**PKA and PKC partially rescue long QT type 1 phenotype by restoring channel-PIP$_2$ interactions**

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Long-QT syndrome causes torsade de pointes arrhythmia, ventricular fibrillation, and sudden death. The most commonly inherited form of long-QT syndrome, LQT1, is due to mutations on the potassium channel gene KCNQ1, which forms one of the main repolarizing cardiac K$^+$ channels, IKs. IKs has been shown to be regulated by both β-adrenergic receptors, via protein kinase A (PKA), and by Gq protein coupled receptors (GqPCR), via protein kinase C (PKC) and phosphatidylinositol 4,5-bisphosphate (PIP$_2$). These regulatory pathways were shown to crosstalk, with PKA phosphorylation increasing the apparent affinity of IKs to PIP$_2$. Here we study the effects of LQT1 mutations in putative PIP$_2$-KCNQ1 interaction sites on regulation of IKs by PKA and GqPCR. The effect of the LQT1 mutations on IKs regulation was tested for mutations in conserved, positively charged amino acids, located in four distinct cytoplasmic domains of the KCNQ1 subunit: R174C (S2-S3), R243C (S4-S5), R366Q (proximal c-terminus) and R555C (distal c-terminus). Mutations in the c-terminus of IKs (both proximal and distal) enhanced channel sensitivity to changes in membrane PIP$_2$, levels, consistent with a decrease in apparent channel-PIP$_2$ affinity. These mutant channels were more sensitive to inhibition caused by receptor mediated PIP$_2$-depletion and more sensitive to stimulation of PIP$_2$ production, by overexpression of phosphatidylinositol-4-phosphate-5-kinase (PI5-kinase). In addition, c-terminus mutants showed a potentiated regulation by PKA. On the other hand, for the two cytoplasmic-loop mutations, an impaired activation by PKA was observed. The effects of the mutations on PKC stimulation of the channel paralleled the effects on PKA stimulation, suggesting that both regulatory inputs are similarly affected by the mutations. We tested whether PKC-mediated activation of IKs, similarly to the PKA-mediated activation, can regulate the channel response to PIP$_2$. After PKC activation, channel was less sensitive to changes in membrane PIP$_2$ levels, consistent with an increase in apparent channel-PIP$_2$ affinity. PKC-activated channel was less sensitive to inhibition caused by block of synthesis of PIP$_2$ by the lipid kinase inhibitor wortmannin and less sensitive to stimulation of PIP$_2$ production. Our data indicates that stimulation by PKA and PKC can partially rescue LQT1 mutant channels with weakened response to PIP$_2$ by strengthening channel interactions with PIP$_2$.

**Introduction**

Long QT syndrome (LQT) is the most common form of inherited cardiac arrhythmia. It is estimated that one of 5,000 to 7,000 newborns have the disease and it may cause sudden death in 3,000 to 4,000 children and young adults each year in the US. The most common form of LQT syndrome (Romano-Ward syndrome) is a heterogeneous, autosomal dominant genetic disease caused by mutations of cardiac ion channel genes. This syndrome is associated with delayed ventricular repolarization and is manifested by syncope and sudden death from ventricular arrhythmias. LQT is identified by abnormal QT interval prolongation on the electrocardiogram (ECG). The QT prolongation may arise from either a decrease in repolarizing membrane currents or an increase in depolarizing currents. QT prolongation produced by delayed repolarization due to reductions of the slow repolarizing cardiac K$^+$ currents (IKs) is associated with the most common form of LQT, LQT1.

