Requirement of a Macromolecular Signaling Complex for β Adrenergic Receptor Modulation of the KCNQ1-KCNE1 Potassium Channel

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Sympathetic nervous system (SNS) regulation of cardiac action potential duration (APD) is mediated by β adrenergic receptor (βAR) activation, which increases the slow outward potassium ion current (I_{KS}). Mutations in two human I_{KS} channel subunits, hKCNQ1 and hKCNE1, prolong APD and cause inherited cardiac arrhythmias known as LQTS (long QT syndrome). We show that βAR modulation of I_{KS} requires targeting of adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (PKA) and protein phosphatase 1 (PP1) to hKCNQ1 through the targeting protein yotiao. Yotiao binds to hKCNQ1 by a leucine zipper motif, which is disrupted by an LQTS mutation (hKCNQ1-G589D). Identification of the hKCNQ1 macromolecular complex provides a mechanism for SNS modulation of cardiac APD through I_{KS}.

Cardiac function is regulated by the SNS; stimulation of βARs increases heart rate and contractility and shortens APD (*1*). βAR-mediated regulation of I_{KS} is particularly important because patients with KCNQ1 or KCNE1 mutations have a form of LQTS (*2, 3*) in which fatal cardiac arrhythmias are precipitated by increased SNS activity associated with exercise and startling (*4–6*).

Because βAR signaling results in PKA activation, we sought to determine whether PKA and a phosphatase were components of an hKCNQ1 macromolecular signaling complex. In immunoprecipitations of hKCNQ1 from cardiac homogenates, we found that PKA catalytic and regulatory (RII) subunits, protein phosphatase 1 (PP1) (but not PP2A), and the PKA- and PP1-targeting protein yotiao [which also targets PKA and PP1 to *N*-methyl-D-aspartate (NMDA) receptors in the brain (*7*)] coimmunoprecipitated with hKCNQ1 from human cardiac homogenates (Fig. 1A). Thus, the PKA catalytic and regulatory subunits, PP1, and yotiao are components of the hKCNQ1 macromolecular complex.

Microcystin Sepharose beads (which bind to PP1) and cAMP Sepharose beads (which bind to the PKA regulatory subunit) specifically associated with hKCNQ1 when incubated with cardiac homogenates from a transgenic mouse (TG^+) expressing an hKCNQ1-hKCNE1 fusion protein in the heart. No such association was detected in homogenates from wild-type (WT) mouse hearts, which lack I_{KS} because their heart rate (~500 beats per minute) requires K^+ channels with faster kinetics to control APD (Fig. 1B). PKA catalytic and regulatory (RII) subunits, PP1, and yotiao were also associated with hKCNQ1-hKCNE1 from TG^+ mouse hearts (Fig. 1C).

The binding of kinases and phosphatases to ion channels through targeting proteins can be mediated by leucine zipper (LZ) motifs (*8*). We tested whether a LZ motif in the COOH-terminus of hKCNQ1 (amino acid residues 588 to 616) functioned in targeting PKA and PP1 to hKCNQ1 through yotiao. Glutathione *S*-transferase (GST)–KCNQ1 fusion proteins were incubated with cardiac muscle

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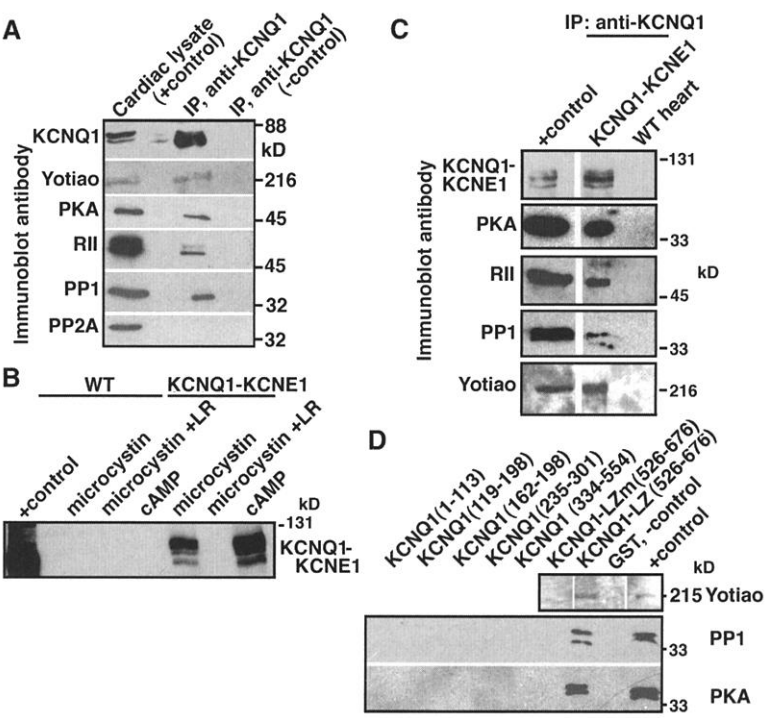

