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I_{Ks} response to protein kinase A-dependent KCNQ1 phosphorylation requires direct interaction with microtubules

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Aims KCNQ1 (alias KvLQT1 or Kv7.1) and KCNE1 (alias IsK or minK) co-assemble to form the voltage-activated K^+ channel responsible for I_{Ks}—a major repolarizing current in the human heart—and their dysfunction promotes cardiac arrhythmias. The channel is a component of larger macromolecular complexes containing known and undefined regulatory proteins. Thus, identification of proteins that modulate its biosynthesis, localization, activity, and/or degradation is of great interest from both a physiological and pathological point of view.

Methods and results Using a yeast two-hybrid screening, we detected a direct interaction between β-tubulin and the KCNQ1 N-terminus. The interaction was confirmed by co-immunoprecipitation of β-tubulin and KCNQ1 in transfected COS-7 cells and in guinea pig cardiomyocytes. Using immunocytochemistry, we also found that they co-localized in cardiomyocytes. We tested the effects of microtubule-disrupting and -stabilizing agents (colchicine and taxol, respectively) on the KCNQ1–KCNE1 channel activity in COS-7 cells by means of the permeabilized-patch configuration of the patch-clamp technique. None of these agents altered I_{Ks}. In addition, colchicine did not modify the current response to osmotic challenge. On the other hand, the I_{Ks} response to protein kinase A (PKA)-mediated stimulation depended on microtubule polymerization in COS-7 cells and in cardiomyocytes. Strikingly, KCNQ1 channel and Yotiao phosphorylation by PKA—detected by phospho-specific antibodies—was maintained, as was the association of the two partners.

Conclusion We propose that the KCNQ1–KCNE1 channel directly interacts with microtubules and that this interaction plays a major role in coupling PKA-dependent phosphorylation of KCNQ1 with I_{Ks} activation.

1. Introduction

KCNQ1 (alias KvLQT1 or Kv7.1) is the α-subunit of a voltage-activated K^+ channel expressed in cardiomyocytes.[1,2] KCNQ1 associates with different β-subunits of the KCNE protein family.[3] In particular, KCNQ1 and KCNE1 (alias IsK or minK) co-assemble to form a voltage-activated K^+ channel characterized by its slow activation and deactivation kinetics. In the human heart, this heteromultimeric channel is responsible for the slow component of the delayed rectifier K^+ current I_{Ks}, a major repolarizing current during the late phase of the action potential,[4,5] especially during β_1-adrenergic stimulation.[6]

There is growing evidence that ion channels are not moving freely along the membrane phospholipid bilayer plane but are instead organized rather intimately through interactions with other proteins. These interactions are instrumental for targeting and regionalizing ion channel proteins, but also for linking regulatory components to channels.[7–9]

KCNQ and KCNE subunits are part of large macromolecular complexes in various tissues. In cardiomyocytes, the KCNQ1–KCNE1 channel associates with T-cap, also called

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telethonin, thereby linking the sarcolemmal channel to myofibrils.[10] As previously shown in different models, β-adrenergic stimulation of I_{Ks} requires the presence of a cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) anchoring protein (Yotiao, alias AKAP9, or other AKAPs) that keeps PKA in the vicinity of its target.[11,12] In cardiomyocytes, Yotiao actively links the β-adrenergic stimulation to the channel activity by (i) keeping the PKA catalytic subunit, its regulatory subunit RII and the phosphatase PP1 close to the channel and (ii) permitting channel activation once phosphorylated.[13,14]

By making use of a yeast two-hybrid assay, we screened for other potential KCNQ1 partners. We found a physical association between KCNQ1 and β-tubulin, which could connect KCNQ1–KCNE1 channels to the cytoskeletal network. In order to evaluate the physiological relevance of this interaction, we investigated the effects of microtubule depolymerization on the channel activity using the permeabilized patch configuration of the patch-clamp technique on transfected COS-7 cells and on guinea pig cardiomyocytes. We observed that microtubule disruption did alter neither the channel activity nor its sensitivity to osmotic challenge. On the other hand, we demonstrated that the PKA-dependence of the current was altered after microtubule depolymerization, whereas channel phosphorylation, Yotiao phosphorylation, and the interaction between both proteins were not affected. Our data suggest that the microtubule cytoskeleton interacts with the KCNQ1 N-terminus and that this interaction is key for the coupling between phosphorylation and activation of KCNQ1–KCNE1 and hence for the regulation by β-adrenergic stimulation.

2. Methods

An expanded Methods section containing details for plasmids, yeast two-hybrid screening, co-immunoprecipitation, western blotting, immunocytochemistry, and electrophysiology is available only in the online data supplement. 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).

2.1 Yeast two-hybrid screening

The KCNQ1 N-terminal domain (NtKCNQ1) was used as a bait. The yeast reporter strain L40 that contains the reporter genes *lacZ* and *HIS3* downstream the binding sequences for LexA, was sequentially transformed with the pVJL10-NtKCNQ1 plasmid and with a mouse cDNA library, using the lithium acetate method[15] and subsequently treated as described.[16]

2.2 Co-immunoprecipitation and western blotting

African green monkey kidney-derived COS-7 cells (ATCC, Manassas, VA), guinea pig whole heart, rabbit anti-β-tubulin polyclonal antibody (Santa Cruz Biotechnology), goat anti-KCNQ1-C-terminus (Ct-KCNQ1) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-KCNQ1 polyclonal antibody (Alomone Labs, Jerusalem, Israel), rabbit anti-Yotiao antibody (Zimed Laboratory Inc. or Fusion Antibodies), rabbit anti-phospho-S27 KCNQ1 antibody,[17] rabbit anti-phospho-specific Yotiao antibody,[14] mouse anti-vesicular stomatitis virus (VSV) antibody (Sigma–Aldrich, St Louis, MO), and secondary horseradish peroxidase-coupled antibody (Santa Cruz Biotechnology) were used.

