ON-LINE DATA SUPPLEMENT

Title: **I_{Ks} response to PKA-dependent KCNQ1 phosphorylation requires direct interaction with microtubules**

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MATERIALS AND METHODS

All procedures performed on animals were approved by the local committee for care and use of laboratory animals, and were performed according to strict governmental and international guidelines on animal experimentation. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Plasmids – To proceed to the two-hybrid screen, a bait corresponding to amino acids 1-121 of KCNQ1 (NtKCNQ1) cDNA was constructed. The plasmid pVJL10-NtKCNQ1 was made using the EcoR1-Xmn1 5'-end of the human KCNQ1 coding sequence[1] inserted into pVJL10[2] to generate the bait in frame with lexA-binding domain (LexA BD). A cDNA library from mouse BALB/c poly(A)+ RNA was fused to the GAL4 activation domain (GAL4 AD), by insertion into pGAD1318[3] using the Stratagene cDNA synthesis kit.

All constructs were checked by direct sequencing.

Yeast two-hybrid screening – The yeast reporter strain L40, which contains the reporter genes *LacZ* and *HIS3* downstream the binding sequence for LexA, was sequentially transformed with the pVJL10-NtKCNQ1 plasmid and with a mouse cDNA library, using the lithium acetate method[4] and subsequently treated as previously described[5]. Cells were plated on synthetic medium lacking histidine, leucine and tryptophan; the plates were incubated at 30°C for 2-6 days. His+ colonies were

patched onto selective plates. Plasmid DNA was prepared from colonies displaying a His+ phenotype and used to transform E. coli DH5α. Clones were screened by PCR with pGAD1318 specific primers and plasmids from positive cells were tested for specificity by co-transformation into L40 with pVJL10-NtKCNQ1-hybrid or pVJL10-PCTAIRE-1. Sequence identification was performed using the Basic Local Alignment Search Tool (BLAST) provided by NCBI Entrez protein site.

Co-immunoprecipitation and Western blotting – The African green monkey kidney derived COS-7 cells (obtained from ATCC), were transiently transfected with 2 or 3 µg of pCI-KCNQ1[6] or pcDNA3-yotiao[7] or 50 % pCI-KCNQ1 and 50 % pcDNA3-yotiao expression vector using JetPEI according to the standard protocol (Polyplus-transfection). For each experimental condition, confluent cells were harvested after 2 days and incubated in ice-cold lysis buffer [in mmol/L: 150 NaCl, 5 EDTA, 20 Tris-HCl, pH 7.4 supplemented with 1% Triton X100, 1 minitab of protease inhibitors (Roche) and 1 mmol/L PMSF (Sigma)] for 1 h at 4°C. The same procedure was used for guinea pig heart except that the tissue was homogenized in lysis buffer on ice in a glass potter. The insoluble fraction was pelleted by centrifugation at 20,000 g for 30 min. The supernatant was then incubated with 1 or 1.5 µg anti-β-tubulin rabbit polyclonal antibody (Santa Cruz Biotechnology), goat anti-Ct-KCNQ1 polyclonal antibody (Santa Cruz Biotechnology), or rabbit anti-KCNQ1 polyclonal antibody (Alomone Labs) for 16 h at 4°C. Fifteen µl of protein-G Sepharose beads (Amersham Biosciences) were added and left for 1 h with constant rotation at 4°C. Beads were centrifuged and washed three times with 500 µL of lysis buffer. The resin was suspended in gel loading buffer, heated for 5 min at 95°C, and subjected to SDS-PAGE. The precipitated proteins were detected by immunoblotting using ECL detection (Amersham Biosciences).