Stress and exercise are known to precipitate arrhythmias associated with the LQT1 syndrome.1 Stimulation of the β1-adrenergic receptor signaling cascade is one of the most important means of increasing cardiac output in response to stress and exercise. IKs is thought to be particularly important in the heart during adrenergic stimulation. Activation of β1-adrenergic receptors is coupled to activation of adenylyl cyclase which catalyzes the conversion of ATP to cAMP and activates protein kinase A (PKA). β-Adrenergic stimulation increases IKs via channel activation by direct phosphorylation by PKA. β-Blockers are commonly used to treat these patients although some patients are refractory to the treatment. Normally, β-adrenergic stimulation of repolarizing cardiac K$^+$ currents suppresses β-adrenergic-induced premature beats and afterdepolarizations which are believed to induced, at least in part, by a concomitant increase in Ca$^{2+}$ currents. G-proteins of the Gq/G11 family (GqPCRs) are also known to mediate positive inotropism in human ventricular myocardium. GqPCRs, when activated, stimulate phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and activate a number of downstream effectors, including protein kinase C (PKC) and Gq protein coupled receptors (GqPCRs), via protein kinase C (PKC) and phosphatidylinositol 4,5-bisphosphate (PIP$_2$). These regulatory pathways were shown to crosstalk, with PKA phosphorylation increasing the apparent affinity of IKs to PIP$_2$. Here we study the effects of LQT1 mutations in putative PIP$_2$-KCNQ1 interaction sites on regulation of IKs by PKA and GqPCR. The effect of the LQT1 mutations on IKs regulation was tested for mutations in conserved, positively charged amino acids, located in four distinct cytoplasmic domains of the KCNQ1 subunit: R174C (S2-S3), R243C (S4-S5), R366Q (proximal c-terminus) and R555C (distal c-terminus). Mutations in the c-terminus of IKs (both proximal and distal) enhanced channel sensitivity to changes in membrane PIP$_2$, levels, consistent with a decrease in apparent channel-PIP$_2$ affinity. These mutant channels were more sensitive to inhibition caused by receptor mediated PIP$_2$-depletion and more sensitive to stimulation of PIP$_2$ production, by overexpression of phosphatidylinositol-4-phosphate-5-kinase (PI5-kinase). In addition, c-terminus mutants showed a potentiated regulation by PKA. On the other hand, for the two cytoplasmic-loop mutations, an impaired activation by PKA was observed. The effects of the mutations on PKC stimulation of the channel paralleled the effects on PKA stimulation, suggesting that both regulatory inputs are similarly affected by the mutations. We tested whether PKC-mediated activation of IKs, similarly to the PKA-mediated activation, can regulate the channel response to PIP$_2$. After PKC activation, channel was less sensitive to changes in membrane PIP$_2$ levels, consistent with an increase in apparent channel-PIP$_2$ affinity. PKC-activated channel was less sensitive to inhibition caused by block of synthesis of PIP$_2$ by the lipid kinase inhibitor wortmannin and less sensitive to stimulation of PIP$_2$ production. Our data indicates that stimulation by PKA and PKC can partially rescue LQT1 mutant channels with weakened response to PIP$_2$ by strengthening channel interactions with PIP$_2$.

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In proteins shown to interact with PIP$_2$, including Kir channels, several basic residues were shown to form the PIP$_2$ binding site.\textsuperscript{15-18} Several positively charged residues present in cytoplasmic portions of the KCNQ1 channel may come together to form a binding-site for PIP$_2$. Mutations that decrease channel-PIP$_2$ interactions also increase the channel sensitivity to PIP$_2$ depletion.\textsuperscript{14,19-23} Approximately 250 LQT1 mutations have been identified to date (http://www.fsm.it/cardmoc/). About 75% of these mutations are missense mutations, which have been associated with the greatest cardiac risk.\textsuperscript{24} Twenty percent of the missense mutations are associated with positively charged cytoplasmic residues and at least another 30% are in close proximity to these putative PIP$_2$ interaction sites, suggesting a decrease in interactions of the channel with PIP$_2$ may be a common mechanism underlying decrease in function observed in LQT1. Most of these charged residues are highly conserved in other KCNQ channel families, also known to be sensitive to PIP$_2$. Here we studied whether four mutations associated with LQT1, present in positively charged amino acids, conserved among the KCNQ family, in distinct cytoplasmic domains of the channel (S2-S3, S4-S5, proximal c-terminus and distal c-terminus), altered channel regulation by PIP$_2$. Because the strength of channel-PIP$_2$ interactions was shown to determine the sensitivity of Kir channels to these mutations are missense mutations, which have been associated with the greatest cardiac risk.\textsuperscript{24} We investigated the consequences of altered channel-PIP$_2$ sensitivity on PKA and PKC regulation.

Two of the four mutants tested (R366Q, in the proximal c-terminus, and R555C, in the distal c-terminus) show an enhanced sensitivity to changes in membrane PIP$_2$ levels, consistent with channels that have a weaker apparent affinity to PIP$_2$. Function of these c-terminal mutations could be partially rescued by PKA and PKC activation. In contrast, the c-loop mutations tested (R174C and R243C) disrupted the transduction of regulatory inputs that led to channel activation. Our data shows that normal GqPCR regulation is disrupted by LQT1 mutations and that changes in IK$_s$ regulation by GqPCRs may contribute to the pathophysiology of long-QT syndrome.

