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PKC ACTIVATION AND PIP₂ DEPLETION UNDERLIE BIPHASIC REGULATION OF IKs BY Gq-COUPLED RECEPTORS

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Abstract

KCNQ1 is co-assembled with KCNE1 subunits in the heart to form the cardiac delayed rectifier K⁺ current (IKs), which is one of the main currents responsible for myocyte repolarization. The most commonly inherited form of cardiac arrhythmias, long-QT syndrome type 1 (LQT1), is due to mutations on KCNQ1. Gq-coupled receptors (GqPCRs) are known to mediate positive inotropism in human ventricular myocardium. The mechanism of IKs current modulation by GqPCRs remains incompletely understood. Here we studied the molecular mechanisms underlying Gq regulation of the IKs channel. Heterologously expressed IKs (human KCNQ1/KCNE1 subunits) was measured in *Xenopus* oocytes, expressed together with GqPCRs. Our data from several GqPCRs shows that IKs is regulated in a biphasic manner, showing both an activation and an inhibition phase. Receptor-mediated inhibition phase was irreversible when recycling of agonist-sensitive pools of phosphatidylinositol-4,5-bisphosphate (PIP₂) was blocked by the lipid kinase inhibitor wortmannin. In addition, stimulation of PIP₂ production, by overexpression of phosphatidylinositol-4-phosphate-5-kinase (PIP5-kinase), decreased receptor-mediated inhibition. The receptor-mediated activation phase was inhibited by the PKC inhibitor calphostin C and by a mutation in a putative PKC phosphorylation site in the KCNE1 subunit. Our results indicate that the depletion of membrane PIP₂ underlies receptor-mediated inhibition of IKs and that phosphorylation by PKC of the KCNE1 subunit underlies the GqPCR-mediated channel activation.

INTRODUCTION

Cardiac function is regulated by a diverse array of hormones and neurotransmitters, many of which exert their physiological effects through G-protein coupled receptors (GPCRs). There are three main GPCR families: Gs, Gi and Gq/G11. G-proteins of the Gq/G11 family, when activated, stimulate phosphatidylinositol-specific phospholipase C (PLC). The substrate for PLC is phosphatidylinositol 4,5-bisphosphate (PIP₂), so that agonist stimulation of the receptor causes reduction of PIP₂ in the plasma membrane. Hydrolysis of PIP₂ generates inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). Stimulation of IP₃ receptors by IP₃ releases Ca²⁺ from intracellular stores. In addition, DAG activates protein kinase C (PKC).

GqPCRs such as α 1 adrenergic, angiotensin II AT1 and endothelin receptor are known to mediate positive inotropism in human ventricular myocardium [1]. Their stimulation contributes to the increase in contractility in early stages of heart failure. Angiotensin-

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converting enzyme (ACE) inhibitors modulate GqPCR function, are widely used for treatment of cardiomyopathies, and have been shown to have antiarrhythmic properties. ACE inhibitors are thought to decrease the incidence of atrial fibrillation [2] and ventricular arrhythmias in patients with decreased left ventricular function (for review see [3]).

The literature on GqPCR modulation of IKs is seemingly contradictory. GqPCRs have been reported to both inhibit and activate the native IKs current [4–7]. The mechanism underlying the GqPCR regulation of the channel is not known. IKs channels have been suggested to be regulated by downstream GqPCR signaling pathways. These include PKC phosphorylation [8,4,9,10], increases in intracellular Ca^{2+} [4,11] and more recently by PIP_2 [12,13]. This is the first report to study the role of each of these pathways in GqPCR regulation of IKs.

We show that IKs is regulated by several GqPCRs in a biphasic manner. Our data shows that PKC phosphorylation underlies the activation phase. In addition, we show that PIP_2 depletion, due to GqPCR stimulation, inhibits IKs. Our data suggest that changes in IKs regulation by GqPCRs may contribute to the pathophysiology of cardiac disease for long-QT syndrome and during heart failure where GqPCR signaling is up-regulated.