2.3 Immunocytochemistry

Immunostaining was performed on COS-7 cells and on freshly isolated cardiomyocytes from guinea pigs using rabbit anti-β-tubulin, mouse anti-β-tubulin, goat anti-Ct-KCNQ1 polyclonal, or rabbit anti-KCNQ1 antibodies and Alexa A488-conjugated anti-rabbit or Alexa A568-conjugated anti-goat secondary antibodies (Molecular Probes). Metamorph (Roper Scientific SAS, Princeton Instruments, Tucso, AZ), Amira (Mercury Computer Systems SAS, Chelmsford, MA) and WCIF ImageJ (NIH) softwares were used for analysis.

2.4 Electrophysiology

Patch-clamp studies were performed on COS-7 cells transiently expressing KCNQ1, KCNE1 (pCI-KCNQ1 and pRC-KCNE1, respectively),[18] Yotiao (pcDNA3-Yotiao),[11] and GFP (pEGFP, Clontech, Palo Alto, CA) and on freshly isolated guinea pig cardiac myocytes using the permeabilized-patch configuration with amphotericin B at $35 \pm 1^{\circ}C$. I_{Ks} was measured as the deactivating current at -40 mV in both cell types. For each set of experiments, untreated, taxol-, or colchicine-treated cells from the same transfected or isolated cell batch were investigated at the same time.

2.5 Statistics

The Costes randomization method (co-localization test plugin, ImageJ) was used to evaluate the significance of the confocal images.[19] Protein phosphorylation variations on western blots were evaluated with the non-parametric Wilcoxon signed rank test. Patch-clamp results are presented as mean ± SEM of current densities. Statistical significance of the observed effects was assessed by an unpaired or paired Student's *t*-test or, when indicated, by one-way or two-way analysis of variance for repeated measures when appropriate, followed by a Tukey test when needed. A value of $P < 0.05$ was considered significant.

3. Results

3.1 The N-terminus of the KCNQ1 K^+ channel interacts with β-tubulin in yeast

The yeast two-hybrid method was used to screen a mouse cDNA library using the N-terminal cytoplasmic domain of the KCNQ1 channel (amino acids 1–121, NtKCNQ1) as bait. Twenty interacting cDNA clones were isolated. DNA sequencing and database searching revealed that the nucleotide sequence of clone 5 encoded for about two-thirds of the length of the tubulin β2 isoform (amino acids 145–445). The specificity of the interaction was confirmed by co-transformation of clone 5 and bait into yeast in comparison with controls (Gal4-AD or LexBD-PCTAIRE-1; *Figure 1A*).

3.2 β-Tubulin interacts with KCNQ1 K^+ channel in transfected COS-7 cells and in the heart

In the quest for biochemical evidence for the interaction between the full-length KCNQ1 and β-tubulin, we immunoprecipitated the complex from the IGEPAL extract of KCNQ1-transfected COS-7 cells (*Figure 1B*) using antibodies against the KCNQ1 C-terminus or against β-tubulin. Western blotting analysis of KCNQ1 immunoprecipitates with a β-tubulin-specific antibody revealed co-precipitation of endogenous tubulin. Conversely, KCNQ1 was co-precipitated from a COS-7 cell lysate using a β-tubulin-specific antibody. Importantly, KCNQ1 and β-tubulin interaction were also consistently detected in the guinea pig heart by the same method ($n = 3$; *Figure 1B*).

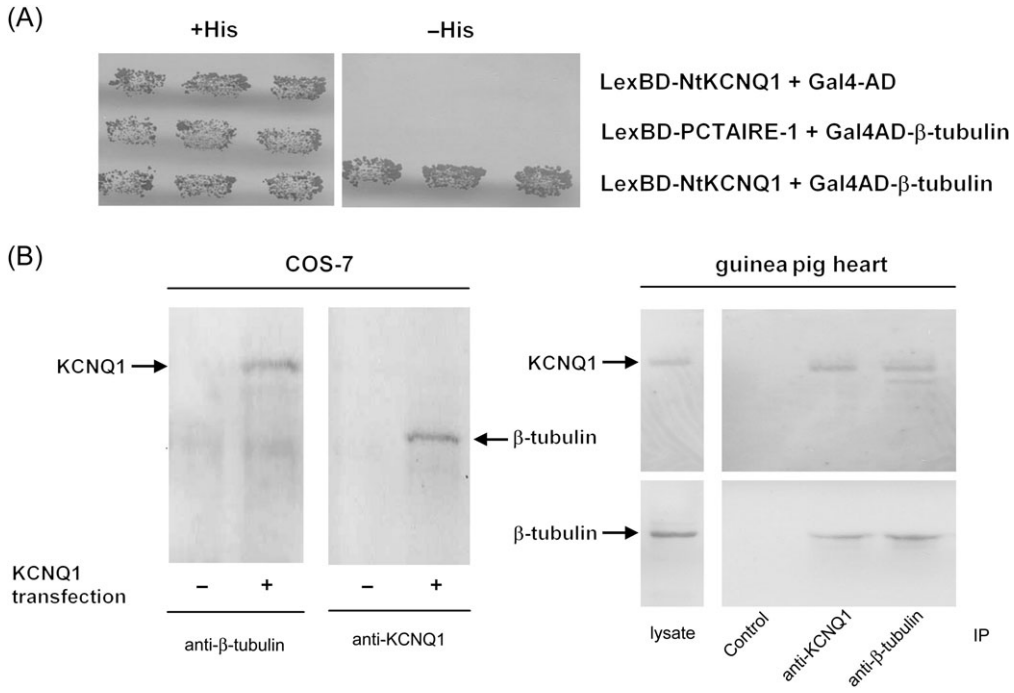

Figure 1 β-tubulin associates with KCNQ1. (*A*) KCNQ1 N-terminus (NtKCNQ1) and β-tubulin binding in yeast. Yeast cells were co-transformed with plasmid pairs coding for the indicated proteins fused to LexA BD or Gal4 AD. Transformants were plated on synthetic medium with or without histidine (His). Each patch represents an independent transformant. (*B*) Interaction of full-length KCNQ1 and β-tubulin. *Left*, COS-7 cells were transfected (+) or not (−) with a KCNQ1 coding vector. Cell extracts were incubated with anti-β-tubulin or anti-KCNQ1 antibody. The immunoprecipitated protein complexes were examined by immunoblotting with anti-KCNQ1 (*left*) or anti-β-tubulin (*right*) antibody. *Right*, Guinea pig heart extract was incubated with anti-KCNQ1 or anti-β-tubulin antibody (IP). The lysate and immunoprecipitated protein complexes were examined by immunoblotting with anti-KCNQ1 (*top*) or anti-β-tubulin (*bottom*) antibody. Control lane: the immunoprecipitation was performed with the corresponding antibody exhausted with the appropriate blocking peptide.