**Results**

**LQT1 related mutations affect IK$_s$ regulation by PIP$_2$.** The inhibitory phase of the IK$_s$ channel regulation by GqPCRs has been shown to be due to PIP$_2$ depletion of the channel.\textsuperscript{13,14} We studied the effect of four naturally occurring LQT1 mutations on PIP$_2$-mediated inhibition of IK$_s$. The LQT1 mutations studied here were neutralizing mutations in positively charged cytoplasmic residues highly conserved among the KCNQ channel family. Because PIP$_2$ has been shown to activate all KCNQ channel family members, these four residues are putative PIP$_2$ interaction sites. To assess the effect of the LQT1 mutations on the channel, we expressed either the wild-type or the mutant KCNQ1 subunit together with KCN€1 at a ratio 1:1 (KCNQ1:KCN€1) in Xenopus oocytes. To determine the voltage dependence of IK$_s$, we constructed isochronal (t = 6 s) activation curves. We measured the IK$_s$ tail current at -40 mV after depolarization to a series of voltage steps from -60 to +80 mV. A Boltzmann fit of this data was used to determine the V$_{1/2}$ and the maximal conductance (Gmax) of activation. IK$_s$ and LQT1 mutant channels did not reach a steady level even after long depolarizations at room temperature. Experiments using depolarizing pulses of varying length (18 s and 2.7 s) showed that V$_{1/2}$ of activation varied with the length of the depolarization pulse and the duration of the observation period.