Fig. 1. Presence of PKA, RII, PP1, and the targeting protein yotiao in the KCNQ1 macromolecular complex. (**A**) KCNQ1 immunoprecipitated from human cardiac homogenates using anti-KCNQ1 (*22*). Positive control for immunoblotting was human cardiac homogenate; negative control for immunoprecipitation was anti-KCNQ1 preabsorbed with the antigenic peptide (lane 3). (**B**) For pull-down assays, either microcystin- or cAMP-Sepharose beads were incubated with cardiac homogenates from wild-type (WT) or TG^+ mice (expressing hKCNQ1-hKCNE1) (*23*). Bound proteins were immunoblotted with anti-hKCNQ1. Specificity of the microcystin pull-down was demonstrated using free microcystin LR. "+control" is anti-hKCNQ1 immunoblot of TG^+ cardiac homogenate that recognizes the hKCNQ1-hKCNE1 fusion product of the transgene, which migrates at ~125 kD. (**C**) Proteins were immunoprecipitated from homogenates of WT or hKCNQ1-hKCNE1 TG^+ mouse hearts with anti-hKCNQ1 and immunoblotted with anti-hKCNQ1, anti-PKA (catalytic subunit), anti-RII (PKA regulatory subunit), anti-PP1 (catalytic subunit of PP1), and anti-yotiao. "+ control" is TG^+ cardiac homogenate. (**D**) Proteins from cardiac homogenates (200 μg) that associated with GST-hKCNQ1 fusion proteins (*24*) were immunoblotted with anti-yotiao, anti-PKA, and anti-PP1 (*25*). "+ control" is TG^+ cardiac homogenate. Data shown are representative of three or more experiments.

homogenates from human or TG$^+$ (hKCNQ1-hKCNE1) mouse hearts. GST–KCNQ1-LZ, containing the LZ motif, coprecipitated PP1, PKA, and yotiao (Fig. 1D). This interaction was specific, as neither GST alone nor GST-KCNQ1 fragments from other intracellular regions without the LZ motif coprecipitated PP1, PKA, or yotiao (Fig. 1D). GST–KCNQ1-LZ did not directly bind recombinant PP1 or PKA catalytic subunits (*9*), indicating that the binding of PP1 and PKA to hKCNQ1 requires yotiao. Moreover, a mutant peptide (KCNQ1-LZ$_m$) containing alanine substitutions (Leu^{602} → Ala, Ile^{609} → Ala) that disrupt the LZ motif did not coprecipitate PP1, PKA, or yotiao. Yotiao contains multiple coiled coils that can form LZs but has not been reported to bind to targets via LZs. Kinase and phosphatase binding to ion channels through specific targeting proteins provides a mechanism regulating phosphorylation at the subcellular level (*10, 11*). For example, PP1 is targeted to the type 2 ryanodine receptor–calcium release channel on the cardiac sarcoplasmic reticulum through spinophilin (*8*) and is now shown to interact through yotiao with hKCNQ1 in the plasma membrane.

Addition of cAMP, in the absence of exogenous PKA, induced phosphorylation of immunoprecipitated KCNQ1 (Fig. 2A). Using an alanine substitution, we identified Ser^{27} as the unique site of PKA phosphorylation on hKCNQ1 (Fig. 2B). We did not detect AKAP 79 or AKAP 15-18 (other PKA targeting proteins) with KCNQ1. Indeed, our data show that yotiao is the only protein that targets PKA and PP1 to hKCNQ1 complex, because incubation of cardiac homogenates with a peptide containing the KCNQ1 LZ motif (which binds yotiao) prevented coimmunoprecipitation of yotiao, PKA, and PP1 with KCNQ1 (Fig. 2C). The corresponding mutant peptide containing a disrupted LZ (GST–KCNQ1-LZ$_m$) did not disrupt complexes of KCNQ1 with yotiao, PKA, or PP1 (Fig. 2C).