MATERIALS AND METHODS

Molecular Biology

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). cRNA was injected at approximately concentrations: 2 ng for KCNQ1, 0.4 ng for KCNE1, 2 ng for IP3-phosphatase and 2 ng for M1, AT1 and BK2 receptors. Wild-type and mutant PI5-kinase RNA were injected into oocytes one day after receptor and channel subunits at a concentration of 0.7ng/oocyte. Detailed information about constructs is provided in the supplementary data.

Electrophysiology

IKs whole-oocyte currents were measured after 2s depolarization to +60mV from –80mV holding potential, unless otherwise indicated. Detailed methods are found in the supplementary data.

Currents were evaluated 5–10 min after oocytes impalement. Cl^- -free solutions were used to inhibit endogenous Ca^{2+} -activated Cl^- currents for Ca_i^{2+} -release experiments and contained (in mM): 82.5 Na-acetate, 2 K₂O, 1 Mg-sulfate, 1.8 Ca-acetate, 5 NaOH/HEPES, pH 7.5. Ohmic leak currents calculated from the current measured at –80mV were subtracted for all time course currents presented.

Current regulation model

An schematic model of current regulation was produced by a sum of three components: two sigmoidal activation components of the form: $\text{PKC}_{\text{slow}} = 1 - 1/(1 + (t/300)^3)$ and $\text{PKC}_{\text{fast}} = 1 - 1/(1 + (t/20)^{1.2})$; and an exponential inhibition of the form: $\text{InhPIP}_2 = 1 - 1 * \exp(t/150)$. Where t was the time after agonist application. Total current regulation was calculated as $\text{IReg} = a * \text{PKC}_{\text{slow}} + b * \text{PKC}_{\text{fast}} + \text{InhPIP}_2$. The constants a and b were chosen to fit the data in figure 1B and figure 2B.

Error bars represented standard-error of the mean. All experiments were performed independently at least 3 times. 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. Changes in current levels were measured after normalizing the current in each batch of oocytes by the average of the control current on the day of the experiment. 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).

RESULTS

Agonist stimulation of Gq-coupled receptors shows biphasic regulation of IKs

In order to study IKs regulation by GqPCRs, we expressed the channel subunits with a number of representative Gq-coupled receptors. Human KCNQ1 and KCNE1 subunits were co-expressed in *Xenopus* oocytes with muscarinic type 1 (M1), angiotensin type 1 (AT1a) and bradykinin type 2 (BK2) receptors. Regulation of IKs by the GqPCR α -adrenergic receptor is particularly important because exercise is known to trigger syncope and sudden death in patients with mutations in the KCNQ1 and KCNE1 subunits [14]. Unfortunately, in our hands, α -adrenergic receptors did not express in oocytes. Nonetheless regulation by other GqPCR receptors, also potentially important in cardiac regulation, showed conserved features. Stimulation of the M1 and AT1 receptor by acetylcholine (ACh) and angiotensin II (ang II) respectively showed two phases of regulation: first, an increase of the current, followed by current decrease (Fig.1A). Stimulation of BK2 receptor by bradykinin (BK) showed mainly an inhibitory phase of regulation, suggesting that either activation is not present or it is concurrent with the inhibition phase. Current inhibition was significantly lower for M1 receptors when compared to both AT1 and BK2 receptors. Effects were measured in the activating current, the same results were observed in the tail current (data not shown). No significant change in the kinetics of agonist mediated activation or inhibition were observed among the receptors. For most cells current was stable after the inhibition phase even for long agonist applications (10–20min), for some cells a slow activation phase was observed at long agonist applications. Cl^- free external solutions were used to prevent the contribution of the endogenous Ca^{2+} -dependent Cl^- channel (see methods).

Intracellular Ca^{2+} -release regulates agonist modulation of IKs

Intracellular Ca^{2+} has been shown to activate IKs [4,11,15,16], although the role of the rise in calcium upon GqPCR stimulation has not been studied. To study the contribution of Ca^{2+} to the biphasic regulation observed, we compared the regulation in the presence and in the absence of intracellular Ca^{2+} -release. We co-expressed KCNQ1, KCNE1 and the receptor with IP3-phosphatase (IP3phosp) to inhibit intracellular Ca^{2+} -release. Experiments were also performed with oocyte injection of 50nl of a 50mM EGTA solution to inhibit intracellular Ca^{2+} -release with equivalent results (data not shown).