**Figure 1 (See opposite page).** PIP$_2$ regulation is modified by LQT1 mutants. (A) Typical IK$_s$ response in oocytes expressing either KCNQ1 or mutant subunit together with KCN€1 subunits. Currents were measured to depolarized steps from -60 to +80 mV from a -80 mV holding potential followed by a -40 mV step. (B) Left: summary data for current measured after 4 s depolarization to +40 mV for wild-type and mutant channels. Current was normalized to wild-type current for each batch of oocytes (WT: 1.00 ± 0.05, n = 30; R174C: 0.47 ± 0.21, n = 14; R243C: 0.12 ± 0.06, n = 22; R366Q: 0.22 ± 0.12, n = 23; R555C: 0.08 ± 0.06, n = 24). Middle: V$_{1/2}$ for current activation for wild-type and mutant channels. V$_{1/2}$ was calculated from a Boltzmann fit of the tail current data. Tail currents were measured at -40 mV after 6 seconds depolarizing pulses from -60 mV to +80 mV from -80 mV holding potential (in mV: WT: 17 ± 2, n = 25; R174C: 34 ± 2, n = 11; R243C: 84 ± 5, n = 6; R366Q: 46 ± 2, n = 14; R555C: 52 ± 2, n = 16). Right: Normalized Gmax for current activation calculated from Boltzmann fit for WT and mutant channels in mV: WT: 1.00 ± 0.04, n = 28; R174C: 0.52 ± 0.06, n = 9; R243C: 0.56 ± 0.19, n = 6; R366Q: 48 ± 0.06, n = 16; R555C: 0.20 ± 0.04, n = 18. (C–F) Effect of BK2 receptor stimulation on the KCNQ1(R174C)/KCNE1, KCNQ1(R243C)/KCNE1, KCNQ1(R366Q)/KCNE1 and KCNQ1(R555C)/KCNE1 mutant channel currents when compared to WT regulation. Depolarizing steps to +40 mV from -80 mV. Left: typical current response to bradykinin 1 μM before (a) and during the inhibition (b) induced by agonist stimulation. Right: time course of the effects of agonist stimulation measured at the end of the depolarizing pulse. (G) Summary data. The inhibition phase of the regulation was disrupted for three of the mutants tested. Regulation for each mutant was compared to wild-type regulation measured in same oocyte batches. Inhibition for the R174C mutant channel was lower than wild-type (R174C: 39 ± 4%, n = 8; WT: 55 ± 5%, n = 9); for R243C it was the same as wild-type channels (R243C: 48 ± 3%, n = 7; WT: 46 ± 7%, n = 8); for R366Q (R366Q: 72 ± 3%, n = 9; WT: 52 ± 6%, n = 9) and R555C (R555C: 48 ± 5%, n = 8, WT: 24 ± 2%, n = 8) inhibition were stronger than wild-type channels. (H) Effect of expression of PI(3)kinase on wild-type and mutant currents measured at +40 mV after 4 s depolarization from -80 mV. Currents were normalized to the current without PI(3)kinase expressed. Current in cells expressing PI(3)kinase was higher than in those without PI(3) kinase being expressed for WT for R366Q (3.5 ± 0.3, n = 18) and R555C (2.0 ± 0.1, n = 8) mutant channels and lower for R174C (0.9 ± 0.1, n = 20) when compared to wild-type. For R243C (1.7 ± 0.1, n = 27) activation was not significantly different than wild-type (1.5 ± 0.1, n = 17).
Figure 1. For figure legend, see page 4.
of IKs is dependent on the length of the depolarizing pulse, but relative shifts in the voltage dependence persist, independent of the length of the pulse. A typical response to depolarizing voltages is shown in Figure 1A for each of the mutants tested. All four mutants showed a decrease in function when compared to the wild-type channel (Fig. 1B). In addition, all mutants tested showed a significant right shift in the voltage dependence of activation and a decrease in Gmax (Fig. 1B). In order to test whether mutations associated with LQT1 affect channel regulation by PI(4)P₂, we used two approaches. First, we co-expressed the GqPCR bradykinin type 2 (BK2) receptor together with KCNE1 and KCNQ1 mutant subunits. Bradykinin-mediated inhibition of IKs was shown to be mediated by PI(4)P₂ depletion. For other GqPCRs, a PKC-mediated activation follows the inhibition phase (Fig. 3). This PKC-mediated activation is strongly decreased for the BK2 receptor, allowing the study of the PI(4)P₂ contribution in isolation (Fig. 1C). We measured channel regulation upon stimulation by BK for four LQT1 mutants (Fig. 1D and E). The inhibition measured for each mutant was compared to the inhibition observed in wild-type channels expressed in the same batch of oocytes. For three of the mutant channels tested, BK regulation was affected. Mutations had similar effects on agonist-induced inhibition observed after stimulation of another GqPCR, the muscarinic type 1 receptor (M1) (data not shown). Agonist-inhibition could not be assessed for the R366Q mutant after M1 receptor stimulation because it was masked by the dramatic increase in PKC activation (Fig. 3). For KCNQ1(R174C)/E1, the inhibitory phase was blunted. For KCNQ1(R243C)/E1 there was no significant effect on the inhibition phase. For the KCNQ1(R366Q)/E1 and KCNQ1(R555C)/E1 mutant channels, the inhibition phase was potentiated. KCNQ1(R555) residue has been suggested to directly interact with PI(4)P₂. Our results show that mutations in the R366 and R555 increased channel sensitivity to agonist mediated PI(4)P₂-depletion.

As a second approach to assess changes in channel regulation by PI(4)P₂ in the mutant channels, we increased membrane PI(4)P₂ levels by expressing PI(4)5-kinase (PI5-kinase), one of the enzymes responsible for PI(4)P₂ production. Overexpression of PI5-kinase in atrial myocytes or in sympathetic neurons tonically increases membrane PI(4)P₂ levels and dramatically reduces PI(4)P₂ mediated regulation for PI(4)P₂-sensitive channels. It decreases desensitization of Kir3 channels and muscarinic modulation of endogenous M current. Overexpression of PI5-kinase increased channel current for wild-type, R243C, R366Q and R555C mutant channels, but not R174C, suggesting that the latter is tonically saturated by basal PI(4)P₂ levels (Fig. 1H). R366Q and R555C channels show a larger PI5-kinase-mediated increase in current when compared to wild-type channels. Increased sensitivity to changes in membrane PI(4)P₂ levels indicate a decrease in the apparent affinity of PI(4)P₂ for the R366Q and R555C channels. The changes observed are not consistent with an exclusive decrease in PI(4)P₂ efficacy, but our results do not preclude efficacy changes in addition to affinity changes. Binding to PI(4)P₂ is necessary for IKs channel activity. Mutations in residues interacting with PI(4)P₂ are not expected to abolish lipid binding but to decrease PI(4)P₂ affinity to the channel. A decrease in the channel affinity to PI(4)P₂ is expected to increase sensitivity to changes in membrane PI(4)P₂ levels, as seen for a number of PI(4)P₂-sensitive channels. Our results are consistent with both R366 and R555 being PI(4)P₂-interacting sites.

