

HERG K⁺ Channel Activity Is Regulated by Changes in Phosphatidyl Inositol 4,5-Bisphosphate

Jinsong Bian, Jie Cui, Thomas V. McDonald

Abstract—Autonomic stimulation controls heart rate and myocardial excitability and may underlie the precipitation of both acquired and hereditary arrhythmias. Changes in phosphatidyl inositol bisphosphate (PIP2) concentration results from activation of several muscarinic and adrenergic receptors. We sought to investigate whether PIP2 changes could alter HERG K⁺ channel activity in a manner similar to that seen with inward rectifier channels. PIP2 (10 μmol/L) internally dialyzed increased the K⁺ current amplitude and shifted the voltage-dependence of activation in a hyperpolarizing direction. Elevated PIP2 accelerated activation and slowed inactivation kinetics. When 10 μmol/L PIP2 was applied to excised patches, no significant change in single channel conductance occurred, indicating that PIP2-dependent effects were primarily due to altered channel gating. PIP2 significantly attenuated the run-down of HERG channel activity that we normally observe after patch excision, suggesting that channel run-down is due, in part, to membrane depletion of PIP2. Inclusion of a neutralizing anti-PIP2 monoclonal antibody in whole cell pipette solution produced the opposite effects of PIP2. The physiological relevance of PIP2–HERG interactions is supported by our finding that phenylephrine reduced the K⁺ current density in cells coexpressing α1A-receptor and HERG. The effects of α-adrenergic stimulation, however, were prevented by excess PIP2 in internal solutions but not by internal Ca²⁺ buffering nor PKC inhibition, suggesting that the mechanism is due to G-protein–coupled receptor stimulation of PLC resulting in the consumption of endogenous PIP2. Thus, dynamic regulation of HERG K⁺ channels may be achieved via receptor-mediated changes in PIP2 concentrations. (*Circ Res.* 2001;89:1168–1176.)

Key Words: HERG ■ potassium channel ■ phospholipids ■ G-protein–coupled receptor

Heart rate and contractility are continually altered in response to changing cardiovascular demands and stresses by means of autonomic stimulation. Some autonomic receptors (muscarinic M1 and α-adrenergic α1A) are coupled to Gαq proteins and, when activated, stimulate phosphatidyl inositol-specific phospholipase C (PLC). The substrate for PLC is phosphatidyl 4,5 bisphosphate (PIP2), a phospholipid that is hydrolyzed to 1,4,5 inositol trisphosphate (IP3) and diacyl glycerol (DAG). Thus, as these receptors are activated there is an increase in the second-messengers [IP3]_i and [DAG]_i with a simultaneous reduction of [PIP2] within the plasma membrane.

PIP2 itself plays an important role in such processes as the organization of actin cytoskeleton and vesicular transport,^{1,2} Na⁺/Ca²⁺ exchanger activity,³ IP3 receptor Ca²⁺ channel activity,⁴ and nonselective cation channels.⁵ PIP2 also regulates the activity of the ATP-sensitive potassium channel (K_{ATP}), a channel whose activity is gated by [ATP]_i/[ADP]_i changes.^{6–9} Other related K⁺ channels (GIRK) of the inward rectifier family are complexly regulated by the βγ-subunits of G-proteins and by simultaneous PLC-mediated changes in [PIP2].^{10–13}

Because changes in autonomic stimulation of the heart appear to be linked to the precipitation of both hereditary and

acquired ventricular arrhythmias,¹⁴ we decided to investigate whether changes in [PIP2] could affect HERG/I_{Kr}, one of the K⁺ channels that governs the rate of repolarization during the cardiac action potential. The human *ether-a-go-go*-related gene (HERG) encodes the pore-forming subunit of the channel that produces the cardiac rapidly activating delayed rectifier K⁺ current, I_{Kr}, and mutations in HERG are responsible for the LQT2 form of hereditary long QT syndrome (LQTS). HERG/I_{Kr} is of the voltage-gated K⁺ channel family with a structure that is substantially different from those K⁺ channels that are known to be regulated by PIP2. No data, however, are available regarding the effect of [PIP2] changes on voltage-gated K⁺ channel activity.

Here, we report the effects of PIP2 on HERG channel activity. We show that modification of [PIP2] results in altered HERG/I_{Kr} current density and gating kinetics. Moreover, we show that stimulation of adrenergic α1A receptors affects coexpressed HERG channels in a manner expected for the PLC-mediated PIP2 consumption. These results support a model where autonomic stimulation of cardiac GPCRs may alter I_{Kr}-dependent repolarizing forces and thus, may be potentially arrhythmogenic.

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Materials and Methods

Cell Culture and Transfection

CHO (from American Type Culture Collection, Manassas, Va) and HEK293 (a gift from Dr Craig January, Madison, Wis) cell lines were cultured in Ham's F12 (Cellgro, CHO) and RPMI 1640 (Cellgro, HEK283) supplemented with L-glutamine, 10% fetal calf serum (BioWhittaker), and penicillin/streptomycin (Cellgro). Cultured cells were maintained in 5% CO₂ humidified air at 37°C. For coexpression of HERG and GPCRs, 4 μg of α1A-adrenergic receptor cDNA was transiently cotransfected together with 4 μg of HERG cDNA and 2 μg of GFP cDNA by electroporation. Cells were electroporated in a 2-mm gap cuvette using a BTX ECM600 with a capacitance=180 μF; resistance=72 Ω; voltage=225 V. Cells were then plated sparsely on sterile glass cover and used for electrophysiological studies 24 to 72 hours after electroporation.