The endogenous Ca^{2+} -dependent Cl^- currents present in oocytes was used to monitor intracellular Ca^{2+} -release and the efficiency of the IP3phosp expression. Stimulation of all three receptors without IP3phosp expression produced robust and equivalent activation of the Ca^{2+} -dependent Cl^- currents (data not shown) suggesting equivalent Ca^{2+} -release upon receptor activation. Oocytes expressing IP3phosp showed no Ca^{2+} -dependent Cl^- currents implying no intracellular Ca^{2+} -release. The expression of IP3phosp did not affect the IKs current ($I_{\text{IP3}}/I = 1.0 \pm 0.1$, $n=9$, $p=0.83$)

In the absence of intracellular Ca^{2+} -release, the kinetics of IKs modulation upon receptor activation changed. The current was first inhibited and then activated for all three receptors (Fig. 2A). The extent and the kinetics of channel activation were not receptor-dependent but

the kinetics of channel inhibition were receptor dependent: the time to inhibit 30% of the initial current (T_{30}) was significantly faster for the BK receptor (53 ± 10 s, $n=6$), than for the AT1 receptor (80 ± 10 s, $n=12$) and the ACh receptor (107 ± 12 s, $n=10$). In addition, inhibition by ACh in the absence of Ca^{2+} was smaller than the Ang II- and BK- induced inhibition.

PKC phosphorylation underlies agonist-induced activation of IKs

PMA, a PKC activator, has been shown to activate human IKs [8,4,9,10]. The role of PKC activation on the GqPCR regulation of IKs is largely unknown. To test whether PKC activation upon receptor stimulation underlies the activation phase of the channel regulation we expressed the channel with either M1 or AT1A receptors. The current activation in the presence and absence of Ca^{2+} could be blocked by calphostin-C, a PKC inhibitor (Fig. 3A and B). Treatment with calphostin-C did not change the basal IKs current either in the presence or absence of IP3phosp (with IP3 phosp: $I_{\text{treated}}/I_{\text{untreated}} = 0.9 \pm 0.1$, $p=0.25$, $n=19$ or without $I_{\text{treated}}/I_{\text{untreated}} = 1.1 \pm 0.1$, $p=0.35$, $n=18$). Calphostin treated and control experiments were done on oocytes from the same batches.

We co-expressed with KCNQ1 the mutant KCNE1(S102A), where a conserved PKC site has been suggested to underlie the PMA inhibition of the channel seen in mice and rats [4]. The increase in activity observed upon stimulation of the receptor was significantly reduced both in the presence and in the absence of intracellular Ca^{2+} -release (Fig. 3C/D). Expression of KCNE1(S102A) did not affect unstimulated IKs current ($I_{Q1+S102A}/I_{WT} = 1.1 \pm 0.1$, $p=0.47$, $n=24$). Our results suggest the phosphorylation of the KCNE1(S102) residue is responsible for most of the current increase observed. Our results suggest that PKC phosphorylation underlies current activation both in the presence and in the absence of intracellular Ca^{2+} -release.