When hKCNQ1 and yotiao were coexpressed in Chinese hamster ovary (CHO) cells, PKA and PP1 were coimmunoprecipitated with hKCNQ1 (Fig. 2D). Without yotiao, PKA and PP1 did not coimmunoprecipitate with hKCNQ1 (Fig. 2D). Disrupting the hKCNQ1 LZ with alanine substitutions (KCNQ1-LZ$_m$) or with the LQTS-associated KCNQ1-G589D mutation (Gly^{589} is the first "e" position in the COOH-terminal hKCNQ1 LZ motif) prevented binding of yotiao, PKA, or PP1 to the channel (Fig. 2D). Thus, the LZ motif is required for yotiao-mediated targeting of PKA and PP1 to hKCNQ1.

The membrane-permeant cAMP analog 8 Br-cAMP (300 μM) significantly increased hKCNQ1-hKCNE1 tail currents after +60 mV prepulses 1.8 ± 0.1–fold (n = 9) (Fig. 3A) in myocytes from mice expressing the human proteins. cAMP has similar effects on native I_{Ks} recorded from guinea pig ventricular myocytes (*12*). hKCNQ1-hKCNE1 channel activity (assayed from tail currents after +60 mV prepulses) was significantly enhanced (3.6 ± 0.7 fold, n = 5) (Fig. 3A) by cAMP (300 μM) plus okadaic acid (OA, 1 μM), a PP1 inhibitor that binds to the phosphatase catalytic site. OA alone did not significantly increase hKCNQ1-hKCNE1 currents (n = 5) (Fig. 3A). Thus, kinase (PKA) activity potently regulates I_{Ks} and inhibiting phosphatase (PP1) activity enhances this regulation.

Expression of yotiao was required to reconstitute cAMP-dependent regulation of the hKCNQ1-hKCNE1 channel in a heterologous expression system (CHO cells). In the absence of yotiao, there was no significant effect of intracellular cAMP (200 μM) on hKCNQ1-hKCNE1 currents either in the absence or presence of OA (0.2 μM n = 5) (Fig. 3B). With yotiao, hKCNQ1-hKCNE1 tail current (after +60 mV conditioning pulses) was doubled by cAMP [without cAMP, 24.3 ± 7.5 pA/pF; with cAMP (200 μM), 50.7 ± 9.2 pA/pF; n = 5] and OA (0.2 μM) plus cAMP (200 μM) increased the hKCNQ1-hKCNE1 current ~4-fold (89 ± 15.6 pA/pF, n = 5) (Fig. 3B).

Substituting Ala for Ser^{27} (S27A) in KCNQ1, which is phosphorylated in response to cAMP, eliminated cAMP-dependent enhancement of hKCNQ1-hKCNE1 current (n = 6, Fig. 3C). Expression in CHO cells of hKCNE1 and yotiao with mutant hKCNQ1 (hKCNQ1-LZ$_m$) or the G589D mutant linked to LQTS (*13*), both of which disrupt the KCNQ1 LZ motif (Fig. 2D), ab-