The entire coding region of adrenergic-α1A receptor cDNA (a generous gift from Dr Ken Minneman, Emory University) was subcloned into the EcoR I site pCDNA3 (Invitrogen). HERG and GFP expression plasmids have been previously described.¹⁵

Patch Clamp Recording

Patch pipettes were pulled and polished to obtain a tip resistance of 2 to 3 mΩ in the external bath solution. Fresh external bath solution was constantly flowed during all experiments to prevent accumulation of perimembrane K⁺ that would alter amplitude and kinetics of *I*_{Kr}. All experiments were carried out at room temperature (20 to 22°C). Cells were studied on an inverted microscope equipped with electronic patch-pipette micromanipulators and epifluorescence optics. Axopatch-1D or 200B patch clamp amplifiers (Axon Instruments) were used for voltage clamp measurements. Voltage clamp protocols were controlled via PC using pClamp8 (Axon Instruments) acquisition and analysis software. To elicit HERG K⁺ currents depolarizing, voltage pulses were applied to various levels from a holding potential of −70 mV for 4.5 seconds followed by stepwise repolarization to −40 mV and then to −120 mV to measure outward and inward tail currents. To study the activation, inactivation, and deactivation of the HERG, channel-specific pulse protocols were used as described in the corresponding figure legends. Signals were analog-filtered at 2000 Hz and sampled at 5 to 10 000 Hz. Voltage-dependent activation data were fitted to Boltzmann relation $I = 1 / (1 + \exp[(V_{1/2} - V)/k])$, where *I* is the measured tail current, *V* is the applied membrane voltage, *V*_{1/2} is the voltage at half-maximal activation, and *k* is the slope factor. To control for transfection efficiency, current densities were normalized to the control group daily based on average current density of cells before application of PIP2.

Whole-cell pipette solution consisted of (in mmol/L) KCl 126, MgSO₄ 2, CaCl₂ 0.5, EGTA 5, Mg-ATP 4, and HEPES 25 (pH 7.2; osmolality: 280±10 mmol/L). External bath solution consisted of (in mmol/L) NaCl 150, CaCl₂ 1.8, KCl 4, MgCl₂ 1, glucose 5, and HEPES 10 (pH 7.4; osmolality: 320±10 mmol/L). To buffer the increased [Ca²⁺]_i during α1A-receptor stimulation, 15 mmol/L BAPTA was added to the pipette solution, and the [CaCl₂]_o was decreased to 0.5 mmol/L in the bath solution.

Single-channel recordings were performed in the inside-out and on-cell patch configuration using an Axopatch 200B amplifier (Axon Instruments). Single-channel data were digitally filtered at 500 or 600 Hz (low-pass) prior to analysis. Patch pipettes were made from thick-walled glass and coated with Sylgard to reduce noise and capacitance. Pipette solution for single channel recording consisted of (in mmol/L) KCl 120, MgCl₂ 1, CaCl₂ 1, and HEPES 25 (pH 7.4). Cells were bathed in a solution consisting of (in mmol/L) KCl 120, NaCl 10, MgCl₂ 2, glucose 5, and HEPES 10 (pH 7.4).

[Ca²⁺]_i Measurement

Cells were loaded with fluo-3 (Molecular Probes) at a final concentration of 2 μmol/L in RPMI medium. After incubation at 37°C for 45 minutes, the unincorporated dye was removed by washing the cells twice in the whole cell recording bath solution. The [Ca²⁺]_i was measured in an inverted microscope (Nikon) that was outfitted with

an excitation light source (excitation wavelength=480 nm). Fluorescent signals were obtained at 540 nm with a digital CCD camera (Quantix, Photometrics) controlled by commercial software (Axon Imaging Workbench 2.2, Axon Instruments). [Ca²⁺]_i changes were expressed as fluorescence measured over basal unstimulated fluorescence (F/F₀).

Materials

PIP2 and chelerythrine were from Calbiochem. To prepare PIP2, it was dispersed by sonication in water (at 0.5 mmol/L) for 30 minutes on ice and then divided into aliquots and keep at −80°C. For each experiment, a new aliquot was thawed and used only once. PIP2 was diluted to 10 μmol/L in the electrophysiology bath solution (for single-channel recording) or pipette solution (for whole-cell recording) and sonicated again for 10 to 30 minutes. This procedure results in the formation of a suspension of mostly small micelles of PIP2 that can be readily absorbed by lipid membranes.¹⁶ PIP2-specific antibody (Assay Designs Inc) was diluted to 60 nmol/L in the pipette solution.^{10,11} To decrease PIP2-Ab binding to the wall of the pipette, 100 μmol/L BSA was added to the pipette solution. Chelerythrine was first dissolved in DMSO as stock solutions and then used at the desired final concentration in the bath solution such that the final DMSO concentration was less than 0.5%. BAPTA was from Sigma.

Statistics

Values presented are mean±standard error of mean (SEM). Student's *t* test was used to compare the difference between 2 groups. Significance level was set at a value of *P*<0.05.

Results

PIP2 Regulation of HERG K⁺ Channel Activity

We compared the HERG/*I*_{Kr} currents immediately after establishing whole cell and 3 minutes after allowing PIP2 (10 μmol/L) to dialyze into the cell (Figures 1A and 1B). We observed that 10 μmol/L PIP2 increased HERG/*I*_{Kr} current amplitude by 20% to 80%. The maximal current density as measured in *I*-*V* relationship increased from 3.58±0.83 to 4.72±1.05 pA/pF (*P*<0.01, *n*=15) (Figure 1C). Relative current amplitude differences were greatest between test voltages between −20 and +10 mV (PIP2-induced increase in current density 81% at −20 mV, 55% at −10 mV, 39% at 0 mV, and 20% at 10 mV). As shown in Figure 1D inset, when the data were normalized to unity, a 10 mV hyperpolarizing shift in voltage-dependent activation was seen (*V*_{1/2} HERG=−14.7±2.7 mV, *V*_{1/2} PIP2=−25.4±2.1 mV, *P*<0.05, *n*=15). The *I*-*V* relationship for the test pulse also showed a similar hyperpolarizing shift in voltage dependence of both the positive and negative slope conductances (Figure 1C inset, left). The PIP2 augmentation of HERG/*I*_{Kr} current was most pronounced at voltages negative to 0 mV (Figure 1C inset, right). The PIP2-dependent effects occurred rapidly and lasted at least 15 minutes (Figure 1E). There were no significant changes in membrane surface area (as measured by cell capacitance measurements) produced by PIP2 application to account for changes in current density.