Agonist stimulation of M1 receptor shifts voltage-dependence of IKs

In order to study in more detail the biophysical effects of agonist stimulation, we measured changes in the voltage dependence of activation and maximal conductance upon agonist stimulation. To determine the voltage dependence of IKs, we constructed isochronal ($t=4$ s) activation curves, because IKs does not reach a steady level even after long depolarizations at room temperature. A longer depolarizing pulse could not be used because of the relative fast kinetics of the changes observed. Experiments using longer depolarizing pulses (18 s and 2.7 s) showed that length of the depolarizing pulse affects the voltage dependence of IKs, but relative shifts in the voltage dependence persist, independent of the length of the pulse [17]. We measured the IKs tail current at -40 mV after depolarization to a series of voltage steps from -40 to $+80$ mV (Fig. 4A). A Boltzmann fit of this data was used to determine the maximal conductance (G_{max}) and $V_{1/2}$ of activation. IKs regulation upon agonist stimulation was due to both to a modulation in the voltage dependence of activation and maximum conductance (Fig. 4B/C and D). As a control we measured time dependent changes of isochronal curves for IKs without agonist stimulation. After initial current stabilization, currents were stable during experiments where no ACh was added (Fig. 4C/D). IP3phosp was expressed to inhibit Ca_i^{2+} -release. Note the changes observed are consistent with the ones shown in Figure 2 when ACh was applied. Similar results were found for cells in the presence of Ca_i^{2+} -release (data not shown). We compared changes in isochronal activation curves for wild-type and KCNQ1/KCNE1(S102A) mutant channel. Consistent with our hypothesis that PKC regulates the activation phase and that phosphorylation of the S102 residue is involved in the regulation, the negative shift in the voltage dependence of activation was decreased by this mutation (Fig. 4E). There was no shift in unstimulated $V_{1/2}$ caused by the mutation ($V_{1/2}(\text{WT})$: 15 ± 2 mV, $n=17$; $V_{1/2}(\text{Q1/S102A})$: 14 ± 2 mV, $n=18$; $p=0.57$).

PIP₂ is produced in the plasma membrane by sequential phosphorylation of PI by PI4-kinase and phosphatidylinositol-4-phosphate-5-kinase (PIP5-kinase). Regulation of either enzyme

expression or activity will affect membrane PIP₂ levels and PIP₂ production. We tested whether the PI4-kinase inhibitor wortmannin (WMN) could shift the voltage-dependence of activation for the channel. At low concentrations WMN inhibits PI3-kinase (nM) and at higher (μM) it inhibits PI4-kinase and consequently the replenishment of PIP₂ in the membrane [18]. Inhibition of PIP₂ replenishment by treatment with WMN has been shown to decrease PIP₂ membrane levels [12]. We measured isochronal activation curves for cells treated with WMN at increasing concentration. Consistent with PIP₂ depletion underlying the inhibitory phase, WMN treatment shifted the voltage dependence of activation to more positive values in a dose dependent manner (Fig. 4E). Leftward shifts upon addition of exogenous PIP₂ to the membrane have also been shown to occur, consistent with our results [19].

PIP₂ hydrolysis underlies agonist-induced inhibition of IKs

PIP₂ directly activated recombinant IKs currents when applied to inside-out patches and poly-Lys, a PIP₂ scavenger, inhibits IKs currents [12], nonetheless, the role of PIP₂ in GqPCR regulation of the channel has not been studied. To test whether depletion of PIP₂ from the channel underlied the IKs inhibition observed upon GqPCR receptor stimulation, we used BK2 regulation of the channel because the smaller activation phase observed upon BK2 stimulation in the presence of intracellular Ca²⁺-release (Fig. 1A).

WMN has been shown to inhibit current and recovery from Gq-coupled receptor inhibition for a number of PIP₂ sensitive channels [20,21,12,22,23]. We constructed a WMN dose-response of IKs inhibition using 1 hour treatment with the drug. No inhibitory effect was observed at low WMN concentrations (up to 100nM, a concentration that completely inhibits PI3-kinases). Progressive inhibition was observed, however, at higher concentrations (Fig. 5A, IC₅₀=4.2 ±0.8μM), suggesting that PI4-kinase function and the depletion of membrane PIP₂ is responsible for the WMN inhibition. WMN effect was abolished in cells expressing PI5-kinase. WMN does not inhibit the channel when PI5-kinase was co-expressed, and membrane PIP₂ levels increased, suggesting both specificity of the effect and that WMN does not directly inhibit the IKs channel (5 μM WMN 45min treatment, 43±2% inhibition for control and 0.5 ±0.7% for PI5kinase expressing cells). PI5 kinase expressing cells have both an increase in PIP₂ levels and stronger drive to make PIP₂ from PI4P. The effect of WMN applications in these cells is expected to be weaker because a stronger inhibition of PI4-kinase is necessary in order to deplete membrane PIP₂ levels to levels that inhibit IKs. These results suggest that the IKs current is sensitive to changes in membrane PIP₂ levels.