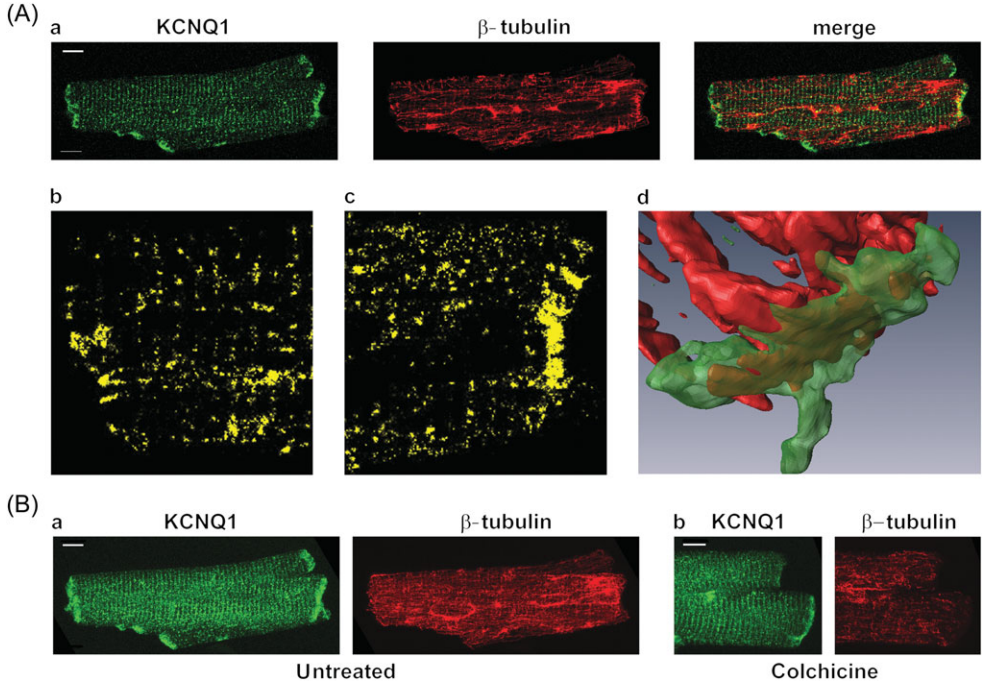

Figure 2 KCNQ1 and β-tubulin localization in guinea pig cardiomyocytes. (*A*) Co-localization probed with KCNQ1- and β-tubulin-specific primary antibodies followed by fluorescence-labelled secondary antibodies. Single confocal images (a); KCNQ1 is shown in green and β-tubulin in red. Co-localization alone (yellow) is shown on the magnified views (b and c). Three-dimensional reconstruction of the intercalated disk zone (d). (*B*) Stack of 46 images for each protein in untreated (a) and colchicine-treated (b, 30 μM for 2 h) cardiomyocytes. Scale bars: 5 μm.

We determined the subcellular distribution of KCNQ1 and β-tubulin using confocal microscopy in guinea pig cardiomyocytes. As illustrated in *Figure 2A*a, KCNQ1 is detected in the intercalated disks, t-tubules, and sarcolemma. Stacking of all confocal images of the same cell allows better visualization of KCNQ1 localization (*Figure 2Ba*, KCNQ1).

Control experiments performed using a 10-fold excess of antigenic KCNQ1 peptide did not exhibit any labelling (not shown). Immunolabelling of β-tubulin revealed a microtubule network in cardiomyocytes (*Figure 2A*a and *B*a, β-tubulin) that partly co-localized with KCNQ1 (four independent experiments; *Figure 2A*a–c, merge). A three-dimensional reconstruction of KCNQ1 and β-tubulin allowed better visualization of the protein co-localization at the intercalated disks (*Figure 2A*d and see Supplementary material online). In order to quantify the co-localization, Pearson and Mander coefficients were calculated on the stack of 46 images of *Figure 2B* (see Supplementary material online, *Table S1*). To test the significance of the co-localization, the Pearson coefficient for the whole image (R_{total}: 0.17) was compared with the one obtained from 100 randomized images (mean R_{rand}: 0.014). R_{total} was always higher than R_{rand} obtained on each of the 100 iterations ($R_{total} > R_{rand}$: 100%).[19] When non-specific co-localization was excluded by automatic thresholds for each fluorescence, a Pearson coefficient (R_{coloc}) of 0.66 was calculated. The Mander coefficient for KCNQ1 (tM1) indicates that 60% of the channels co-localized with β-tubulin.

The effects of microtubule depolymerization on KCNQ1 localization were evaluated. After a 2-h pretreatment with 30 μM colchicine, KCNQ1 staining was not modified (*Figure 2Bb*).

Altogether, our results demonstrate the presence of a physical interaction between the KCNQ1 channel and β-tubulin *in situ* in cardiomyocytes, and that microtubule depolymerization does not modify KCNQ1 membrane expression. Therefore, we investigated the functional consequences of the KCNQ1 and β-tubulin interaction.

3.3 Microtubule targeting agents do not modify KCNQ1—KCNE1 K^+ channel basal activity in COS-7 cells

The permeabilized-patch configuration was chosen over the conventional ruptured-patch configuration to avoid run-down of KCNQ1–KCNE1 channel activity.[20–22] In basal conditions, 1–2 h pretreatment with 10 μM colchicine did not modify the K^+ current density measured at −40 mV after a depolarization to +40 mV (*Table 1*), despite microtubule depolymerization as visualized on confocal images (*Figure 3A* and *B*, insets). Neither the half-activation time used to estimate the activation kinetics, nor the deactivation time constant showed any change after colchicine treatment. Similarly, 10 μM taxol treatment neither modified the current amplitude nor its biophysical characteristics (*Table 1*). Microtubule alteration did not alter the channel gating or trafficking.