PIP2 Acceleration of HERG K⁺ Channel Activation

We examined in detail the effect of increased [PIP2] on activation kinetics of HERG/*I*_{Kr}. PIP2 (10 μmol/L) produced an acceleration of the time constants at all membrane potentials tested (Figure 2A). The time constant for the outward current activation at −40 mV was accelerated by PIP2 from

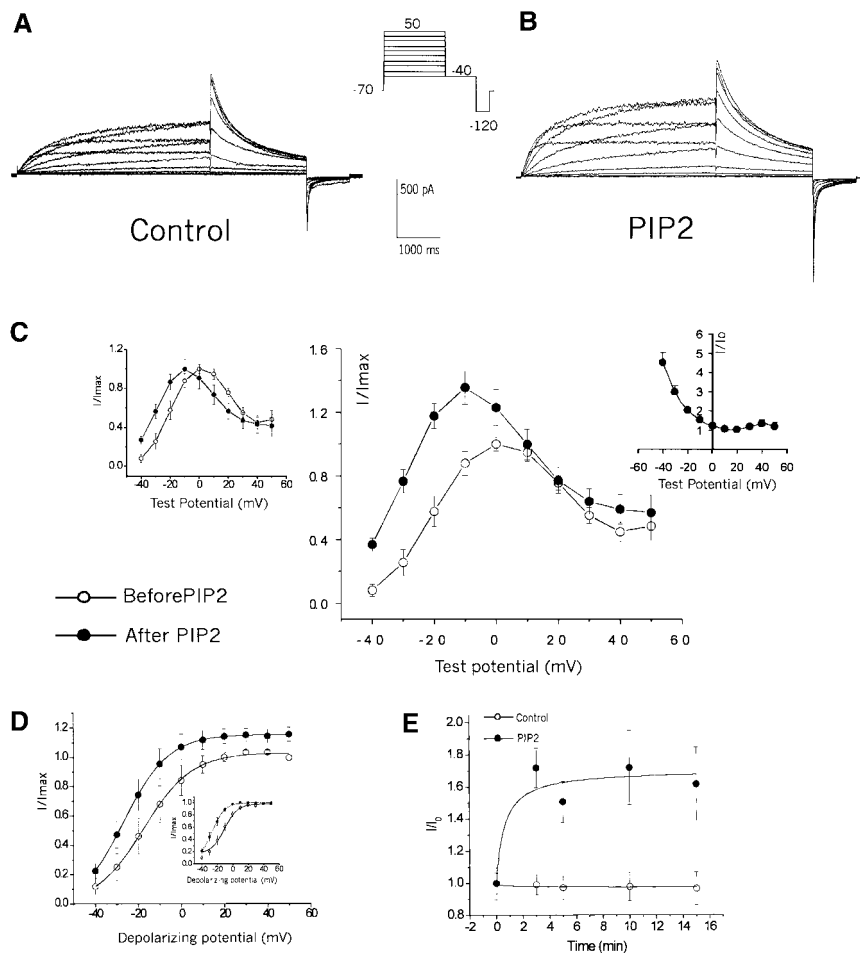


Figure 1. PIP2-mediated effects on HERG K⁺ currents. A and B, Representative current tracings in response to a series of membrane depolarizations in a CHO cell expressing HERG. A, Current immediately after establishing whole-cell access (0 minutes). B, Current from the same cell 3 minutes after whole-cell access with 10 μmol/L PIP2 in the pipette solution. Currents were elicited by 4.5-second depolarizing steps to various levels (between 50 and -40 mV) followed by repolarizing steps to -40 and -120 mV. Scale bars, 500 pA and 1000 ms. C, Current-voltage (I-V) relation curve plotted from current measured at the end of depolarizing test pulses. (Relative current amplitude, I/I_{\max} , during depolarizing steps plotted against the step potential, I_{\max} =maximal current before PIP2.) Left inset, Data were normalized to unity before and after PIP2. Right inset, Incremental increase in current amplitude plotted against voltage with the greatest increase at potentials negative to 0 mV. D, Voltage-dependent activation curves plotted from peak tail currents during a repolarizing step to -40 mV after depolarizing to various voltages. Inset, Same data normalized to the maximum current in the same curve. Open circles: before (0 minutes) PIP2 treatment; Solid circles: 3 minutes after treatment of PIP2. E, Time course of the effect of PIP2 (mean±SEM; n=15).

2.34±0.47 to 1.67±0.22 seconds ($P<0.05$, $n=8$). As measured by rate of rise of outward current, this acceleration could be due to more rapid activation or slowing of voltage-dependent inactivation. To distinguish PIP2 effects on activation versus inactivation, we measured the time course of activation using a voltage clamp protocol varying the duration of the depolarizing pulse and measuring the peak inward tail current on repolarizing to -110 mV (Figures 2B and 2C). Because the inactivation of HERG channels is released at a faster rate than the channels deactivate,¹⁷ this is a more specific measure of activation. Figures 2B and 2C show the representative time course of the peak inward current on depolarization to different voltages (20 to 50 mV) in the control and PIP2 treatment group (Figures 2B and 2C). The half time to maximal rise in tail currents ($T_{1/2}$) for both control and PIP2 treatment groups are summarized in Figure 2D. PIP2 significantly accelerated activation of current at all voltages measured.

PIP2 Effects on Inactivation and Deactivation of HERG

The amplitude of outward I_{K_r} and its rate of rise are determined by the relative degree of voltage-dependent activation and inactivation. Because both positive and negative slope conductances were shifted in response to increased [PIP2] (Figure 1C), voltage-dependent inactivation could be affected

in addition to its effects on activation. To determine if [PIP2] changes altered inactivation kinetics, we used voltage clamp protocols to specifically measure voltage-dependence of steady-state inactivation in the procedure described by Smith et al.¹⁷ Our results show that increased [PIP2] significantly enhanced the current amplitude on release of inactivation (Figure 3A and 3B). Normalized data shows that there is a small but nonsignificant leftward shift in voltage-dependent inactivation (Figure 3B, inset). We also measured the rate of onset of inactivation before and after application of PIP2 (Figures 3C and 3D). Channels were activated by prolonged depolarization to +20 mV followed by a brief hyperpolarizing step to -120 mV and then depolarization to various potentials. The outward current reflects the open channel conductance prior to the onset of inactivation and onset of inactivation is seen in its declining phase to the steady state. Elevated [PIP2] significantly prolonged the time course of HERG inactivation, suggesting that PIP2 slows the onset of inactivation.