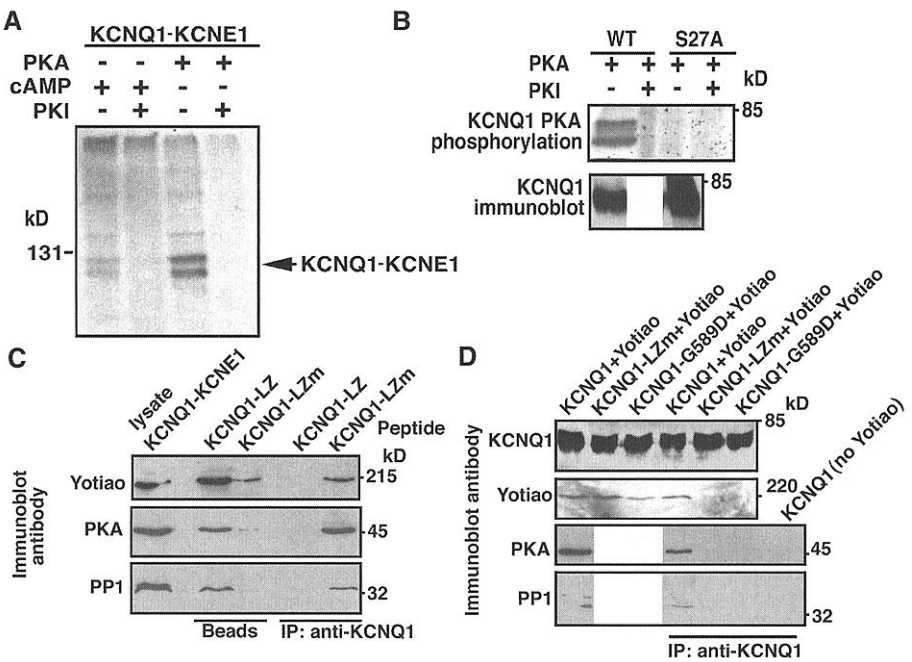

Fig. 2. Phosphorylation of Ser^{27} on KCNQ1 by PKA requires an intact LZ motif in the COOH-terminus of KCNQ1. (**A**) hKCNQ1-hKCNE1 immunoprecipitated from TG$^+$ mouse heart was incubated with cAMP (10 μM) or exogenous PKA (5 units) and [γ-^{32}P]ATP. The immunoprecipitates were size-fractionated, followed by autoradiography. The specificity of the PKA phosphorylation was established using the PKA inhibitor PKI (500 nM). (**B**) Identification of Ser^{27} as the PKA phosphorylation site on hKCNQ1 by alanine substitution (S27A). WT hKCNQ1 and hKCNQ1-S27A expressed in CHO cells were immunoprecipitated with anti-hKCNQ1 and incubated with PKA (5 units) and [γ-^{32}P] ATP. The immunoprecipitates were size-fractionated and phosphorylation was detected by autoradiography. Equivalent amounts of hKCNQ1 and hKCNQ1-S27A were present in immunoprecipitates, as demonstrated by immunoblotting (lower panel). Data are representative of three experiments. (**C**) A fusion peptide containing the COOH-terminal LZ motif of hKCNQ1 (hKCNQ1-LZ) competes off yotiao, PKA, and PP1 from the hKCNQ1-hKCNE1 complex. TG$^+$ cardiac homogenates (200 μg) were incubated with glutathione beads containing hKCNQ1-LZ or hKCNQ1-LZ$_m$. Supernatants from these reactions were immunoprecipitated with anti-hKCNQ1 and the precipitates were blotted for yotiao, PKA, and PP1. The "+" control is TG$^+$ cardiac homogenate. (**D**) WT hKCNQ1, mutant channels with disrupted LZ motifs, hKCNQ1-LZ$_m$, or hKCNQ1-G589D were coexpressed with yotiao in CHO cells. Equal amounts of WT and mutant hKCNQ1, yotiao, PKA, and PP1 were shown by immunoblotting (first three lanes). Proteins were immunoprecipitated from lysates with anti-KCNQ1 and immunoblotted. Immunoprecipitated hKCNQ1 from CHO cells without yotiao is shown in the last lane. Data shown are representative of three or more experiments.