Table 1 Biophysical characteristics of I_{Ks} in untreated, colchicine-, or taxol-treated COS-7 cells

	Untreated	Colchicine 10 μM	Taxol 10 μM
Cell number	16–17	13	8
Tail current density (pA/pF)	8.1 ± 2.0	6.8 ± 1.2	7.3 ± 1.6
$t_{1/2}$ act (ms)	163.5 ± 17.5	151.0 ± 15.5	138.4 ± 13.8
τ_{deact} (ms)	153.4 ± 6.8	166.8 ± 8.6	154.6 ± 8.4

Tail current density at −40 mV; $t_{1/2}$ act denotes half-activation time at +40 mV; τ_{deact} represents deactivation time constant (single exponential fit) at −40 mV. One-way analysis of variance: non-significant for each parameter.

3.4 I_{Ks} response to osmotic challenge does not depend on microtubules

Although the functional link between KCNQ1 and β-tubulin did not appear under basal conditions in COS-7 cells, it may become critical for channel regulation. In ventricular myocytes, cell swelling increases the native I_{Ks} current.[23,24] Interestingly, Grunnet and colleagues[25] showed that an N-terminus deleted KCNQ1 (amino acid 1–95, i.e. missing part of, if not all, the KCNQ1 interacting zone with β-tubulin determined in our study) failed to respond to osmotic challenges in *Xenopus* oocytes. Thus, we investigated the importance of microtubular network integrity on the response of KCNQ1–KCNE1 channels to osmotic challenge in COS-7 cells. The K^+ tail current, elicited by a +40 mV depolarization, increased in hypo-osmolar solution (*Figure 3A* and *C*) and decreased in hyper-osmolar solution, compared with the current recorded in iso-osmotic condition. Activation kinetics was also modified by osmolarity changes (*Figure 3D*). After microtubule depolymerization with colchicine, the same changes in response to osmotic shock were observed (*Figure 3B–D*), as in untreated cells. The deactivation kinetics was similar in all conditions (see Supplementary material online, *Table S2*). These data indicate that the β-tubulin interaction with KCNQ1 is not involved in current modulation by cell volume changes.

3.5 I_{Ks} response to PKA-dependent stimulation depends on microtubules

When Yotiao was cloned, it was suspected to associate with the cytoskeleton.[26] Since then, Yotiao has been shown to target PKA and protein phosphatase to KCNQ1 and to be mandatory for cAMP-dependent I_{Ks} regulation.[11,27] A potential role of KCNQ1–β-tubulin interaction would be that microtubules could consolidate the KCNQ1–Yotiao complex. Therefore, we evaluated the effects of microtubule depolymerization on the PKA-activated K^+ current. These experiments were first conducted in COS-7 cells transfected with KCNQ1, KCNE1, and Yotiao. In this condition, the basal I_{Ks} current was not modified by colchicine (*Figure 4*). The density of the K^+ current related to KCNQ1–KCNE1 expression was more than doubled by adding 400 μM cpt–cAMP, 10 μM forskolin, and 0.2 μM okadaic acid (*Figure 4A* and *C*). PKA stimulation of the KCNQ1–KCNE1 current was lost in the presence of colchicine (*Figure 4B* and *C*). Interestingly, the activation kinetics were significantly accelerated in both conditions by cAMP enhancement (*Figure 4D*), indicating that channel phosphorylation still occurs in colchicine-treated cells. The fact that only the amplitude response to PKA is altered, suggests that amplitude and activation kinetics are not linearly correlated (such as in the channel regulation by PIP_2[21]) and that an efficient coupling gives rise first to acceleration of the activation, then to an increased amplitude. Another possibility is that amplitude