LQT1 related mutations affect IKs regulation by PKA. To assess the PKA effects on the channel, we applied forskolin (50 μM), which activates PKA through direct stimulation of adenyl cyclase, and measured its effects on the activity of KCNQ1/KCNQ1 channels (Fig. 2A and B). KCNQ1/KCNQ1 currents were potentiated by forskolin. To determine the voltage dependence of IKs, we constructed isochronal (Δt = 10 s) activation curves. Forskolin applications significantly shifted the voltage of activation of the IKs channel to more hyperpolarizing voltages. These findings are consistent with data published for the PKA regulation of the IKs channels. In addition, direct phosphorylation by PKA has been shown biochemically for KCNQ1/KCNQ1 channels.

We tested whether the four LQT1 associated mutations affected PKA regulation of the channel. Note the effects of the mutations shown in Figure 1B are opposite to the effect of channel regulation by PKA (Fig. 2A). As in Figure 2A we applied forskolin (50 μM) and measured its effects on the activity of wild-type and mutant channels subunits co-expressed with KCNE1. For the two mutations present in the cytoplasmic loops of the channel, R174C and R243C (Fig. 5), there was no significant increase in current after forskolin treatment (Fig. 2B). In addition, there was no shift in the voltage dependence of activation caused by forskolin (Fig. 2F). For the two mutations in the c-terminus of the channel, R366Q and R555C, the shift in the voltage dependence of activation caused by forskolin was not affected by the mutation, and activation by forskolin was increased when compared to wild-type (Fig. 2B and C).

LQT1 related mutations affect IKs regulation by PKC. To assess the PKC-mediated effects on the channel, we co-expressed KCNQ1 and KCNE1 subunits with muscarinic type 1 (M1) receptors. Stimulation of the M1 showed two phases of regulation: first, a decrease of the current, followed by current increase (Fig. 3C). The activation phase of this response was significantly reduced by the PKC inhibitor calphostin C (2 μM) (Fig. 3E). These findings are consistent with data showing that PKC phosphorylation underlies IKs activation upon GqPCR stimulation. For these experiments we co-expressed KCNQ1, KCNE1 and the M1 receptor with IP3-phosphatase (IP3phosp) to inhibit intracellular Ca²⁺-release and endogenous Ca²⁺-dependent Cl⁻ currents present in oocytes.

In order to test whether the mutations also affected PKC-mediated activation upon GqPCR stimulation of the channel, we co-expressed M1 receptors together with KCNE1 and KCNQ1 mutant subunits. We measured channel activation upon stimulation by ACh for three LQT1 mutants. For KCNQ1(R174C) and KCNQ1(R243C) the activation phase was blunted (Fig. 3A–D). For the KCNQ1(R366Q) mutant channel the activation was potentiated. PKC activation effects paralleled the effect of the mutation on the PKA activation for the three mutants tested.

PKC and PI(4)P₂ regulation of IKs crosstalk. We previously showed that PKA regulation can alter response of the IKs channel to PI(4)P₂ in both heterologous and native systems. To test whether PKC-mediated activation of the channel also depended
on PIP₂, we studied dependence of PKC activation on membrane PIP₂ levels. PIP₂ is produced in the plasma membrane by sequential phosphorylation of PI by PI4-kinase and PI(4)5-kinase (PI5-kinase). Altering either the expression level or activity of either enzyme will markedly affect membrane PIP₂ levels. We measured IKs channel regulation by ACh and studied the effect of changes of PIP₂ levels in the PKC-mediated activation (Fig. 4). Treatment of the cells with the PI4-kinase inhibitor wortmannin (5 μM) (WMN) for 30–60 min has been shown to inhibit the IKs channel by decreasing membrane PIP₂ levels. Here we show that 5 μM WMN, for 30–60 min potentiated the PKC-mediated activation phase (Fig. 4). WMN treatment inhibited channel current by 48 ± 13% (n = 6). We assessed effects of increasing membrane PIP₂ levels by overexpressing PI5-kinase. An inactive, truncated PI5-kinase was used as a negative control. Overexpression of PI5-kinase, but not the inactive PI5-kinase mutant, and consequent increase in membrane PIP₂ levels, blunted PKC-induced IKs activation (Fig. 4B). Currents were also larger when PI5-kinase was expressed (Fig. 4C) suggesting the unstimulated channels in the absence of PI5-kinase expression, are not saturated by PIP₂.