We examined whether elevation of [PIP2] exerted any effects on HERG/ I_{K_r} deactivation using relaxation of tail current analyses (Figure 4A). The I-V relationship determined in this manner show the typical rectification with PIP2 producing increased amplitude, seen most prominently at potentials negative to -90 mV (Figure 4B). When relaxation time constants were examined from the tail currents, we

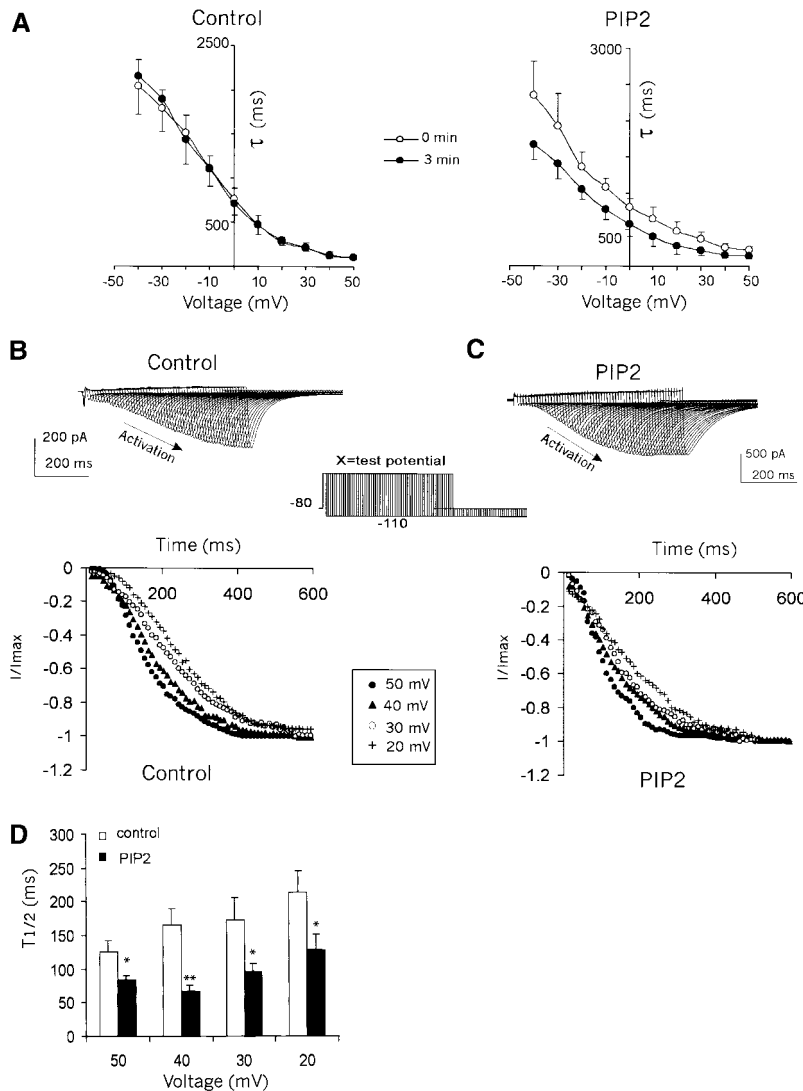


Figure 2. Effects of PIP2 on the activation of HERG K⁺ currents. **A**, Summary of activation kinetic analysis as determined by time constants fitted to depolarization-induced outward currents shows PIP2-dependent acceleration of activation in a voltage-dependent fashion. Left: control group, n=9; right: PIP2 group, n=8. **B** and **C**, Analysis of activation kinetics with voltage clamp protocols of depolarizing steps to the test voltages (20, 30, 40, or 50 mV) for varying durations followed by a hyperpolarizing step to -110 mV; peak inward tail currents represent accumulated current activation. **B**, Time course of the peak inward current amplitude in control cells. Top, Representative currents; Bottom, Kinetic time courses. **C**, Same analysis as in **B** for cells treated with PIP2. **D**, Summary of activation kinetics shows PIP2-dependent acceleration of activation at all voltages between 20 and 50 mV. Open bars: control; Solid bars: PIP2 group (mean±SEM; n=8 to 17).

observed no significant change in either the fast or slow rates of deactivation on application of [PIP2] (Figures 4C and 4D).

Effects of PIP2 on HERG/I_{Kr} in Excised Membrane Patches

To determine if the augmentation of whole cell current density in response to elevated [PIP2] was due to changes in single channel conductance, we examined HERG channels in excised membrane patches (Figure 5A). The slope conductance under these conditions was 10.7 ± 2.3 pS (n=7) between the voltages of -40 and -120 mV in the absence of added PIP2. When the membrane patch was excised into solutions containing 10 μ mol/L PIP2 the conductance was 10.2 ± 0.8 pS (n=7), a value not significantly different from control (Figure 5B).

HERG channel activity in membrane patches consistently and quickly ran down after excision into the cell-free configuration (Figure 6A, upper traces). The run-down of the currents in excised patches has been reported for a wide variety of channels.^{10,18–24} Although the mechanisms for run-down are different for each channel and not entirely understood for all, one obvious explanation is the diffusion of

necessary cytoplasmic cofactors on patch excision from the cell. We tested for loss of PIP2 as an explanation of HERG channel run-down. The integral of current amplitude and time was determined in multi-channel patches before and after excision to express the run-down of the channel activity. When 10 μ mol/L PIP2 was included in the solution bathing the cytoplasmic surface of the patch there was less channel run-down (Figures 6A, lower traces, and 6B). Summary data for rates of channel run-down show a 5-fold reduction in loss of channel activity with PIP2 (Figure 6C). Loss of PIP2 through membrane associated phospholipases or diffusion from excised patches excision therefore, is at least partly responsible for the run-down of HERG channel activity and is consistent with PIP2 effects on channel rundown from other K⁺ channels.^{10–12}

Effects of PIP2-Ab on the HERG Channel Activity

To test for the specificity of our observed PIP2 affects HERG/I_{Kr}, we used a neutralizing monoclonal PIP2-specific antibody that has been shown to disrupt PIP2 interactions with other K⁺ channel proteins.^{10,11,13} Inclusion of anti-PIP2