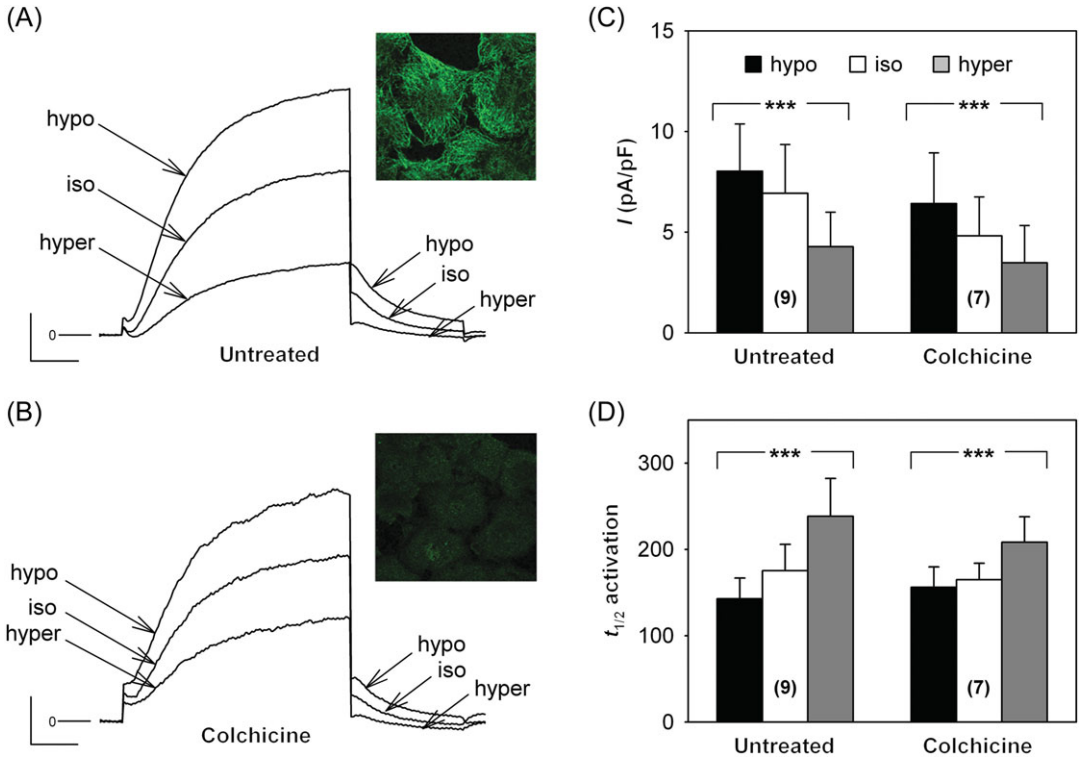

Figure 3 Effects of osmotic challenge on I_{Ks} current in COS-7 cells. (*A*) Representative current traces recorded in a cell expressing KCNQ1–KCNE1 channels exposed to hypo-osmolar (hypo: −80 mOsm), hyper-osmolar (hyper: +80 mOsm), or iso-osmolar solution (iso). The membrane was depolarized from −80 mV to +40 mV for 1000 ms and repolarized to −40 mV for 500 ms during which the tail current was measured (scales: 100 pA and 200 ms). (*B*) Representative current traces from a colchicine-treated cell (10 μM) during the same voltage protocol and challenged with the same solutions (scales: 50 pA and 200 ms). *Insets*: β-tubulin immunostaining showing β-tubulin dilution in treated cells. Tail current density (*C*) and half-activation time ($t_{1/2}$ activation in milliseconds; (*D*) measured in untreated and colchicine-treated COS-7 cells (colchicine). Untreated and colchicine-treated cells exhibited similar I_{Ks} responses to osmotic challenge [two-way analysis of variance (ANOVA), non-significant for both parameters; one-way ANOVA, $^{***}P < 0.001$ for both parameters with or without treatment]. Numbers between brackets indicate the number of cells tested.

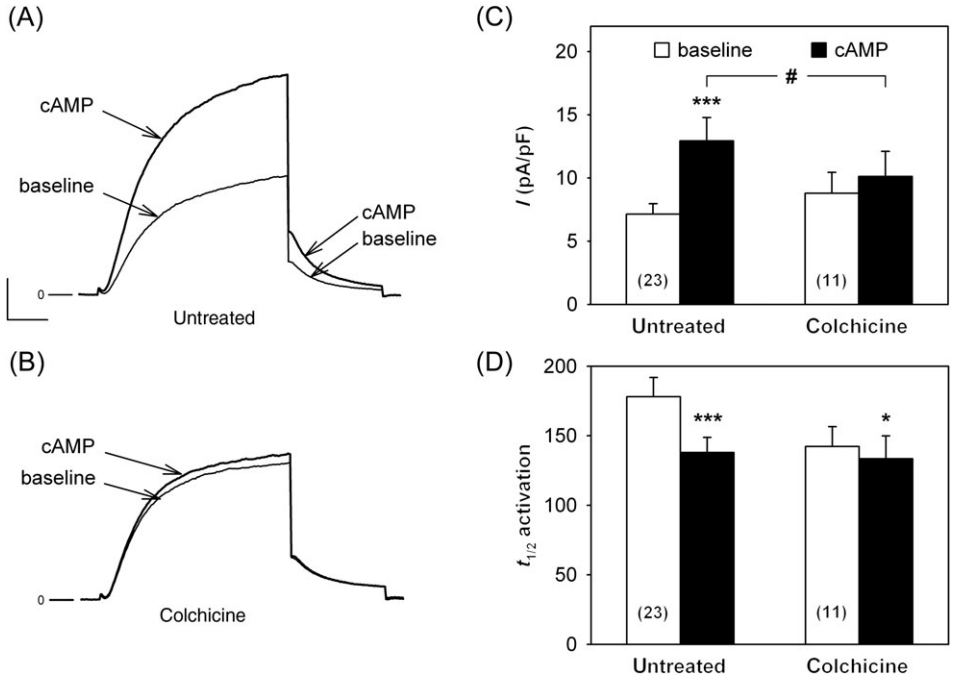

Figure 4 I_{Ks} response to protein kinase A (PKA)-dependent stimulation depends on microtubule polymerization state. Representative current traces recorded in an untreated (*A*) and a colchicine-treated (10 μM, *B*) COS-7 cells expressing KCNQ1, KCNE1, and Yotiao, in basal conditions (baseline) and exposed to 400 μM cpt–cAMP, 10 μM forskolin, and 0.2 μM okadaic acid (cAMP). Voltage protocol as in *Figure 3A*—scales: 100 pA and 200 ms; (*B*) same scales as (*A*). (*C*, *D*) Tail current density and half-activation time (ms) measured in untreated and colchicine-treated cells, respectively. PKA-dependent I_{Ks} response was significantly decreased in colchicine-treated cells [two-way analysis of variance (ANOVA): $^{\#}P < 0.05$; Tukey test: untreated: $^{***}P < 0.001$ vs. baseline value; colchicine-treated: non-significant (NS)], but the activation acceleration owing to PKA remained (two-way ANOVA: NS; *t*-test: untreated: $^{***}P < 0.001$ vs. baseline value; colchicine-treated: $^{*}P < 0.05$). Deactivation kinetics was not altered in all conditions (see Supplementary material online, *Table S2*).

and activation kinetics alterations reflect distinct mechanisms (such as voltage sensing and gate opening). Nevertheless, these results suggest that microtubules are implicated in the I_{Ks} response to PKA-dependent stimulation.

To further test the hypothesis that microtubules participate to the KCNQ1 and Yotiao connection, we checked whether Yotiao is associated with microtubules as other PKA-anchoring proteins, like AKAP75[28] and MAP2B.[29] As illustrated in *Figure 5A*, we did not observe any co-immunoprecipitation of native β-tubulin and transfected Yotiao in COS-7 cells in the absence of KCNQ1 and KCNE1 ($n = 3$).

One can argue that Yotiao can interact with microtubules only when phosphorylated and that this PKA-dependent association is required for subsequent I_{Ks} activation. However, we show that PKA activation leading to Yotiao phosphorylation did not induce any interaction with β-tubulin, ruling out this hypothesis (*Figure 5A*).

Secondly, we reasoned that if microtubules are involved in Yotiao and KCNQ1 interaction, their depolymerization should (i) limit KCNQ1 and Yotiao interaction and/or (ii) limit the channel phosphorylation. As illustrated in *Figure 5B*, colchicine treatment did not modify KCNQ1 and Yotiao interaction, revealed by co-immunoprecipitation in COS-7 cells ($n = 3$). Furthermore, KCNQ1 phosphorylation was not significantly altered ($n = 4$, *Figure 5C*).