To perform immunoprecipitation of KCNQ1 complex of the plasma membrane, COS-7 cells were transfected with 50% pcb6-VSV-KCNE1-KCNQ1, a VSV-tagged KCNE1-KCNQ1 fusion protein[7] and 50 % pCDNA3-yotiao. After treatment with cpt-cAMP (400 µmol/L), forskolin (10 µmol/L) and okadaïc acid (0.2 µmol/L) for 10 min, intact cells were incubated with a mouse anti-VSV antibody (1/500, Sigma Aldrich) or a goat anti-Ct-KCNQ1 polyclonal antibody (1/ 500, Santa Cruz Biotechnology) for 1h at 4°C before lysing the cells. After centrifugation, protein-G Sepharose beads were added to the supernatant, left one hour and washed as described above. Beads were suspended in Laemmli sample buffer and proteins were denatured for 30 min at room temperature before separation by SDS-PAGE. Blots were probed with a rabbit anti-KCNQ1 polyclonal antibody (Alomone Labs) and a HRP-coupled anti-rabbit antibody (1/10000, Santa Cruz Biotechnology) and revealed using ECL detection. To evaluate KCNQ1 and yotiao phosphorylation state, COS-7 cells were transiently transfected with 20% pCI-KCNQ1, 40% pRC-KCNE1 and 40% pCDNA3-yotiao or 100% pEGFP (total: 2 µg) using Fugene according to the standard protocol (Roche). Treatment with colchicine (10 µmol/L, 2h) and/or cpt-cAMP, forskolin and okadaïc acid (0.2 µmol/L or 1 µmol/L for yotiao) for 10 min were realized before lysing the cells as described above, 24 to 48h after transfection. The proteins were denatured for 30 min at room temperature in Laemmli sample buffer or boiled 5 to 10 min at 95°C with 100 mM DTT for yotiao denaturation, before separation by SDS-PAGE. Rabbit anti-KCNQ1 antibody (1/2000, Alomone Labs) or rabbit anti-phospho-S27 KCNQ1 antibody[8] (1/250), rabbit yotiao antibody (1/200, Fusion antibodies) or rabbit phospho-specific yotiao antibody[9] (1/100) and secondary HRP-coupled antibody (1/10000, Santa Cruz Biotechnology) were used to detect total KCNQ1, yotiao or their phosphorylated forms. The experiments to evaluate KCNQ1 and yotiao

phosphorylation state were performed four times for each protein and the band intensity was quantified using WCIF ImageJ (NIH) software. For KCNQ1, each phosphorylated-KCNQ1 band was normalized to that of total KCNQ1 of the same lysate and the cAMP-induced increase was calculated using these values. For yotiao, the absence of band in baseline condition prevented to use the same calculation. Therefore, the band of phosphorylated yotiao after PKA stimulation was normalized to the non-specific 200-kDa band, in untreated and treated cells.

Immunocytochemistry – Thirty-six to 48 h after transfection, COS-7 cells grown on glass coverslips were rinsed in PBS buffer (in mmol/L: 4 KH_2PO_4, 16 Na_2HPO_4, 115 NaCl) and fixed in freshly prepared 3.7%-formaldehyde PBS. Cells were rinsed in PBS, permeabilized with 0.2%-Triton X-100 PBS for 10 min. After 30 min incubation in PBS containing 1% BSA, cells were incubated overnight at 4°C with rabbit anti-β-tubulin antibody (1/100). Following incubation, cells were rinsed in PBS and stained for 1 h at room temperature with a 1/5000 dilution of Alexa A488-conjugated anti-rabbit antibody. Coverslips were rinsed with PBS and mounted with Vectashield immunofluorescence medium.

Guinea-pig (Hartley, males) ventricular myocytes were dissociated by standard enzymatic digestion, using both collagenase (350 U/mL, Worthington type 2, Serlabo) and protease (0.6 U/mL, Sigma). Animals were anesthetized with intra-peritoneal injection of sodium pentobarbital (55 mg/kg) and dislocated, in accordance with the institutional guidelines for animal use in research. Single ventricular myocytes were obtained by gentle agitation in a high K^+, low Cl^- solution (in mmol/L: 70 K-glutamate, 10 taurine, 25 KCl, 10 KH_2PO_4, 0.5 EGTA, 22 glucose, pH 7.4 with KOH).

Cardiomyocytes were fixed for 20 min in 2% paraformaldehyde and then permeabilized for 20 min in PBS containing 0.025% Triton X100 and 3% gelatin fish.

They were then incubated with rabbit anti-KCNQ1 (1/250; Alomone) and mouse anti-β-tubulin (1/250; Sigma Aldrich) antibodies over night at 4°C. After washing, they were incubated with the appropriate Alexa-488 and Alexa-568 conjugated secondary antibodies (1/1,000, Molecular Probe). The cells were observed on a Leica LSM confocal microscope and the acquired image stacks were analyzed using Metamorph (Roper Scientific SAS, Princeton Instruments) and Amira (Mercury Computer Systems SAS) softwares. Co-localization was quantified using Colocalization Threshold plugin of the WCIF ImageJ software and random images were realized with the Colocalization Test plugin.

Electrophysiology – For patch-clamp experiments, cells were mounted on an inverted microscope and an Axopatch 200A amplifier (Axon Instruments, Inc.) was used. To prevent I_{Ks} run-down, the permeabilized-patch configuration was used. Stimulation, data recording through an A/D converter (Tecmar TM100 Labmaster, Scientific Solutions; 5 kHz filtering, 25 kHz sampling) and analysis were performed with Acquis1 software (Bio-Logic).