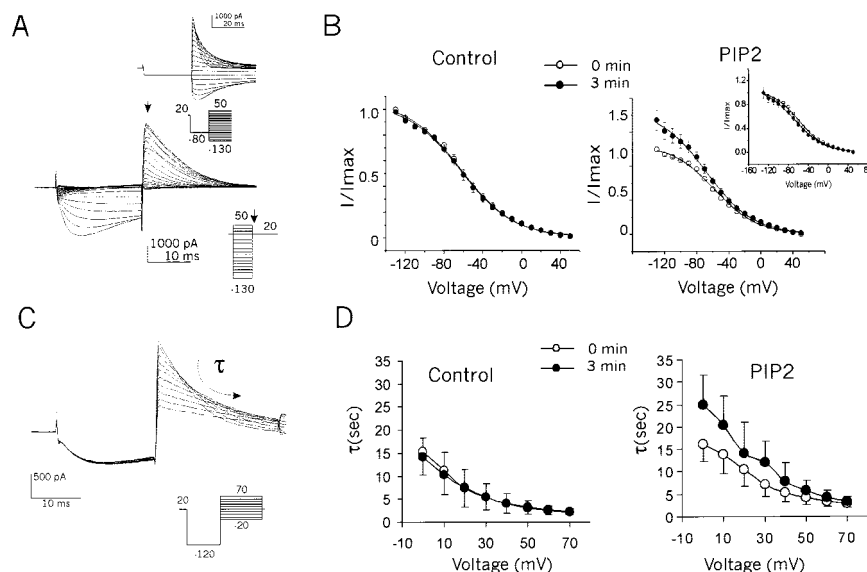


Figure 3. Effects of PIP2 on voltage-dependent inactivation HERG K^+ current. A, Representative HERG current tracing from steady-state pulse protocol. (Inset, pulse protocol used for correcting for simultaneous deactivation by the method of Smith et al.¹⁷) Channels are activated and inactivated by holding at 20 mV. Brief repolarizing steps to various potentials relieves inactivation before deactivation takes place, and resulting currents are measured when the voltage is returned to 20 mV (marked by the arrow). B, Summary data of corrected steady-state inactivation graphed as recovered current plotted against the repolarizing step potential showing increased current amplitude and left shift (seen in normalized curves, Inset) in voltage dependence resulting from PIP2 (right graph, $n=7$). Left graph, Control group ($n=6$). C, Representative current tracing from inactivation onset protocol. Activated channels are released from activation as in A after which the mem-

brane potential is returned to various potentials and rates of onset of inactivation are taken from the relaxation of the tail currents. D, Summary data of voltage-dependence of rates of inactivation showing that PIP2 slows the rate of inactivation in a voltage-dependent fashion. Open circles: (0 minutes) immediately after establishing whole cell. Solid circles: 3 minutes after internal dialysis. Left graph, control cells; right graph, PIP2-treated cells ($n=7$ for each).

monoclonal antibody in whole cell pipette solution produced a significant reduction of HERG K^+ current density (Figures 7A and 7B). Normalized data show a 7 mV depolarizing shift in voltage-dependent activation was seen ($V_{1/2}$ HERG = -18.1 ± 1.6 mV, $V_{1/2}$ PIP2-Ab = -11 ± 2.2 mV, $n=7$) (Figure 7B, inset). Because PIP2-Ab produced the inverse results that we obtained with elevated [PIP2], we conclude that the antibody bound to and neutralized the endogenous PIP2, thereby effecting a reduction of [PIP2] and its effects on HERG/ I_{Kr} . Moreover, these results support the specificity of the PIP2 effects on the channel activity.

Effects of $\alpha 1A$ -Adrenergic Receptor Stimulation on HERG K^+ Current

To test whether physiological receptor-mediated alterations of [PIP2] could affect HERG/ I_{Kr} , we cotransfected $\alpha 1A$ -adrenergic receptor and HERG cDNAs into HEK293 cells. To verify that adequate receptor expression was achieved and whether they coupled to G α_q and PLC, we measured $[Ca^{2+}]_i$ in response to receptor stimulation with phenylephrine. Receptor-mediated PLC activity will generate IP₃, which releases $[Ca^{2+}]_i$ from internal stores. The $\alpha 1A$ -adrenergic agonist, phenylephrine (10 μ mol/L), rapidly increased

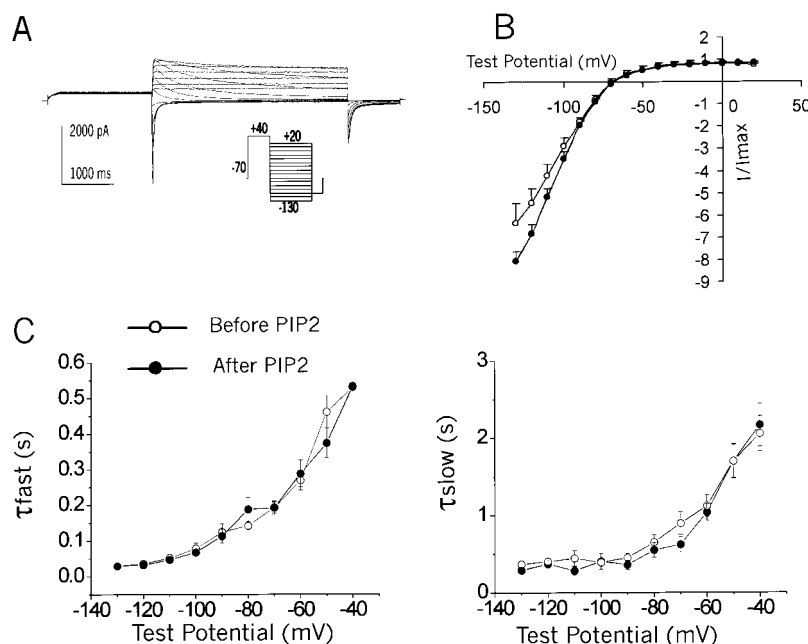


Figure 4. PIP2 has no effects on the deactivation of HERG K^+ currents. A, Representative currents stimulated by a 2-second prepulse to +40 mV were followed by test pulses ranging from -130 to +20 mV for 3.76 seconds. B, I - V data plotted from peak tail currents during various repolarizing steps showing increased maximal current amplitude but no changes in reversal potential (relative current amplitude, I/I_{max} , I_{max} =maximal current before PIP2). C and D, Summary of deactivation kinetic analysis showing no significant change in the fast time constant (C) or slow time constant (D) of deactivation. Open circles: before (0 minutes) PIP2 treatment; Solid circles: 3 minutes after treatment of PIP2 (mean \pm SEM; $n=12$).