Yotiao phosphorylation is a prerequisite for PKA-dependent activation of KCNQ1.[14] Therefore, we also tested the effects of microtubule disruption on Yotiao phosphorylation. As shown in *Figure 5D*, colchicine treatment did not affect PKA-induced Yotiao phosphorylation ($n = 4$).

These results, together with the absence of β-tubulin and Yotiao co-immunoprecipitation in baseline conditions or after PKA activation, suggest that microtubules play no role in Yotiao-dependent KCNQ1 phosphorylation but modulate the influence of KCNQ1 phosphorylation on the channel activity.

Another hypothesis to explain the loss of current increase in colchicine-treated cells is that PKA stimulation recruits sub-membrane KCNQ1 pools to the membrane, as previously reported for $Na_v1.5$,[30] and microtubule depolymerization alters this trafficking. Alternatively, cAMP may decrease KCNQ1 channels endocytosis as recently reported on the potassium channel $K_v1.2$,[31] by stabilizing the channels at the membrane. We thus tested whether the current increase observed in untreated cells was associated with an increased amount of KCNQ1 channel at the plasma membrane. COS-7 cells were transfected with the fusion protein KCNE1–

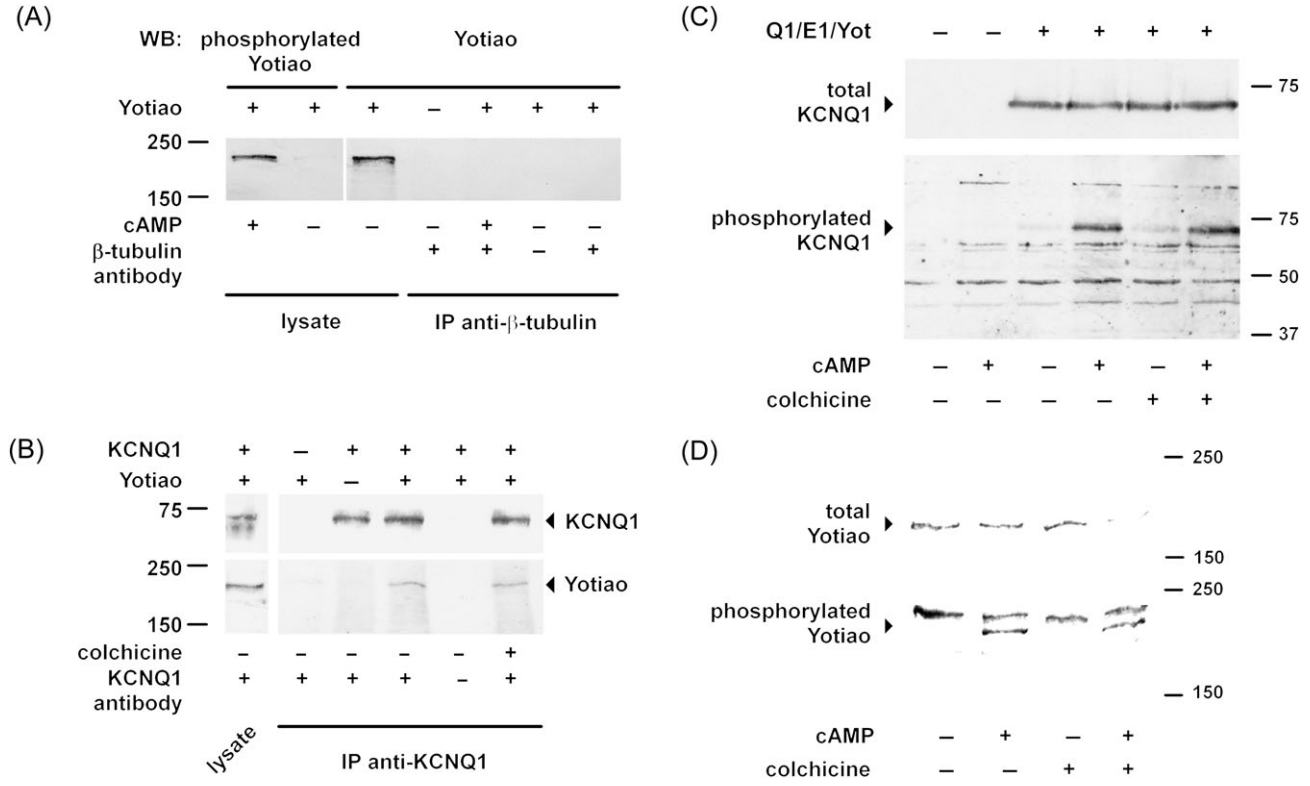

Figure 5 Microtubules interfere neither with the KCNQ1 and Yotiao interaction nor with their protein kinase A (PKA)-dependent phosphorylation. (*A*) Yotiao and β-tubulin do not interact in transfected COS-7 cells. Lysates of untreated cells or those exposed to 400 μM cAMP, 10 μM forskolin, and 1 μM okadaic acid, were immunoprecipitated with anti-β-tubulin antibody and the presence of Yotiao in the complex was examined by western blot. Phosphorylation efficiency was examined in lysates with anti-phosphorylated Yotiao antibody. (*B*) KCNQ1 interacts with Yotiao in COS-7 cells. Transfected cells were either treated or not with 10 μM colchicine for 2 h. KCNQ1 was immunoprecipitated and presence of Yotiao in the complex was examined by western blot. (*C*) KCNQ1 phosphorylation is not impaired by microtubule depolymerization in COS-7 cells. Western blot of cells either transfected with KCNQ1, KCNE1, and Yotiao (Q1/E1/Yot) or GFP alone, treated with 400 μM cpt–cAMP, 10 μM forskolin, and 0.2 μM okadaic acid, and probed with anti-KCNQ1 (Top) or anti-phospho-S27 KCNQ1 antibody (Bottom). The increase of phosphorylated KCNQ1 was similar in untreated and treated cells (4.4 ± 1.4-fold and 4.0 ± 1.2-fold, respectively; $n = 4$; Wilcoxon-signed rank test: NS). (*D*) Yotiao phosphorylation is not impaired by microtubule depolymerization in COS-7 cells. Western blot of cells transfected with KCNQ1, KCNE1, and Yotiao, treated with 400 μM cpt–cAMP, 10 μM forskolin, and 1 μM okadaic acid, and probed with anti-Yotiao (Top) or anti-phosphorylated Yotiao antibody (Bottom). The normalized density of the phosphorylated Yotiao-specific band was similar for untreated and treated cells ($n = 4$, Wilcoxon-signed rank test: NS).