We studied the effect of PKC activation on the PIP₂-mediated WMN inhibition and the PI5-kinase activation of the wild-type channel. Both inhibition by WMN and activation by PI5-kinase were blunted in PKC-activated channels (Fig. 4C). These results suggest a crosstalk between PIP₂ and PKC regulation of IKs as previously seen for the PKA and PIP₂, signaling. PIP₂ activates the channel in a dose dependent manner. For a given membrane PIP₂ level, a channel with higher PIP₂ affinity will be closer to saturation and less sensitive to changes in membrane PIP₂ level. For channel with a weaker apparent affinity for PIP₂, both depletion of membrane PIP₂ and increase in membrane PIP₂ levels are expected to cause an increase in the fractional of change of the current. For channels with only changes in efficacy of PIP₂ activation, the fractional WMN-inhibition and activation by PI5-kinase are expected to be the same. Our data is consistent with the PKC activated channel having an increase in the apparent affinity to PIP₂, and it is compatible with PKC strengthening KCNQ1 interactions with PIP₂.

To test whether the PKC regulation of response to PIP₂ was abolished for the PKC-insensitive mutant R243C, we measured the effect of PKC activation on PIP₂ levels using forskolin. Figure 2. PKA regulation is modified by LQT1 mutants. (A) Typical response to forskolin treatment in oocytes expressing KCNQ1/KCNE1 subunits. Currents were measured to depolarized steps from -80 to +80 mV from a -80 mV holding potential followed by a -40 mV step. (B) Left: summary data for current activation by forskolin measured after 4 s depolarization to +40 mV and voltage dependence of activation for cells either treated or untreated with 50 μM forskolin for 60–90 min (untreated: 1.00 ± 0.05, n = 25; treated 1.33 ± 0.05, n = 24). Forskolin was present during the measurement of the currents for treated cells. Right: V1/2 for current activation for cells either treated or untreated with forskolin (as in A). V1/2 was calculated from a Boltzmann fit of the tail current data. Tail currents were measured at -40 mV after 6 s depolarizing pulses from -60 mV to +80 mV from -80 mV holding potential. (C) Typical KCNQ1(R366Q) response to forskolin treatment. Currents were measured to 6 s depolarized steps from -80 to +80 mV from a -80 mV holding potential followed by a -40 mV step. (D) Summary currents for KCNQ1/KCNE1 wild-type and mutant channels (n = 7–21). Whole-oocytes currents were measured after 4 s depolarization to +40 mV from a -80 mV holding potential for wild-type channels and each of the mutants indicated. Forskolin potentiation: WT: 1.38 ± 0.05, n = 24; R174C: 1.0 ± 0.7, n = 7; R243C: 1.2 ± 0.12, n = 12; R555C: 1.68 ± 0.10, n = 16. Currents were normalized to the average untreated current. (E) Shift in V1/2 for current activation for cells treated with forskolin compared to untreated cells (as in A). V1/2 was calculated from a Boltzmann fit of the tail current data measured at -40 mV (in mV): WT: -13.3 ± 1.4, n = 25; R174C: 1.0 ± 0.7, n = 7; R243C: 4.6 ± 7.1, n = 8; R366Q: -13.3 ± 2.0, n = 13; R555C: -11.1 ± 3.5, n = 11). Channels expressing R174C and R243C mutant subunits had no significant increase in current or shift in V1/2 after forskolin treatment. Regulation of wild type channels were compared to mutant channel regulation in the same batches of oocytes.
both blunting and potentiating regulation. Our data indicates that mutations that alter channel sensitivity to PIP₂ can be rescued by regulatory inputs and that one mutation in the S2-S3 and another in the S4-S5 loop impair channel regulation. We suggest that effects on channel regulation can be important in the risk stratification of LQT patients and may have important implications for disease treatment. In addition, this work is the first to suggest that changes in Gq-coupled regulation underlie the pathophysiology of this disease.