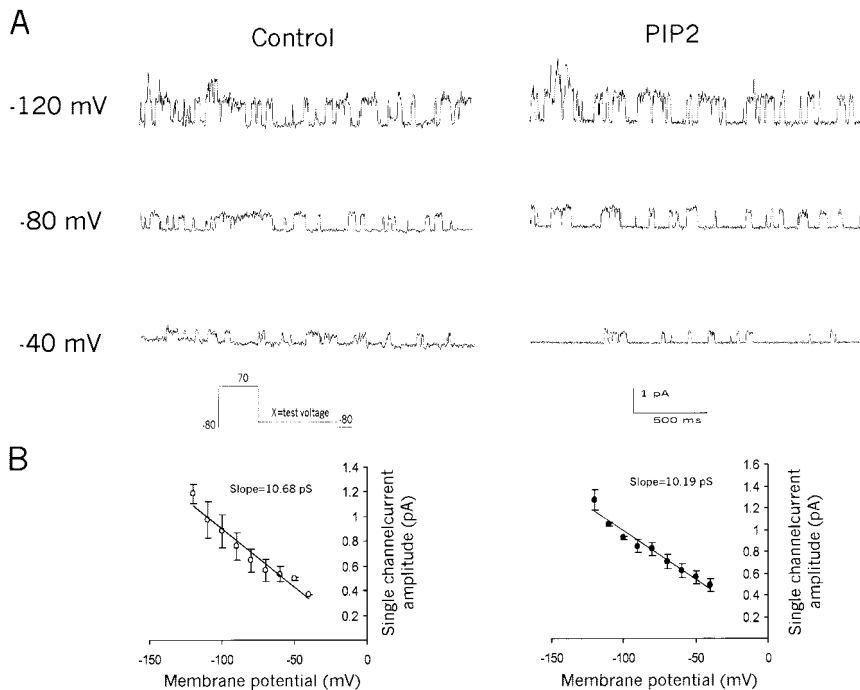


Figure 5. Single-channel HERG currents with and without PIP2 administration. A, Examples of current records during repolarizing pulses to the indicated membrane potentials. Voltage protocol and scale bars are shown below the current traces. B, Single-channel current-voltage curves showing unitary conductances for HERG treated with or without PIP2 (n=8).

[Ca²⁺]_i, demonstrating that the signaling pathway was intact (Figure 8A).

Activation of α 1A-adrenergic receptor with 10- μ mol/L phenylephrine resulted in a significant decrease in current amplitude and a small depolarizing shift in the voltage-dependence of activation (Figures 8B and 8C). The phenyl-

ephine-dependent reduction in current occurred rapidly and was sustained (Figure 8B, inset). Activation of PLC generates the second messengers IP₃ and DAG, which may alter channel behavior. To exclude the possibility that these effects are due to IP₃-mediated [Ca²⁺]_i elevation, BAPTA (15 mmol/L) was included in the whole cell pipette solution and the [Ca²⁺]_o was reduced to 0.5 mmol/L. Under these conditions, we observed a comparable phenylephrine-mediated reduction in current amplitude (Figure 8E). To exclude the possibility of DAG-mediated activation of protein kinase, we treated cells with the PKC inhibitor chelerythrine (1 μ mol/L) for 1 hour prior to patch clamp study. Similarly, under these conditions the phenylephrine-mediated reduction of HERG/I_{Kr} was intact (Figure 8F). As a further test for PIP2-specificity of the α -adrenergic effect, 10 μ mol/L PIP2 internal was able to abolish current amplitude and voltage shifts from phenylephrine (Figure 8G). These data support the hypothesis that stimulation of G-protein-coupled receptors that activate PLC can alter endogenous [PIP2] sufficiently to modify the behavior of HERG K⁺ channels.

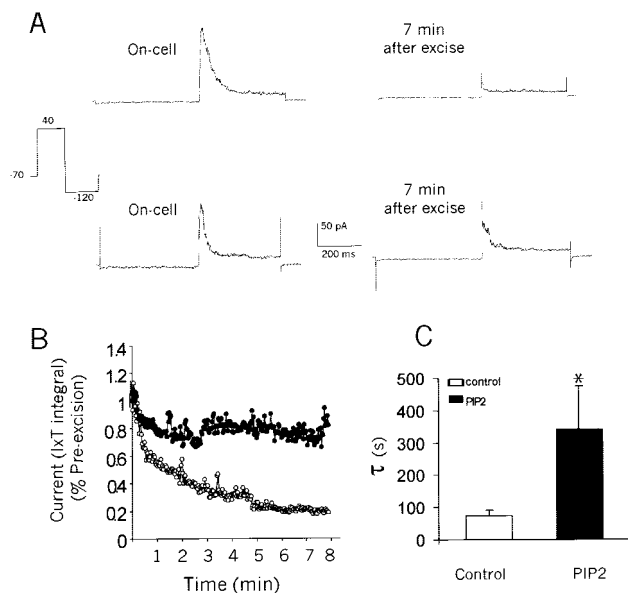


Figure 6. PIP2 prevents run-down of HERG K⁺ channel activity in excised patches. A, Example current tracings from multichannel patches in the cell-attached mode (left traces) and the same patches 7 minutes after excision (right traces). The patch in the lower traces was excised into solution containing 10 μ mol/L PIP2. B, Time course of curves of HERG channel activity after excise in the presence and absence of PIP2. Open circle: control group. Filled circle: PIP2 treatment group. C, Statistical summary of the run-down data shows the time constant of the rundown of the channel (mean \pm SD; n=6).

Polyvalent Cations Inhibited the PIP2 Effects on HERG K⁺ Channel

To investigate the possibility that the anionic head of PIP2 interacts with charged amino acids in HERG, we studied polyvalent cations ability to screen those charges. Although 100 μ mol/L Gd³⁺ or La³⁺, when internally dialyzed, had no significant effect on HERG K⁺ current (data not shown), either could significantly inhibit the effect of PIP2 (Figure 8H). This is consistent with the previous studies of KATP channels.^{6,10,25}

Discussion

PIP2 has been shown to regulate the activity of several different K⁺ channels. Fan and Makieski⁶ first described