To test whether PIP₂ depletion underlied BK induced inhibition, we treated cells with WMN and measured agonist inhibition and recovery from inhibition. Inhibition was significantly increased after WMN treatment (Fig. 5B). An increase in PIP₂-dependent inhibition is expected because less PIP₂ is available to activate the channel and agonist mediated PIP₂ hydrolysis is expected to cause a larger fractional decrease in the current. A PIP₂ independent mechanism of inhibition would be expected to have the same fractional decrease and a smaller magnitude of decrease in current. In addition, recovery from inhibition after a short BK application (60s) was significantly inhibited after WMN treatment (Fig. 5C).

We also co-expressed the channel with PIP5-kinase, which has been shown to specifically increase membrane PIP₂ levels in cells [24–27]. Overexpression of PI5-kinase in atrial myocytes or in sympathetic neurons tonically increases membrane PIP₂ levels and dramatically reduces desensitization of Kir3 channels and muscarinic modulation of endogenous M current [24,28]. Co-expression of the kinase decreased current inhibition upon agonist stimulation (Fig. 5D). PI5 kinase expression increased basal current. Currents were larger when PI5-kinase was expressed (72±10% increase relative to control, n=11) suggesting the channels not to be tonically saturated by PIP₂.

In addition, we co-expressed a truncated form of the PIP5-kinase that lacks residues 1 to 238 and is catalytically inactive [29,27]. The truncated PIP5-kinase had no significant effect on BK-induced IKs inhibition. A similar result was observed for the IKs inhibition upon ACh stimulation of the M1 receptor. This data is complementary to the BK inhibition data in the presence of WMN, and indicates that the inhibition of the channel is due to PIP₂ depletion. A decrease in PIP₂-dependent inhibition is expected because more PIP₂ is available to activate the channel and agonist mediated PIP₂ hydrolysis is expected to cause a smaller fractional decrease in the current. A PIP₂ independent mechanism of inhibition would be expected to have the same fractional decrease and a larger magnitude of decrease in current. In order to test whether the PIP₂ depletion also underlied the ACh mediated inhibition we co-expressed PI5kinase and M1 receptors. The ACh-inhibition observed when cells were over-expressing PI5-kinase (8±2%, n=15) was lower than for control (40±3%, n=16).

DISCUSSION

Here we show that Gq-coupled receptors regulate IKs in a biphasic manner. Although downstream signaling molecules from GqPCR activation have been suggested to activate the channel [9,10,16,15,30], their physiological role in GqPCR stimulation has not been established. We studied the role of intracellular Ca²⁺-release, PKC phosphorylation and PIP₂ depletion for Gq-coupled receptor regulation of IKs. We show that the activation phase is mediated by PKC phosphorylation and that PIP₂ depletion underlies channel inhibition. This is the first time that evidence for a molecular mechanism is presented to explain Gq-coupled receptor inhibition of IKs. The work we present here is the first to study regulation by Gq-coupled receptors on channels formed by KCNQ1 and KCNE1 subunits in intact cells and suggests that this is an important pathway of heart rhythm regulation.

The kinetics of channel regulation observed in figure 1 and figure 2 for the stimulation of the three receptors is complex. Using a simple model we can simulate the basic kinetic parameters and magnitude of activation and inhibition of the channel with 3 components: one inhibitory component and two activation components (Fig. 6A). The inhibitory component corresponds to PIP₂ depletion, modeled by an exponential inhibition. The activation components correspond to a fast and a slow/delayed activation, modeled by sigmoidal functions. Our data suggest that most of the activation phase is mediated by PKC, but they do not preclude a small contribution from other signaling pathways.

The change in kinetics of activation observed when comparing figure 1 and figure 2 requires the faster activation component to be more prominent in the presence of intracellular Ca²⁺ release (Fig. 6B). Changes in the inhibitory component are not necessary to fit the data, but are not precluded. Nonetheless, activation of endogenous Ca²⁺-dependent Cl⁻ currents were the same for the receptors tested (data not shown), suggesting equivalent Ca²⁺-release upon receptor activation and hydrolysis of PIP₂. For simplicity, the model assumes a full inhibition by PIP₂. A strong inhibition by PIP₂ is necessary to explain the average inhibition observed in the concomitant presence of an activating signal. A complete inhibition is likely not achieved because of activation of lipid kinases in response to PIP₂ depletion.