For experiments devoted to osmotic challenge of I_{Ks}, cells were transfected with the plasmids (2 µg, 20% pCI-KCNQ1, 40% pRC-KCNE1, and 40% pEGFP; Clontech) complexed with JetPEI. Eight to 24 h after transfection, cells were isolated and plated at low density on glass coverslips. Twenty-four to 72 h post-transfection, K^+ currents were recorded. The cells were continuously perfused by Tyrode solution containing (in mmol/L) 145 NaCl, 4 KCl, 5 HEPES, 1 $CaCl_2$, 1 $MgCl_2$ and 5 glucose (pH 7.4 with NaOH). To record K^+ currents, pipettes (1.5-3.5 MΩ; Kimble) were filled with the following solution (in mmol/L): 120 K-gluconate, 25 KCl, 10 HEPES, 1 EGTA, 0.8 µg/mL Amphotericin B (pH 7.2 with KOH). The iso-osmolar solution, applied locally, contained (in mM): 145 Na-gluconate, 4 K-gluconate, 4 $Mg_{1/2}$-gluconate (1 mmol/L

free Mg^{2+}), 7 $Ca_{1/2}$-gluconate (1 mmol/L free Ca^{2+}), 5 HEPES, 5 glucose, 20 mannitol (pH 7.4 with NaOH). The hypo-osmolar solution contained (in mmol/L) 80 Na-gluconate, 4 K-gluconate, 4 $Mg_{1/2}$-gluconate (1 mmol/L free Mg^{2+}), 7 $Ca_{1/2}$-gluconate (1 mmol/L free Ca^{2+}), 5 HEPES, 5 glucose, 50 mannitol (pH 7.4 with NaOH) and the hyper-osmolar solution contained (in mmol/L) 205 Na-gluconate, 4 K-gluconate, 4 $Mg_{1/2}$-gluconate (1 mmol/L free Mg^{2+}), 7 $Ca_{1/2}$-gluconate (1 mmol/L free Ca^{2+}), 5 HEPES, 5 glucose (pH 7.4 with NaOH).

The PKA-dependent I_{Ks} response was evaluated in COS-7 cells transfected with 20% pCI-KCNQ1, 30% pRC-KCNE1, 30% pCDNA3-yotiao and 20% pEGFP using JetPEI. PKA-dependent response was elicited by the iso-osmolar solution containing 400 µmol/L cpt-cAMP, 10 µmol/L forskolin and 0.2 µmol/L okadaic acid.

The permeabilized-patch configuration was used to record I_{Ks} in guinea-pig cardiomyocytes also. The cells were continuously perfused by Tyrode solution containing (in mmol/L) 132 NaCl, 4 KCl, 5 HEPES, 1.8 $CaCl_2$, 1.2 $MgCl_2$ and 5 glucose (pH 7.4 with NaOH). Pipettes were filled with the following solution (in mmol/L): 120 K-gluconate, 20 KCl, 10 HEPES, 0.8 µg/mL Amphotericin B (pH 7.3 with KOH). The solution applied locally, contained (in mmol/L): 132 NaCl, 4 KCl, 5 HEPES, 1.2 $MgCl_2$, 5 glucose and 5 mannitol (pH 7.4 with NaOH). Four µmol/L nifedipine and 5 µmol/L E-4031 were added in order to inhibit the L-type Ca^{+} current and the K^{+} current I_{Kr}, respectively. For PKA-dependent response, the same drugs as for COS-7 cells were used. I_{Ks} is the time-dependent component of the tail current and I_{K1} the steady-state residual current measured after a depolarization from –60 mV to –40 mV, while I_{Ks} is not activated.

Some COS-7 cells or cardiomyocytes were pre-treated with colchicine (10 or 30 µmol/L) or taxol (10 µmol/L) for 1 to 3 h before patch-clamp experiments.

All products were purchased from Sigma. Mg^{2+} and Ca^{2+} activities were calculated using a software designed by G. L. Smith (University of Glasgow, Glasgow, UK). These experiments were performed at 35.0 ± 1.0°C.

References

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Supplemental table 1: co-localization analysis on cardiac myocyte (stack of 46 confocal images)

using thresholds			whole images (except zero-zero pixels)				
R_{coloc}	tM1 (green)	tM2 (red)	R_{total}	R_{rand}	$R_{total}>R_{rand}$	M1 (green)	M2 (red)
0.66	0.60	0.48	0.17	0.014	100 %	0.86	0.76

R_{coloc} : Pearson correlation coefficient for pixels above threshold. The thresholds for KCNQ1 (green) and β-tubulin (red) are automatically determined.

tM1 and tM2 : Mander co-localization coefficients for KCNQ1 (channel 1) and β-tubulin (channel 2), using thresholds.