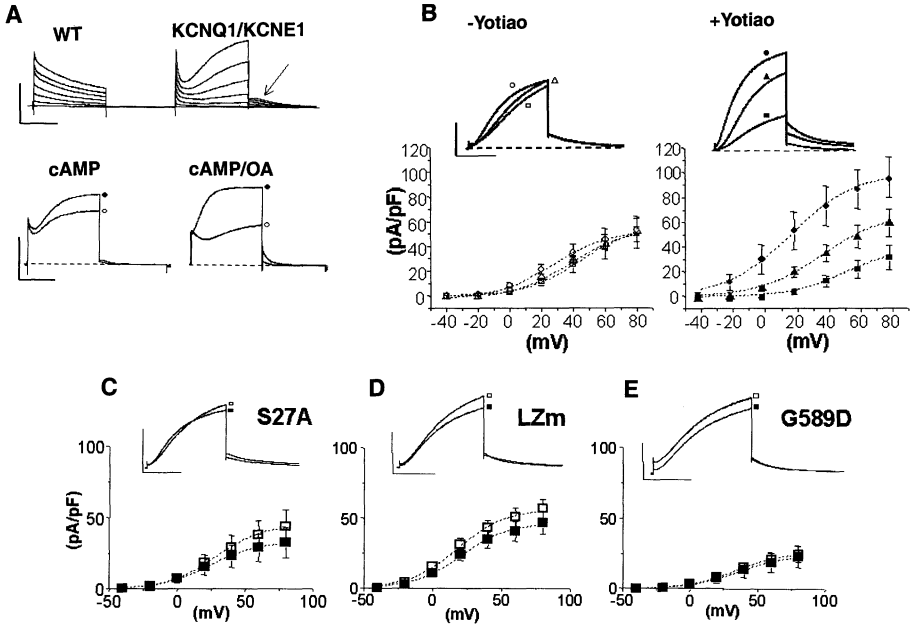

Fig. 3. Requirement of Ser[27], the KCNQ1 LZ motif, and yotiao for PKA-dependent regulation of I_{KS}. **(A)** Whole-cell patch clamp currents recorded from WT and TG$^+$ (hKCNQ1-hKCNE1) murine cardiomyocytes (*26*). Tail current after +60 mV pulses was significantly increased by cAMP ($P < 0.01$, Student's t test, pre-cAMP versus cAMP, $n = 9$) and by cAMP plus OA ($P = 0.01$, Student's t test, pre-cAMP + OA versus cAMP + OA, $n = 5$). Currents shown in upper traces were activated by a series of 2-s voltage pulses from −40 mV to +80 mV (20-mV increments, 0.067 Hz) from a −65 mV holding potential and deactivated by returning to −40 mV. hKCNQ1-hKCNE1 channel current is evident as slowly activating outward current during pulses and deactivating current "tails" upon repolarization detectable in TG$^+$ (arrow) but not TG$^-$ cells. Myocytes were treated with cAMP and OA (bottom two traces) and hKCNQ1-hKCNE1 currents were activated by depolarization (+60 mV). Currents recorded before (open circles) and after (solid circles) a 5-min external application of 8-Br-cAMP (300 μM) or 8-Br-cAMP (300 μM) plus OA (1.0 μM). Scale: 20 pA/pF and 1 s. **(B)** Requirement of yotiao for cAMP and OA dependent modulation of hKCNQ1-hKCNE1 channels in CHO cells. Shown are mean currents ($n = 5$) (+60 mV pulse, −40 mV return) as well as plots of mean tail current ± SEM vs. pulse voltage. Tail currents (after +60 mV) were significantly different by ANOVA $P < 0.05$. CHO cells were transfected with hKCNQ1-hKCNE1 without (left) or with (right) yotiao. Internal solutions without cAMP (control); with 200 μM cAMP (cAMP); or with 200 μM cAMP plus 0.2 μM OA (cAMP/OA) were dialyzed at room temperature for 13 min before measurements were made. Open symbols, no yotiao; solid symbols, with yotiao. Squares, control; triangles, cAMP; circles, cAMP/OA. Scale: 100 pA/pF, 1 s. **(C)** Substituting Ala for Ser[27] in KCNQ1. I_{KS} was measured in CHO cells cotransfected with yotiao, hKCNE1, and hKCNQ1-S27A ($n = 6$). Scale: 100 pA/pF, 1 s. **(D)** Disruption of the KCNQ1 LZ motif by Ala substitution. CHO cells were transfected with yotiao, hKCNE1, and hKCNQ1-LZm ($n = 5$). Scale: 100 pA/pF, 1 s. **(E)** Disruption of the KCNQ1 LZ motif with the LQTS mutation (G589D). CHO cells were transfected with yotiao, hKCNE1, and hKCNQ1-G589D ($n = 5$). Scale: 50 pA/pF, 1 s. In (C) to (E), cells were dialyzed with cAMP/OA-containing (solid squares) or control (open squares) internal solutions. Currents and plots as in (B).

lated cAMP-dependent regulation of I_{KS} ($n = 5$) (Fig. 3, D and E). Thus, assembly of the macromolecular complex via LZ-mediated binding of yotiao to hKCNQ1, as well as PKA phosphorylation of Ser[27], are necessary to reconstitute PKA- and PP1-dependent regulation of hKCNQ1-hKCNE1 channels.