The role of PIP₂ as a second messenger molecule has become increasingly important through its modulation of a growing number of ion channels and transporters (reviewed in ref. 32). For Kir channels, several stimuli exert their effects through modulating interactions of the channel with PIP₂. Mutations of the inward rectifiers Kir2.1 and Kir1.1 have been shown to cause disruptions in the channel-PIP₂ interactions leading to both Andersen’s and Bartter’s syndromes. The activity of the channels formed by KCNQ1 and KCNE1 subunits, by KCNQ1 subunits alone, and by all other members of the KCNQ family have been shown to be critically dependent on PIP₂. KCNQ channels were shown to be activated by PIP₂ and channel rundown was linked to PIP₂ hydrolysis. In addition, for channels formed by the KCNQ2 and KCNQ3 subunits, agonist-induced depletion of PIP₂ has been associated with channel inhibition. PIP₂-dependent rundown and exogenous PIP₂ application were shown to shift the voltage dependence of IKs activation. We have shown that GqPCRs inhibit the IKs channel by depleting membrane PIP₂ levels.

Decreases in IKs channel-PIP₂ interaction have been suggested to be associated with LQT1. We tested whether LQT1 mutants modify PKA and GqPCR regulation of IKs. Different mutations can alter regulation in a diverse manner, with WT/E1 and PI5-kinase expression in PKC-stimulated and unstimulated R243C mutant channel. Currents were normalized to cells not expressing PI5kinase (left) or cells without WMN treatment. For this mutant channel, sensitivity to changes in PIP₂ levels was maintained after PKC-activation (Fig. 4D).

**Discussion**

Here we show that several mutations associated with the LQT1 syndrome affect both PKA and GqPCR regulation of IKs. Different mutations can alter regulation in a diverse manner, both blunting and potentiating regulation. Our data indicates that mutations that alter channel sensitivity to PIP₂ can be rescued by regulatory inputs and that one mutation in the S2-S3 and another in the S4-S5 loop impair channel regulation. We suggest that effects on channel regulation can be important in the risk stratification of LQT patients and may have important implications for disease treatment. In addition, this work is the first to suggest that changes in Gq-coupled regulation underlie the pathophysiology of this disease.

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channel activity, strongly suggesting R555 was a PIP₂ interacting site. Our results agree with these results for R555C. The residues tested here affected channel regulation by distinct mechanisms. R174C decreased PKA, PKC and PIP₂ regulation, suggesting that it is important for the transduction of regulatory inputs. R243C decreased PKA and PKC activation, suggesting that the S4-S5 region may be necessary to transduce PKA and PKC regulation to the channel gate. Indeed, the S4-S5 linker region has been suggested to interact with KCNE1.37 We also previously showed the residue in KCNE1 (S102) is important for channel regulation by PKC.13 In addition, KCNE1 mutations have been shown to affect IKs regulation by PKA without affecting channel phosphorylation.38 For the R366Q mutant channel, a stronger inhibition by PIP₂-depletion and activation by increases in membrane PIP₂.

Figure 4. Changes in PIP₂ levels modulate PKC-mediated activation. Current activation induced by 10 μM ACh measured as in Figure 3. (A) Typical trace of ACh regulation in cells after 30–60 min WMN treatment and co-expressing PI5kinase. Depolarizing step to +40 mV from -80 mV. (B) Activation phase is stronger with after WMN treatment (control: 152 ± 17%, n = 10; WMN: 298 ± 77%, n = 6), presumably because of lower levels of membrane PIP₂. Activation phase is lower when PI5-kinase was overexpressed, presumably because of higher membrane PIP₂ levels (control: 152 ± 14%, n = 16; PI5-Kinase: 2.9 ± 1.3%, n = 15; PI5-kinaseΔ1-238: 181 ± 23%, n = 6). Significant difference from WT regulation was indicated. In all experiments IP₃phosp was co-expressed to block Ca²⁺-release. (C) Data as in (B) normalized to wild-type control current. Both inhibition by WMN and activation by PI5kinase are abolished after PKC-mediated channel activation. Basal current is measured before ACh stimulation. PKC-stimulated current is measured 800 s after ACh stimulation. Currents were measured after 4 s depolarization to +40 mV from -80 mV. (D) Comparison of regulation of wild-type and R243C mutant channel. PKC-induced effects were abolished for the mutant channel. Left: PI5kinase activation is preserved after PKC activation for R243C channels. Right: WMN inhibition is preserved for the R243C mutant channel (n at least 6 for all experiments).