R_{total} : Pearson correlation coefficient for all the non zero-zero pixels in the image.

R_{rand} : mean of Pearson correlation coefficients obtained from randomized images by Costes method (100 iterations).

$R_{total}>R_{rand}$: number of times the actual Pearson R_{total} is greater than the random R, within the number of iterations performed.

M1 and M2 : Mander co-localization coefficients for KCNQ1 (channel 1) and β-tubulin (channel 2) with >0 as thresholds.

Supplemental table 2: deactivation time constant in various conditions in transfected COS-7 cells.

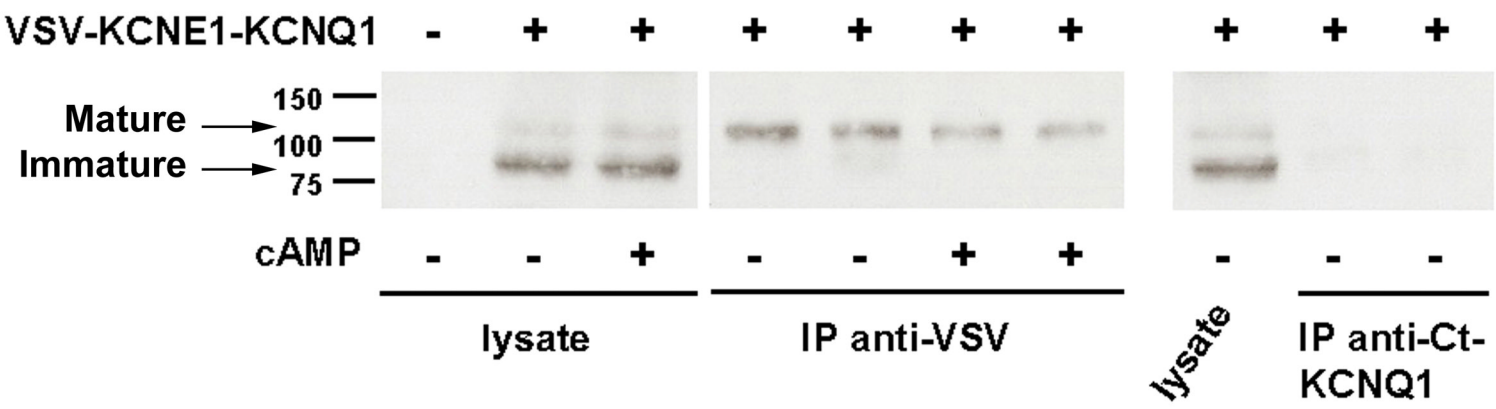
		(number of cells)	τ_{deact} (ms)
control	**hypo**	(9)	136.9 ± 11.4
	iso		136.0 ± 9.8
	hyper		149.3 ± 10.9
	baseline	(22)	175.3 ± 11.3
	cAMP		179.5 ± 12.4
colchicine 10 µM	**hypo**	(7)	177.2 ± 10.1
	iso		180.0 ± 12.9
	hyper		180.6 ± 18.4
	baseline	(11)	155.4 ± 12.1
	cAMP		153.4 ± 9.2

τ_{deact} = deactivation time constant (single exponential fit) at –40 mV; hypo, iso and hyper = in hypo-, iso- and hypertonic conditions; cAMP = during exposure to 400 µM cpt-cAMP, 10 µM forskolin and 0.2 µM okadaic acid

For each set of experiments, untreated and colchicine-treated cells derived from the same transfected cells batch and were investigated at the same time.

Statistics: osmolarity effects: two-way ANOVA, and one-way ANOVA in each condition, NS. cAMP effects: two-way ANOVA and t-test in each condition, NS

Supplemental Fig. The amount of KCNQ1 complex at the plasma membrane is not increased by PKA stimulation. COS-7 cells were transfected with a VSV-tagged KCNE1-KCNQ1 fusion protein (extracellular tag) and yotiao and treated with 400 μM cpt-cAMP, 10 μM forskolin and 0.2 μM okadaic acid when indicated. A mouse anti-VSV antibody was then incubated on intact cells to label the complex at the plasma membrane and the cell lysates were immunoprecipitated. No increase of KCNE1-KCNQ1 signal can be seen in cAMP-treated cells. As a control, some intact cells were incubated with a goat anti-Ct-KCNQ1 antibody directed against KCNQ1 intracellular C-terminus (right). No signal could be seen after immunoprecipitation (n = 2, 3 points each time).


VSV-KCNE1-KCNQ1
- + + + + + + + + +
Mature
Immature
150
100
75
cAMP
- - + - - + + - - -
lysate
IP anti-VSV
lysate
IP anti-Ct-KCNQ1