The finding that the hKCNQ1-G589D mutation prevents cAMP-dependent regulation of I_{KS} suggests that these mutant channels may not respond to βAR-mediated signaling in patients. Along with the reduced expression of I_{KS} in carriers of the hKCNQ1-G589D mutation (*13*), uncoupling of the channel from SNS modulation may exacerbate the defect in APD shortening and further increase the risk of exercise-induced ventricular tachyarrhythmias that cause sudden cardiac death (SCD) in some patients (*6*). Indeed, SCD is associated with SNS stimulation in 81% of individuals with the KCNQ1-G589D mutation in whom a trigger event can be identified (*13*), in contrast to other K^+ channel mutations in which only ~10% of SCD cases are triggered by SNS activation (*4*).

Taken together, our data show that the regulation of hKCNQ1 by PKA-dependent phosphorylation requires a macromolecular complex that includes PKA, PP1, and the targeting protein yotiao and provide a mechanistic link between the sympathetic nervous system and modulation of the cardiac APD through I_{KS}.

References and Notes

1. R. S. Kass, W. Wang, in *Cardiac Electrophysiology from Cell to Bedside*, D. P. Zipes, J. Jalife, Eds. (Saunders, Philadelphia, 2000), chap. 11.
2. M. C. Sanguinetti *et al.*, *Nature* **384**, 80 (1996).
3. J. Barhanin *et al.*, *Nature* **384**, 78 (1996).
4. P. J. Schwartz *et al.*, *Circulation* **103**, 89 (2001).
5. J. A. Towbin, Z. Wang, H. Li, *Drug Metab. Dispos.* **29**, 574 (2001).
6. K. J. Paavonen *et al.*, *Heart* **86**, 39 (2001).
7. J. W. Lin *et al.*, *J. Neurosci.* **18**, 2017 (1998).
8. S. O. Marx *et al.*, *J. Cell Biol.* **153**, 699 (2001).
9. S. O. Marx, S. Reiken, A. R. Marks, unpublished data.
10. P. C. Gray, J. D. Scott, W. A. Catterall, *Curr. Opin. Neurobiol.* **8**, 330 (1998).
11. J. D. Scott, T. Pawson, *Sci. Am.* **282**, 72 (June 2000).
12. K. B. Walsh, R. S. Kass, *Science* **242**, 67 (1988).
13. K. Piippo *et al.*, *J. Am. Coll. Cardiol.* **37**, 562 (2001).
14. A. Feliciello *et al.*, *FEBS Lett.* **464**, 174 (1999).
15. W. Wang, J. Xia, R. S. Kass, *J. Biol. Chem.* **273**, 34069 (1998).
16. J. Gulick, A. Subramaniam, J. Neumann, J. Robbins, *J. Biol. Chem.* **266**, 9180 (1991).
17. Supplemental material on methods used for GST pull-down assays, membrane targeting, and subunit assembly of KCNQ1-G589D is available on *Science* Online at www.sciencemag.org/cgi/content/full/295/5554/496/DC1.
18. R. Mitra, M. Morad, *Am. J. Physiol.* **249**, H1056 (1985).
19. O. P. Hamill, A. Marty, E. Neher, B. Sakmann, F. J. Sigworth, *Pflugers Arch.* **391**, 85 (1981).
20. R. S. Kass, J. P. Arena, *J. Gen. Physiol.* **93**, 1109 (1989).
21. R. An *et al.*, *J. Physiol. (London)* **516**, 19 (1999).
22. Cardiac homogenates (500 μg) were suspended in 0.5 ml of RIPA buffer and incubated with antibody to hKCNQ1 (Santa Cruz) for 2 hours at 4°C. Protein G–Sepharose beads were added, incubated at 4°C for 1 hour, washed with RIPA buffer, and resuspended in SDS sample buffer. Microcystin-sepharose (35 μl; Upstate Biotechnology Inc.) and cAMP-Sepharose (40 μl; Sigma) were incubated with cardiac membrane preparations (200 μg) at 4°C for 1 hour and then washed. Samples were heated to 95°C and size-fractionated by SDS–polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes, immunoblotted with the primary antibody, and detected by enhanced chemiluminescence (ECL, Amersham). Primary antibodies: anti-PKA catalytic subunit (1:1000), anti-PP1 (1:1000) (Transduction Laboratories, Lexington, KY), anti-hKCNQ1 (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-yotiao [gift from M. Sheng (*7*) and from M. E. Gottesman (*14*)]. For preparation of CHO lysates, cells were washed twice 48 hours after transfection, scraped into phosphate-buffered saline, centrifuged at 4°C, and resuspended in 0.5 ml of 20 mM Hepes-NaOH (pH 7.5) containing 1% Triton X-100 and EDTA-free protease inhibitors (Boehringer Mannheim). Lysates were centrifuged at 10,000*g* for 15 min, supernatants were collected, and protein concentration was determined by Bradford assay.
23. hKCNQ1-hKCNE1 (2.6 kb) cDNA (*15*) under the control of the α-MHC-promoter fragment for cardiac-specific expression (*16*) was injected into the pronuclei of fertilized mouse eggs by the Transgenic/Chimeric Mouse Facility. Genotyping of hKCNE1-hKCNQ1 transgenic mice was by the polymerase chain reaction (PCR). Transgenic positive (TG$^+$) mice did not show distinctive phenotypes.
24. hKCNQ1 cDNA template was amplified by PCR (Stratagene) and cloned into pGEX-4T1, -4T2 or -4T3 (Amersham Pharmacia Biotech) for expression as GST fusion proteins in either DH5α or BL21 cells (Stratagene) and affinity-purified with glutathione 4B sepharose (Amersham Pharmacia Biotech). All cDNA constructs were confirmed by sequencing (*17*).
25. hKCNQ1 was immunoprecipitated from TG$^+$ mouse heart homogenates and hKCNQ1-transfected CHO cells. Immunoprecipitates were washed with 1× phosphorylation buffer [8 mM $MgCl_2$, 10 mM EGTA, and 50 mM tris/piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.8], resuspended in 10 μl of a 1.5× phosphorylation buffer containing either PKA catalytic