KCNQ1 with a VSV extracellular tag on KCNE1 N-terminus (VSV–KCNE1–KCNQ1)[12] and Yotiao. An immunoprecipitation was performed with an anti-VSV antibody on intact non-permeabilized cells. These experiments show that the amount of KCNQ1 at the plasma membrane was not increased by cAMP treatment (see Supplementary material online, *Figure S1*). These data suggest that KCNQ1 phosphorylation modulates the biophysical properties rather than the channel trafficking.

To evaluate the physiological relevance of the I_{Ks} regulation by microtubules, we repeated the patch-clamp experiments in guinea pig cardiomyocytes. The PKA-dependent I_{Ks} increase was significantly reduced by more than two-fold after 30 μM colchicine treatment (*Figure 6B*, top and *C*) when compared with untreated cardiomyocytes (*Figure 6A*, top and *C*), whereas activation and deactivation were similarly modified in both conditions (*Figure 6D* and *E*). Unlike I_{Ks}, the inward rectifier K^+ current I_{K1} is decreased by PKA (*Figure 6A* bottom), as already reported for human and guinea pig ventricular myocytes.[32,33] Here, we observed that colchicine did not alter the I_{K1} baseline current or its cAMP-induced decrease (*Figure 6B*, bottom and *F*). The sensitivity to microtubule depolymerization thus seems to be specific for the KCNQ1 channel.

4. Discussion

In the present study, a direct interaction between β-tubulin and KCNQ1 was identified using a yeast two-hybrid screening. Both proteins are not only co-localized in cardiomyocytes, but also co-immunoprecipitate in this model and in COS-7 cells, confirming a physical association. Using patch-clamp experiments, we demonstrated that the PKA-dependent activation of I_{Ks} is modulated by microtubule polymerization in COS-7 cells and cardiomyocytes. However, microtubule disruption does not modify KCNQ1–Yotiao association and does not prevent KCNQ1 and Yotiao phosphorylation. This regulation seems to be specific to I_{Ks}, as I_{K1} current response to PKA activation was not modified by colchicine treatment, suggesting that I_{Ks} modulation by microtubules acts downstream the phosphorylation in the PKA signalling cascade.

Numerous and very different ion channels have been shown to be regulated by microtubules. For most of them,