The lack of activation observed with BK stimulation can be explained by a smaller contribution of the fast activation component for this receptor (Fig. 6A right panel). A decrease in the fast activation component also explains the faster kinetics observed for BK inhibition when compared to ACh and angII in the absence of intracellular Ca²⁺-release (Fig. 6B). The model also explains the slower kinetics of inhibition observed for BK stimulation in the presence of Ca²⁺ when compared to the absence of Ca²⁺ (Fig 6 A and B right panels). Our data does not preclude that the fast-activation component is transient, but that is not necessary to explain the overall data.

We hypothesize that a faster channel response to Ca^{2+} -dependent PKCs occurs in the presence of intracellular Ca^{2+} release. In the absence of intracellular Ca^{2+} -release, a slower response to Ca^{2+} -independent PKCs is more prominent. Our data is consistent with the translocation of the Ca^{2+} -dependent isoforms of PKC being impaired with bradykinin stimulation. Indeed, bradykinin has been shown to activate PKC δ and PKC ϵ but not PKC α in neonatal ventricular cardiomyocytes [31], while angiotensin has been shown to mediate redistribution of PKC ϵ and the Ca^{2+} dependent PKC α and β II in guinea pig heart [32].

PKC is a serine/threonine kinase that has been implicated in many pathological conditions, such as cardiac hypertrophy, arrhythmias, heart failure and ischemic preconditioning [33–37]. Knowledge of the arrhythmogenesis mechanism of PKC is very limited. Use of specific PKC blockers have been suggested as novel treatments for heart failure [36,37]. Channels formed with rat, mouse or human KCNE1 protein together with the endogenous KCNQ subunit in oocytes have been shown to be inhibited by PMA [4,38], a PKC activator. Phosphorylation of the KCNE1 S102 residue, also present in human KCNE1 has been implicated in this effect. More recent experiments on channels formed by human KCNQ1 and KCNE1 subunits showed current activation by PMA. The effect was blocked by the PKC inhibitor calphostin-C [10]. In addition, angiotensin II was suggested to potentiate atrial IKs through activation of PKC [39]. Phosphorylation of the KCNQ1 subunit has also been suggest to be involved in PMA regulation of the IKs channel [40]. Our data does not preclude the phosphorylation of the KCNQ1 subunit, but it suggests that KCNE1(S102) phosphorylation accounts for about half of the PKC-mediated activation observed upon Gq agonist stimulation for the human channel.

Intracellular Ca^{2+} -release controls cardiac contraction. Abnormal release of Ca^{2+} from sarcoplasmic reticulum contributes to contractile dysfunction and arrhythmogenesis in heart failure. In heart failure the sarcoplasmic reticulum has reduced Ca^{2+} content so that the amount of Ca^{2+} released is smaller than under normal conditions [41–43]. Channels formed by co-expression of the KCNQ1 and KCNE1 subunits have been reported to be activated by increases in intracellular calcium with use of the Ca^{2+} ionophore ionomycin [11]. Work done with KCNE1 and endogenous oocyte KCNQ subunits showed that this channel is inhibited by decreases and enhanced by increases in intracellular Ca^{2+} . Phosphorylation of the KCNE1 subunit by calmodulin-dependent kinases were implicated in this effect [4,44] (for review see [45]). However the phosphorylated residue (S40) is not present in the human KCNE1. Recently two papers suggested that basal intracellular Ca^{2+} affects both gating and assembly of IKs channels [16,15]. Our work does not contradict these data, here we studied whether increases of Ca^{2+} above resting level regulate channel gating and does not preclude regulation of channel gating by basal Ca^{2+} . Nonetheless, our data suggests that the activation phase is mediated by PKC and main effect of IP3-dependent Ca^{2+} -release upon GqPCR stimulation is not direct. We suggest that intracellular Ca^{2+} -release regulates PKC-mediated activation, possibly through a Ca^{2+} -dependent PKC isoform. A minor effect of Ca^{2+} -Calmodulin in the early activation phase was not precluded.