Figure 5. Scheme depicting LQT1 mutation location.
levels was observed, consistent with the mutant channel having weaker interactions with PIP$_2$ than the wild-type channel. We also tested the R555C channel, suggested by Park et al. to interact with PIP$_2$, which also shows stronger PIP$_2$-mediated inhibition and PI5-kinase-mediated activation. Our data suggest that mutations in R366 and R555 increase channel sensitivity to changes in membrane PIP$_2$ levels, suggesting a decrease in the apparent affinity of the channel to PIP$_2$ for these mutant channels. That is not the case for R174 and R243.

For the four mutants tested, regulation of the channel was altered. A decrease in the activation both by PKA and PKC such as the one observed for cytoplasmic-loop KCNQ1(R174C) and KCNQ1(R243C) may contribute to the disease phenotype by decreasing normal channel activation upon receptor stimulation. This would be expected to be independent of the QTc prolongation measured at rest for these patients. An increase in the activation such as the one observed for the c-terminal KCNQ1(R366Q) and KCNQ1(R555C) mutants may be protective and decrease the burden of the mutation. Indeed, patients with mutations in the c-terminus region of the KCNQ1 subunit have been found to be less prone to cardiac arrhythmias than patients with mutations in the transmembrane regions, independently of the effect of the mutation on QT interval. In addition, in contrast to most LQT1 patients, patients with KCNQ1(R555C) mutation have been found not to have exercise induced syncopes. Most c-terminus mutations linked to LQT1 occur in or in close proximity to conserved positive amino acids, suggesting this may be a common feature of LQT1 C-terminus mutations. Our data provides a possible mechanistic explanation for the milder clinical phenotype of any mutant that decreases channel sensitivity to PIP$_2$.

For the R366Q and R555C channels, which our results suggest interact with PIP$_2$, regulation of the channel was potentiated, partially rescuing the mutant phenotype. We recently showed that PKA modulated channel response to PIP$_2$ for the IKs channel.

Our results indicate that PKC regulation of IKs is dependent of membrane PIP$_2$ levels. Our data is compatible with a model where PIP$_2$ binding to the C-terminus of the channel maintains channel activity and both PKA and PKC activate the channel by strengthening KCNQ1 interactions with PIP$_2$.

Our results suggest that there is altered GPCR regulation for several mutant channels linked to LQT1. IKs regulation by GqPCRs may prove to be one of the causes of cardiac arrhythmias both in heart failure, where Gq-coupled receptors are particularly important in regulating inotropy, and for long-QT patients. The understanding IKs regulation may allow us to devise innovative strategies to treat patients with these conditions.

Materials and Methods

Molecular biology. Human KCNQ1, KCNE1, M1, AT1a and BK2 clones were subcloned in to the pGEMsh vector (modified from PGEMHE vector) for oocyte expression. Site direct mutagenesis was performed using PFU ultra DNA polymerase (Stratagene). Construct sequences were confirmed by DNA sequencing (Cornell, Ithaca). cRNAs were transcribed using the “message-machine” kit (Ambion) and RNA concentrations were estimated using RNA markers (Gibco).

Electrophysiology. Xenopus oocytes were harvested, dissociated and defolliculated by collagenase type I (Sigma) treatment. cRNA was injected at the approximately concentrations: 2 ng for KCNQ1, 0.4 ng for KCNE1, 2 ng for IP3-phosphatase, AT1a, M1 or BK2 receptors. Wild-type and mutant PI5-kinase RNA were injected into oocytes one day after receptor and channel subunits at a concentration of 0.7 ng/oocyte.

Oocytes were constantly superfused with (in mM): 82.5 NaCl, 2 KCl, 1 MgCl$_2$, 1.8 CaCl$_2$, 5 NaOH/HEPES (pH 7.5). Currents were evaluated 5–10 min after oocytes impalement. Cl$^-$ free solutions were used to inhibit endogenous Ca$^{2+}$-release occur, and contained (in mM): 82.5 Na-acetate, 2 KOH, 1 Mg-sulfate, 1.8 Ca-acetate, 5 NaOH/HEPES, pH 7.5. IKs currents were measured after 2 s depolarization to +60 mV from -80 mV holding potential unless otherwise indicated.

Error bars represented standard-error of the mean. All experiments were performed in at least 3–6 oocytes from the same batch and at least 2–3 oocyte batches were used. Test conditions and control experiments were always done on oocytes from the same batch. Student t-test (two groups) or one-way ANOVA (more than two groups) were applied for the assessment of statistical significance. The investigation conforms with 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). Protocol approval was granted by a university ethics review board (UCAR-2004-275).

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