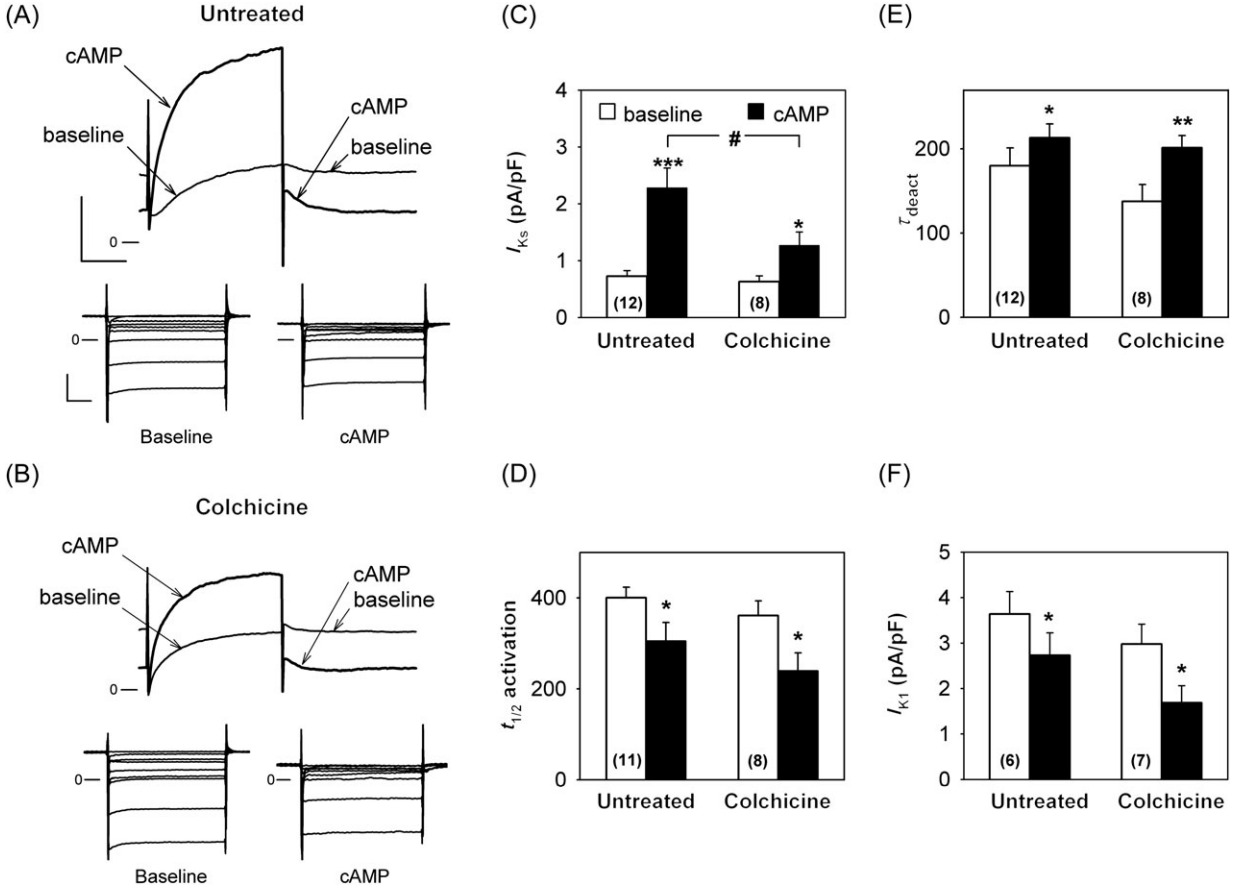

Figure 6 I_{Ks} and I_{K1} response to cAMP-dependent stimulation in guinea pig ventricular myocytes. Representative I_{Ks} (Top) and I_{K1} (Bottom) traces recorded in untreated (*A*) and colchicine-treated (30 μM, *B*) cardiomyocytes in basal conditions (baseline) and exposed to cAMP increase (same as in *Figure 4*). The membrane was depolarized from −40 mV to +40 mV for 1500 ms and repolarized to −40 mV where the deactivating tail current, I_{Ks}, was measured, the remaining outward current being I_{K1} (*A* and *B*, scales: 200 pA and 500 ms). For I_{K1}-specific recording, voltage steps were applied from −60 mV to various potentials from −100 to −20 mV for 500 ms (*A* and *B* scales: 500 pA and 100 ms). I_{Ks} tail current density (*C*), half-activation time (*D*), and deactivation time constant (*E*, single exponential fit, τ_{deact}) in untreated and colchicine-treated cardiomyocytes. (*F*) I_{K1} current measured at the end of a 500 ms pulse from −60 mV to −40 mV, potentials at which I_{Ks} is not activated. Protein kinase A (PKA)-dependent I_{Ks} response was significantly decreased in colchicine-treated myocytes [two-way analysis of variance (ANOVA): $^{\#}P < 0.05$; Tukey test: untreated—$^{**}P < 0.001$ vs. baseline value; colchicine-treated: $^{*}P < 0.05$] despite similar activation acceleration induced by PKA (two-way ANOVA: NS; *t*-test—$^{*}P < 0.05$, vs. corresponding baseline value) and deactivation deceleration (two-way ANOVA: NS; *t*-test—$^{*}P < 0.05$ vs. baseline value and $^{**}P < 0.01$). I_{K1} decrease was maintained in treated cells (two-way ANOVA: NS; *t*-test—$^{*}P < 0.05$ vs. corresponding baseline value).

the role of the microtubular cytoskeleton is to regulate the protein trafficking to the appropriate cellular location.[34–40] Most frequently, the identified mechanism involves a third protein [e.g. GABARAP for the neuronal $GABA_A$ receptor channel[41] or mammalian diaphanous 1 (mDia1) for the mechanosensitive cation channel (PKD2) in the primary cilium of kidney cells[42]]. On the contrary, the Ca^{2+} channel TRPC1 has been demonstrated to bind directly to β-tubulin in retinal epithelium cells.[43] Microtubule disruption leads to a decrease of TRPC1 expression at the plasma membrane of transfected cells. Here, we show that microtubule disruption does not modify the basal I_{Ks} amplitude or KCNQ1 targeting in COS-7 cells and in cardiomyocytes. Altogether these results rule out the involvement of tubulin in KCNQ1 trafficking.

Downstream the channel trafficking, there are several examples of channel activity modulation through tubulin polymerization. One context in which the cytoskeleton may regulate ion fluxes is volume regulation. For instance, the activity and stretch sensitivity of BK_{Ca} channels from rabbit coronary artery smooth muscle depend on microtubule stability.[44] However, I_{Ks} sensitivity to osmotic changes is not dependent upon microtubule polymerization either in cardiomyocytes[24] or in COS-7 cells as observed in our study.

Another way that cytoskeletal changes alter ion conductivity is by altering the efficacy of regulatory elements. It has been shown that microtubule polymerization affects L-type Ca^{2+} channel regulation by phosphorylation.[45] However, this regulation was indirect: the authors proposed that microtubule depolymerization induces release of a guanosine triphosphate, which may lead to a maximal adenylyl cyclase activation[46] and failure of β-adrenergic stimulation to further increase the Ca^{2+} current. The experiments presented here strongly suggest that, for KCNQ1, the participation of the microtubule network is direct: (i) We show that tubulin directly interacts with KCNQ1 and (ii) colchicine treatment spares the whole cascade down to KCNQ1 and Yotiao phosphorylation and only alters the coupling between phosphorylation and channel opening. This suggests that KCNQ1—but not a regulatory protein involved in PKA-dependent regulation (adenylyl cyclase, PKA, etc.)—is directly affected by tubulin polymerization. Furthermore, PKA stimulation does not increase KCNQ1 channel density at the membrane. This rules out the hypothesis of an altered channel trafficking after microtubule depolymerization that would prevent PKA-mediated I_{Ks} increase. Altogether, our data suggest that the current response alteration is caused by a weaker coupling of the channel phosphorylation and its intrinsic activation.

Previous studies showed that the I_{Ks} current response to PKA-dependent stimulation does not only depend on KCNQ1 channel phosphorylation, but also on Yotiao integrity. Indeed, the amplitude of the pseudo-phosphorylated form of the KCNQ1 channel (S27D KCNQ1) is increased only in the presence of Yotiao.[13] In this case, the AKAP Yotiao seems to be required for the post-phosphorylation response. Furthermore, phosphorylation of Yotiao itself by PKA is needed to observe any I_{Ks} increase. In CHO cells, the unphosphorylable S43A Yotiao mutant prevents an I_{Ks} increase despite the conserved KCNQ1 phosphorylation when PKA is activated.[14] In addition to ours, these results indicate that both Yotiao phosphorylation and microtubule integrity are required to maintain the PKA-dependent channel phosphorylation and activation coupling. However, these two factors seem independent because co-immunoprecipitation experiments failed to show any interaction between Yotiao and tubulin.

The duration of the action potential repolarization is known to be heterogeneous across the human ventricular wall. A longer action potential is recorded in cells from the mid-myocardium (M cells).[47] Using different animal models, this difference has been shown to be owing to a smaller I_{Ks} in M cells.[48,49] We have previously shown that the action potential prolongation in human M cells is correlated to higher expression of the dominant-negative alternative isoform of KCNQ1 that results in a smaller I_{Ks}.[50] I_{Ks} plays a major role in human ventricular repolarization mostly under sympathetic stimulation and when the repolarization reserve is reduced.[6] Therefore, a change in I_{Ks} sensitivity to β1-adrenergic stimulation, when microtubules are altered in pathological conditions, may appear to be highly relevant for ventricular repolarization and induction of arrhythmias, in particular, by inducing transmural dispersion alterations.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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