The role of PIP_2 as a second messenger molecule has become increasingly important through its modulation of a growing number of ion channels and transporters (for review see [46]). For Kir channels, several stimuli exert their effects through modifying interactions of the channel with PIP_2 [47–49]. Mutations of the inward rectifiers Kir2.1 and Kir1.1 have been shown to cause disruptions in the channel- PIP_2 interactions leading to both Andersen's and Bartter's syndromes [50]. 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 PIP_2 [19,21,12]. KCNQ channels were shown to be activated by PIP_2 and channel run down was linked to PIP_2 hydrolysis [12]. In addition, for channels formed by the KCNQ2 and KCNQ3 subunits, agonist-induced depletion of PIP_2 has been associated

with channel inhibition [12]. PIP₂-dependent rundown/exogenous PIP₂ application was shown to shift the voltage dependence of IKs activation [19]. Our work indicates that PIP₂ depletion, upon Gq-coupled receptor activation, inhibits channel activity.

Understanding the signaling pathways underlying IKs regulation provides insight into the molecular basis of electrical signaling in the normal heart and the pathophysiology of heart failure and long-QT syndrome. Our results suggest that 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 Long-QT syndrome. Understanding IKs regulation may allow us to devise innovative strategies to treat cardiac arrhythmias.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Biphasic modulation of IKs by Gq coupled receptor stimulation

A. Left: Typical response to GqPCR stimulation in oocytes expressing KCNQ1/KCNE1 subunits, before (a), during the activation (b) and inhibition (c) phase. Cells were co-expressed with either M1, AT1A or BK2 receptors. **Right:** time course of the effects. Dashed line represents zero current level. **B. Summary data. Left:** current activation upon application of 10 μ M ACh ($36\pm3\%$, $n=76$), 100nM angII ($41\pm6\%$, $n=21$) or 100nM BK ($1.1\pm0.3\%$, $n=41$). **Right:** current inhibition phase upon application of ACh ($26\pm2\%$, $n=76$), AngII ($44\pm4\%$, $n=21$) or BK ($43\pm3\%$, $n=41$). Activation was calculated as % increase with respect to the initial current. Inhibition was calculated as % decrease with respect to the maximal current.

Figure 2. Block of Ca_i^{2+} -release changes modulation kinetics for GqPCR receptors

KCNQ1/KCNE1 subunits were co-expressed with either M1, AT1A or BK2 receptors as indicated. **A. Left:** typical KCNQ1/KCNE1 current response, before (a), during the inhibition (b) and activation (c) phase. **Right:** time course of the effects. Dashed line represents zero current level. **B. Left:** summary data of stimulation by 10 μM ACh ($143 \pm 13\%$, $n=57$), 100 nM AngII ($95 \pm 19\%$, $n=20$) or 100 nM BK ($102 \pm 28\%$, $n=8$). **Right:** summary data of inhibition by ACh ($37 \pm 2\%$, $n=57$), Ang II ($50 \pm 4\%$, $n=20$) or BK ($52 \pm 4\%$, $n=8$). Inhibition was calculated as % decrease with respect to the initial current. Activation was calculated as % increase with respect to the minimal current.

Figure 3. Current stimulation phase is mediated by PKC

A. Time course of KCNQ1/KCNE1 response to ACh in the presence and absence of PKC inhibitor calphostin-C (2 μ M) in the presence (left) and absence (right) of Ca_i²⁺-release. Dashed line represents zero current level. **B.** Summary data. Activation phase: *Left*: 10 μ M ACh in the presence (control: 48 $\pm$ 7%, n=25; calphostin-C: 9 $\pm$ 4%, n=16), and absence of Ca_i²⁺-release (control: 190 $\pm$ 23%, n=16; calphostin-C: 55 $\pm$ 10%, n=16). *Right*: 100 nM angII in the presence (control: 29 $\pm$ 8%, n=7; calphostin-C: 4 $\pm$ 2%, n=6), and absence of Ca_i²⁺-release (control: 65 $\pm$ 22%, n=5; calphostin-C: 5 $\pm$ 2%, n=4). **C.** Time course of wild-type and KCNQ1/KCNE1(S102A) channel response. Stimulation was inhibited by KCNQ1/KCNE1(S102A) mutation in the presence (left) and absence (right) of Ca_i²⁺-release. Dashed line represents zero current level. **D.** Summary data of stimulation by 10 μ M ACh in the presence (*left*: WT: 26 $\pm$ 4%, n=37; KCNQ1/KCNE1(S102A): 12 $\pm$ 3%, n=40), and absence (*right*: WT: 117 $\pm$ 15%, n=17; KCNQ1/KCNE1(S102A): 60 $\pm$ 12%, n=15) of Ca_i²⁺-release.