subunit (5 units) or cAMP (10 μM; Sigma) with or without a PKA inhibitor (PKI_{5-24}, 500 nM, Calbiochem). Phosphorylation at room temperature was initiated with 33 μM Mg–adenosine triphosphate (ATP) containing 10% [γ-^{32}P]ATP (NEN Life Sciences, Boston) and terminated after 5 min at room temperature with 5 μl of stop solution (4% SDS and 0.25 M dithiothreitol). Samples were size-fractionated by SDS-PAGE (10% gel).

26. Adult murine cardiomyocytes were isolated (*18*). Cells were plated in petri dishes placed on the stage of an inverted microscope (IMT-2, Olympus), and currents (myocytes and CHO cells) were recorded using the whole-cell patch-clamp technique (*19*) with local perfusion (*20*) and solutions previously described for CHO cells (*15*) and murine myocytes (*21*). Voltage pulse protocols are described in figure legends. Statistical significance was assessed with Student's *t* test for simple comparisons and analysis of variance (ANOVA) for multiple comparisons; differences at $P < 0.05$ were considered to be significant.

27. We thank W. A. Catterall for anti-AKAP 15; M. Sheng for anti-yotiao; M. Gottesman for anti-yotiao; J. D. Scott for yotiao cDNA; M. Beggs for help with statistical analysis; S. A. Siegelbaum for critical review of the manuscript and helpful discussions. Supported by NIH grants RO1-HL44365-07 and RO1-HL56810-05 (R.S.K.), RO1-HL61503, RO1-HL56180, and RO1-AI39794 (A.R.M.), PO1HL67849-01 (A.R.M., R.S.K.), and RO1-HL68093 (S.O.M.); the American Heart Association (A.R.M., J.K., and S.O.M.); and the Whitaker Foundation (S.R.). A.R.M. is a Doris Duke Charitable Foundation Distinguished Clinical Scientist.

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Calcium, Calmodulin, and CaMKII Requirement for Initiation of Centrosome Duplication in *Xenopus* Egg Extracts

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Aberrant centrosome duplication is observed in many tumor cells and may contribute to genomic instability through the formation of multipolar mitotic spindles. Cyclin-dependent kinase 2 (Cdk2) is required for multiple rounds of centrosome duplication in *Xenopus* egg extracts but not for the initial round of replication. Egg extracts undergo periodic oscillations in the level of free calcium. We show here that chelation of calcium in egg extracts or specific inactivation of calcium/calmodulin–dependent protein kinase II (CaMKII) blocks even initial centrosome duplication, whereas inactivation of Cdk2 does not. Duplication can be restored to inhibited extracts by addition of CaMKII and calmodulin. These results indicate that calcium, calmodulin, and CaMKII are required for an essential step in initiation of centrosome duplication. Our data suggest that calcium oscillations in the cell cycle may be linked to centrosome duplication.

