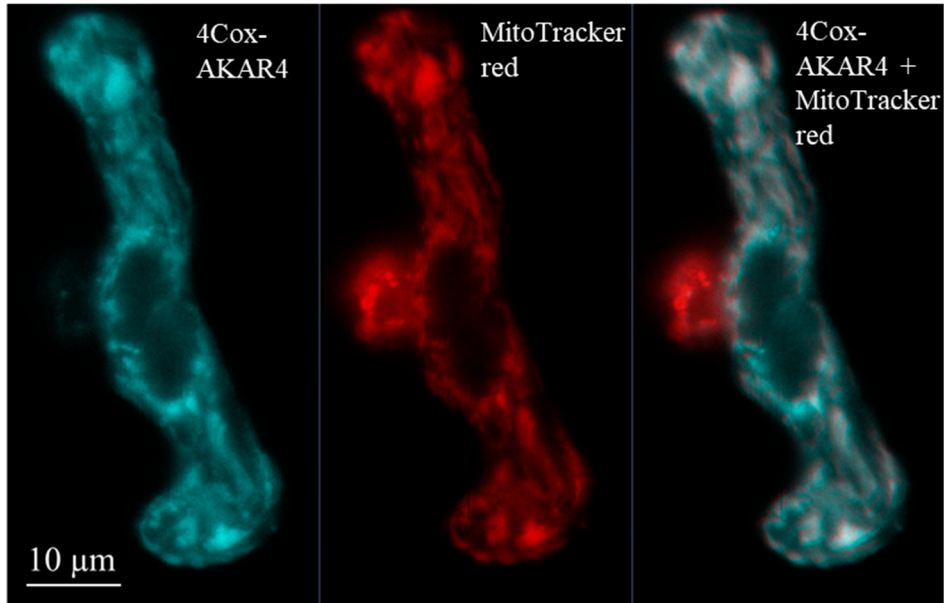
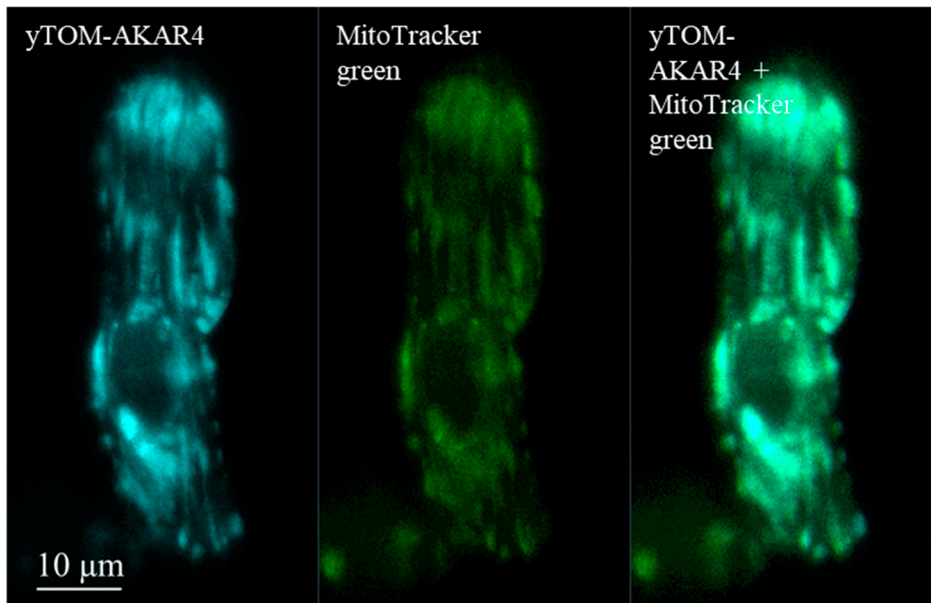
A**B**

Figure S16. Localization of 4Cox8-AKAR4 in the membrane matrix (MM) and yTom-AKAR4 in the outer mitochondrial membrane (OMM) of spontaneously beating SANCs. **(A)** A confocal image of a single pacemaker cell expressing 4Cox8-AKAR4 and loaded with MitoTracker red as the MM indicator, excited alone and together. **(B)** A confocal image of a single pacemaker cell expressing yTom-AKAR4 and loaded with MitoTracker green as the OMM indicator, excited alone and together.