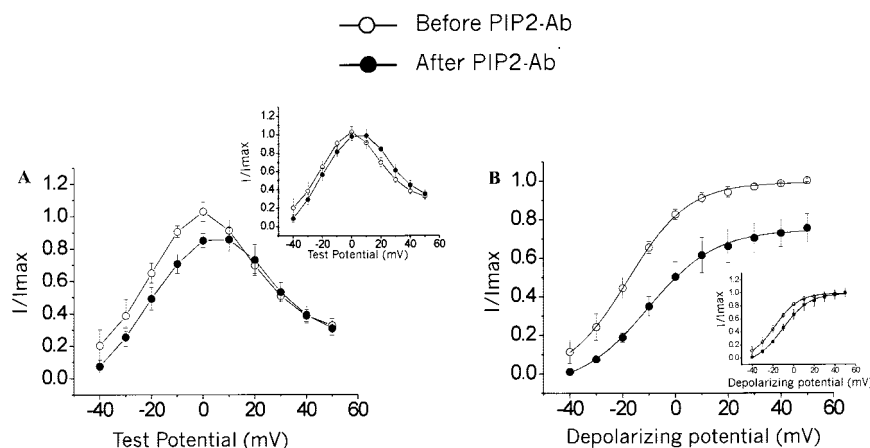


Figure 7. PIP2-neutralizing antibody produces opposite effects of elevated PIP2 on HERG-K⁺. Inclusion of PIP2-specific antibody (60 nmol/L) in whole-cell pipette solutions produces a reduction of K⁺ current amplitude after 10 minutes of perfusion with a concomitant depolarizing shift in voltage-dependence of activation. *I*-*V* curves (A) and voltage-activation curves (B) (normalized data shown in insets). Voltage clamp protocol is the same as Figure 1. Open circles: control at beginning of whole-cell recording; filled circles: 10 minutes after treatment of PIP2 (mean \pm SEM; *n* = 7).

regulation of the KATP potassium channel by anionic phospholipids. Of the lipids tested, phosphatidyl inositol 4,5-bisphosphate (PIP2) was the most potent in augmenting channel activity. They identified positively charged (arginines) portions of the cytoplasmic N- and C-termini that appeared to be responsible for the lipid channel interaction and proposed that several other channels of the related inward rectifier class (ROMK, IRK, GIRK) may be similarly regulated.⁶ Nichols's⁸ and Fakler's⁷ groups then showed that PIP2 stimulated KATP channels by antagonizing the inhibitory effects of ATP_i. Logothetis's²⁶ and Hilgemann's¹⁰ groups showed that the G-protein-gated K⁺ channel (GIRK) required PIP2 for its normal activation by affecting the channel interactions with the $\beta\gamma$ -G-protein subunits. Kobrin's et al²⁷ provided evidence that supports PIP2 as a dynamic physiological regulator of GIRK depending on rates of PIP2 hydrolysis by GPCR activation.

Although no voltage-gated K⁺ channels have been shown to interact with PIP2 to date, a suggestion of such an interaction is supported by the observation that LQTS arrhythmias are often precipitated by stress, presumably due to increased adrenergic stimulation of GPCRs in the heart. Heart rate and contractility are continually regulated by a multiplicity of hormonal and signaling information that impinges on the myocyte to control electrical properties required for different cardiovascular needs. β -Adrenergic-mediated increases in cAMP are the best studied so far of the HERG/I_{Kr} regulators,^{28–30} but other second messengers that are generated or altered via other receptors such as α -adrenergic or muscarinic invite further investigation. Our results show that increases in [PIP2] specifically lead to augmentation of HERG/I_{Kr} activity. This is accomplished by a combination of hyperpolarizing shift in voltage-dependent gating, accelerated activation, and slowed inactivation. Moreover, we show that activation of the α 1A-adrenergic receptor, an activator of phosphatidyl inositol-specific PLC, leads to diminished HERG/I_{Kr} amplitude and a rightward shift in voltage dependence of activation as expected for PIP2 consumption.

The concentration of PIP2 (10 μ mol/L) that we have used in this study is comparable to concentrations that have been used to demonstrate effects on K_{ATP} and inwardly rectifying K⁺ channels. Moreover, the amplitude of the electrophysiological response of HERG/I_{Kr} to PIP2 is likewise comparable

to effects in these other K⁺ channels. Our demonstration that internal polyvalent cations could inhibit the PIP2 effects on HERG are supportive of electrostatic interactions between the oppositely charged portions of the channel and PIP2.^{6,9,10,31}

The molecular mechanism of PIP2-dependent regulation of channels is postulated to be negatively charged phosphate groups of the PIP2 interacting with positively charged amino acids in the channel protein. The region of conserved cationic residues in the proximal C-terminal cytoplasmic domain of the inward rectifier K⁺ channels is considered to be a putative PIP2-binding site⁶ because charge-neutralizing mutations or deletion of this region markedly reduced the ability of channel to bind PIP2.¹⁰ Multiple potential PIP2 binding sites in Kir channels have now been proposed,²⁵ not only in the proximal C-terminal but also in other C-terminal fragments. Three independent sites (aa175–206, aa207–246, aa324–365) that could potentially bind PIP2 were identified in the C-terminal tail of Kir2.1 channels.²⁵ Several polycationic segments exist in the cytoplasmic C-terminus of HERG (611: RYHTQMLRVREFIRFHQIPNPLRQRL637 and 881: RKRKLSFRRRRT895). Although there is no sequence homology between these segments and those documented in K-ATP, ROMK1, and GIRK channels, there is a consistent clustering of 5 to 7 positively charged amino acids (arginines and lysines) within a small region of the C-terminal cytoplasmic tail. Our demonstration that internal polyvalent cations could inhibit the PIP2 effects on HERG is supportive of electrostatic interactions between the oppositely charged portions of the channel and PIP2. Further biochemical studies are needed to determine the exact binding site of PIP2 to the HERG channel.