Centrosome duplication occurs once and only once during the cell cycle, ensuring the formation of bipolar spindles that distribute replicated chromosomes equally to daughter cells. Overduplication of centrosomes causes multipolar spindles that are thought to lead to genomic instability. More than two centrosomes have been observed in many different types of tumor cells (*1*), and the absence of the tumor suppressor protein p53 correlates with the presence of multiple centrosomes in the cell (*2*). Thus, the process of centrosome duplication is likely to be important in the etiology of cancer.

There are links between the cell cycle and the centrosome duplication cycle. Centrosome duplication generally occurs at the G_1-S transition (*3*), and if S phase is prolonged in mammalian cells and in embryonic systems including cycling *Xenopus* egg extracts, centrosomes can reproduce multiple times. Cdk2 is identified as a direct link between the cell cycle and centrosome duplication (*4*–*7*). The cyclin E–Cdk2 complex regulates DNA replication, and its activity is greatest at the G_1 to S phase transition. Cdk2 is also required for multiple rounds of centrosome duplication in *Xenopus* egg extracts and embryos (*4, 5*) and in somatic cells (*6, 7*). Nucleophosmin (also called B23) and the mouse Mps1p-like kinase (mMps1p) were recently identified as possible centrosomal substrates for cyclin E–Cdk2 (*8, 9*).

Calcium and calmodulin (CaM) are required for cells to traverse the G_1-S, G_2-M, and metaphase-anaphase boundaries of the cell cycle (*10*). Calcium/calmodulin–dependent protein kinase II (CaMKII), which has a wide tissue distribution (*11*), has been suggested to be a target of calcium and calmodulin at the G_2-M transition in various cell types (*10*). In addition, periodic calcium oscillations occur in each cell cycle in dividing *Xenopus* embryos (*12*) and in cycling egg extracts (*13*). In particular, calcium oscillations occur at the G_1-S boundary and near the G_2-M transition. These times correlate with centrosome duplication at the G_1-S boundary and centrosome separation at the G_2-M transition. Moreover, calcium-modulated proteins, including CaMKII and centrin [a homolog of Cdc31p, a protein essential for duplication of yeast centrosomes (*14*)], are localized in the centrosome (*15, 16*). Thus, we examined the role of calcium in centrosome duplication using *Xenopus* egg extracts arrested in S phase by aphidicolin in which centrosome duplication starts after addition of sperm nuclei (*4*).

Addition of the calcium chelator BAPTA [1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] (*17*) caused depolymerization of microtubules in the extracts (*18*) and prevented detection of centrosomes by polarization microscopy. Therefore, centrosome duplication in the presence of BAPTA was visualized by immunofluorescence with an antibody to γ-tubulin, a well-characterized component of the centrosome (*19*). Extracts incubated with sperm nuclei were subjected to microtubule depolymerization with nocodazole, and the centrosomes were centrifuged onto a cover slip for staining (*20, 21*). The number of γ-tubulin–staining foci increased during a 6-hour incubation (Fig. 1A), and staining with antibody to either α-tubulin or centrin, a component of the centriole (*14*), confirmed that each γ-tubulin–staining focus (>90%) corresponded to one centriole (Fig. 1B). Because each centrosome contains two centrioles, the number of centrosomes is half the number of γ-tubulin–staining foci. A fourfold increase in centrosomes was detected during the 6-hour incubation (Fig. 1C, control), indicating that centrosomes duplicate twice in these extracts in approximately 6 hours.

In the presence of 2 mM BAPTA, no increase in centrosomes was evident during the 6-hour incubation (Fig. 1C), even though 20 mM EGTA had no effect on centrosome duplication (*18*). Considering that BAPTA chelates calcium more rapidly than does EGTA (*17*), this suggests that rapid oscillations rather than prolonged changes in the concentration of free calcium are necessary for centrosome duplication. Inositol 1,4,5-trisphosphate (IP_3) is a second messenger that releases calcium from intracellular stores (*22*). To determine whether calcium oscillations are mediated by the IP_3 receptor, we used heparin, an IP_3 receptor antagonist (*23*) that blocks calcium oscillations in *Xenopus* egg extracts (*13*). Heparin effectively inhibited centrosome duplication in the extracts (Fig. 1C). Moreover, after centrosomes had

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