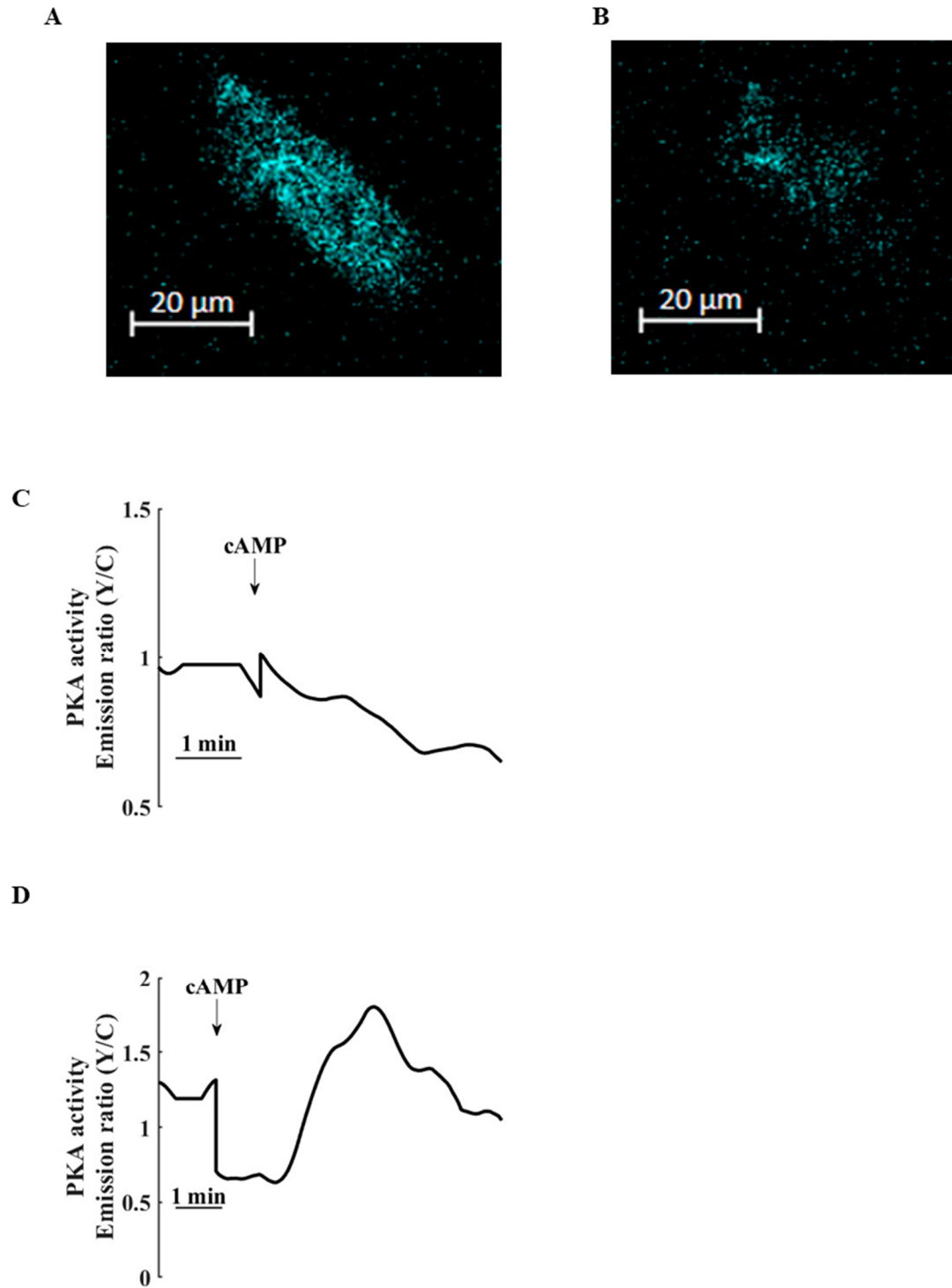


Figure S17. Localization of 4Cox8-AKAR4 in the mitochondrial matrix (MM) and of yTom-AKAR4 in the outer mitochondrial membrane (OMM) of spontaneously permeabilized beating hiPSC-CMs. A representative example of cytosolic PKA activity (AKAR4 emission Y/C) in a single hiPSC-CM, measured before (A) and after (B) the use of 5 μ M digitonin. (C) A representative example of PKA activity (4Cox8-AKAR4 emission Y/C) in a single hiPSC-CM using 25 μ M digitonin in the presence of 10 μ M cAMP. (D) A representative example of PKA activity (yTom-AKAR4 emission Y/C) in a single hiPSC-CM using 25 μ M digitonin in the presence of 10 μ M cAMP.