Figure 4. Biphasic modulation of voltage-dependence of IKs by ACh

A. Typical IxV protocol of 4 seconds depolarizing pulses from -60mV to $+80\text{mV}$ from -80mV holding potential, followed by a step to -40mV . **B. Left:** Typical tail current IxV before (a, squares), after 3 minutes (b, circles) and 25 minutes (c, triangles) ACh application. Tail currents were measured at -40mV , as indicated, using IxV protocols as in A. G_{Max} and $V_{1/2}$ were calculated from a Boltzmann fit of the data (solid lines). Current is normalized to the initial current. **Right:** Tail currents in B normalized to maximal conductance, showing a typical shift in $V_{1/2}$. **C.** Time course of changes in G_{Max} without (squares) and with $10\mu\text{M}$ ACh application (circles) ($n=9$). **D.** Time course of changes in $V_{1/2}$ without (squares) and with ACh application (circles). **E.** Time course of changes in $V_{1/2}$ in the presence of ACh for KCNQ1/KCNE1 (black) and mutant KCNQ1/KCNE1(S102A) (red) channels. The negative shift in $V_{1/2}$ was significantly smaller for the mutant channel when compared to WT (15min ACh application, WT: 7.9 ± 1.0 , $n=9$ and S102A: 4.1 ± 0.8 , $n=9$; $p<0.001$). **F.** WMN treatment shifts $V_{1/2}$ to positive values in a dose-dependent manner ($n=\text{at least } 19$). Cells were treated with WMN for 1–2 hours at each of the specified concentrations.

Figure 5. PIP₂ depletion mediates the agonist-induced current inhibition phase

A. Left: Typical KCNQ1/KCNE1 current in the absence or after 1 hour treatment with WMN (3 μ M). **Right:** dose-response curve of WMN treatment. Dose response was fit with a Hill equation ($IC_{50}=4.2\pm0.8\mu$ M). **B. Left:** time course of KCNQ1/KCNE1 response to BK (100 nM) in untreated cells (Ctrl) and 15–20 min after WMN (10 μ M). **Right:** Summary data. Inhibition for control ($33\pm5\%$, $n=10$) and WMN treated cells ($70\pm5\%$, $n=13$). **C. Left:** time course of KCNQ1/KCNE1 recovery from inhibition in untreated (Ctrl) and WMN treated cells. Note that the current inhibition was normalized to aid better visualization of recovery. **Right:** summary data. Recovery was significantly reduced in WMN treated ($9.0\pm3.3\%$, $n=12$) when compared to untreated cells ($34\pm6\%$, $n=12$). **D. Left:** time course of KCNQ1/KCNE1 response to BK in cells expressing either PIP5-kinase or its catalytic inactive mutant enzyme (PIP5-kinase Δ 1–238) when compared to control cells. **Right:** Summary data. BK-induced inhibition for control ($38\pm3\%$, $n=31$), cells expressing PIP5-kinase ($22\pm3\%$, $n=32$) and PIP5-kinase Δ 1–238 ($37\pm4\%$, $n=25$).

Figure 6. Schematic model of IKs current regulation by M1, AT1 and BK2 receptors

A. In black an exponential inhibition component and two sigmoidal activation components are depicted. The sum of the three components is the red line. The red line models the extent of inhibition and activation of the current seen in figure 1B. The only differences between the right, left and middle panels are the magnitude of the fast and slow activation components, the inhibitory component is the same. **B.** As in A, the red line is the sum of the three regulatory components (black lines). The kinetics of activation and inhibition of all three components is the same as panel A, only the magnitude of each component is varied. The red line models the extent of inhibition and activation of the channel in the absence of intracellular Ca^{2+} release, measured in figure 2B.