Some Kir channels have additional regulators that influence their dependence on PIP2. Kir1.1 (ROMK1) channels are regulated by protein kinase A (PKA) through phosphorylation that seems to enhance the interaction of these channels with PIP2.¹¹ However, in the HERG K⁺ channel, PKA may not be involved in the interaction of PIP2 and HERG channel, because we previously found that phosphorylation of HERG channel by PKA does not increase but decreases HERG K⁺ current.²⁹ This is complicated, however, by the complex regulation of HERG/I_{Kr} by cAMP due to the four PKA consensus sites and cyclic nucleotide-binding domain of

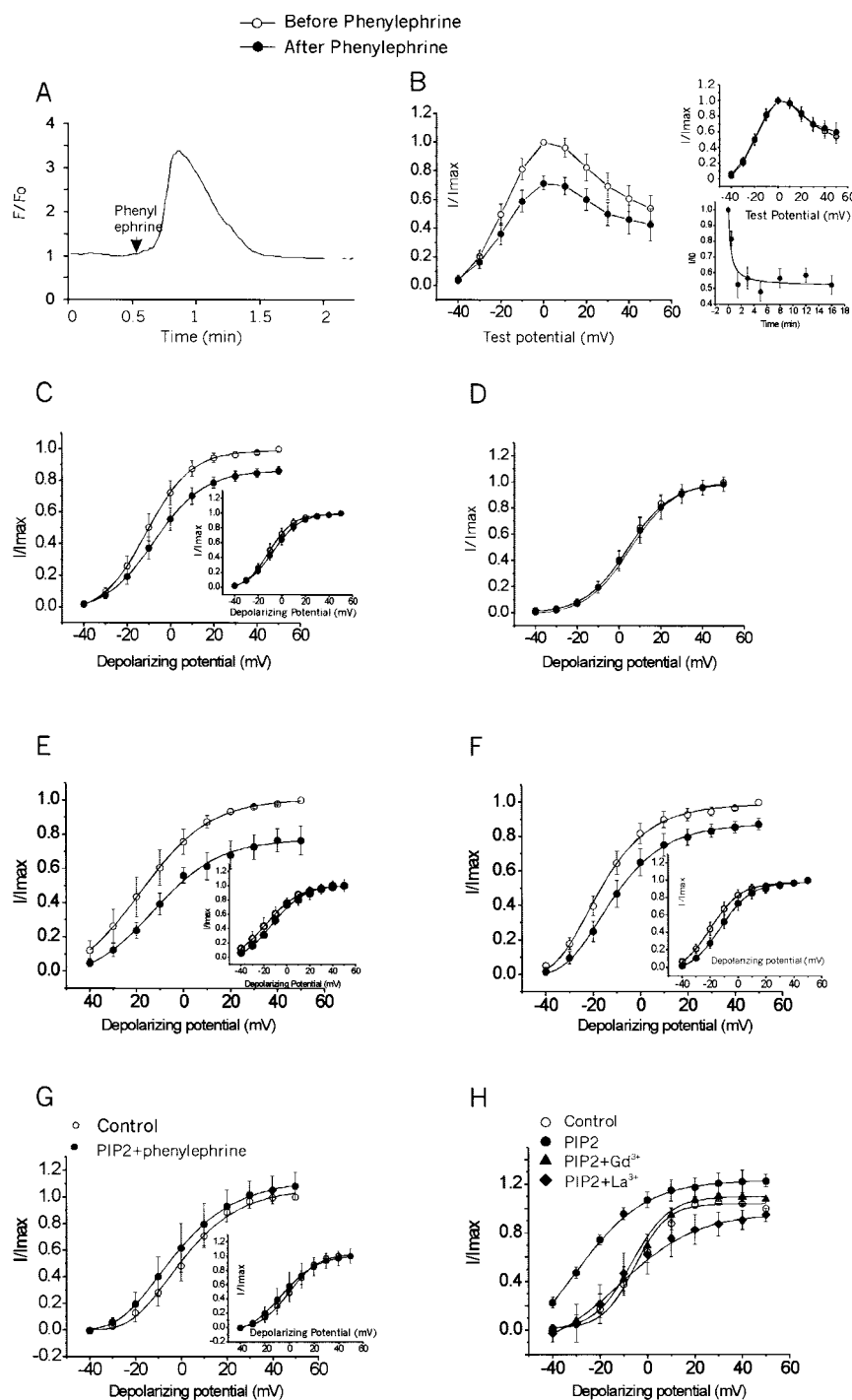


Figure 8. Activation of a G α q-coupled α 1A-adrenergic receptor alters HERG K⁺ channel activity through changes in [PIP2]. A, Verification of the activity of transfected α 1A-adrenergic receptor by measurement of [Ca²⁺]_i signal response to phenylephrine (n=7). B, I-V curves from cells coexpressing HERG and α 1A-adrenergic receptor before (open circles) and after (filled circles) administration of phenylephrine. Top inset, Normalized I-V data; Lower inset, Time course of change in peak current after administration of phenylephrine (n=7). C, Voltage-dependent activation curves before and after phenylephrine (inset, normalized data, n=7). D, Phenylephrine fails to alter HERG K⁺ currents in cells not expressing α 1A adrenergic receptors (n=8). E, Buffering of intracellular Ca²⁺ transients with 15 mmol/L BAPTA fails to prevent the phenylephrine effects on K⁺ currents (n=7). F, One hour preincubation with chelerythrine (1 μ mol/L) fails to prevent the phenylephrine effects on K⁺ currents (n=7). G, PIP2 (10 μ mol/L) in the pipette solution prevents the effect of phenylephrine (n=6). H, Gd³⁺ and La³⁺ (100 μ mol/L) significantly attenuate the effect of PIP2 (n=6).

HERG.^{29,30} Further investigation is needed to explore signaling cross talk between cAMP, PIP2, and HERG.

There is evidence that activation of PLC-linked GPCRs significantly alters cell membrane concentrations of PIP2. Willars et al³² showed that stimulation of the muscarinic M3 receptor reduced the basal PIP2 concentration to 15% of basal levels in a rapid and sustained manner. Moreover, they showed that the pool of receptor-sensitive PIP2 turned over every 5 seconds during sustained stimulation. That these receptor-mediated changes in [PIP2] can affect K⁺ channel function is supported by reports using KATP and GIRK

channels.^{7,27} The effects of short- versus long-term stimulation of receptors and stimulation of receptors in cardiac myocytes will require further investigation.

The heart is presented with continually varying cardiovascular demands that require dynamic responses, both inotropic and chronotropic. Much of the cardiac adaptation that occurs is the result of changes in autonomic/hormonal stimulation involving G-protein-coupled receptors. Under normal situations these adaptations are harmless and meet the needs of the situation at hand; however, under other circumstances they can lead to maladaptive results including ventricular arrhyth-

mias. The results in this study show, for the first time, that PIP₂ regulates the activity of the voltage-gated K⁺ channel, HERG/I_{Kr}. These results provide an additional link between cardiovascular stresses, autonomic stimulation, and arrhythmias, both hereditary and acquired.

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