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SCHOOL OF MEDICINE

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MODULATION OF GIRK CHANNELS BY PROTEIN KINASE C

MSc Thesis

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Abstract

Atrial fibrillation is a cardiac arrhythmia that affects 1 in 10 people over 65 years of age. The main characteristic of the disease is an irregular heart rate that can cause blood clots, stroke and heart failure. The etiology of atrial fibrillation has not been completely defined, however, the constitutive activity of the G-protein gated inwardly-rectifying potassium channel GIRK1/4 in atrial myocytes has been implicated. GIRK channels are critical for the maintenance of the resting membrane potential and inhibitory post-synaptic potentials in the body. One of several modulators of GIRK channel activity is protein kinase C (PKC). PKC isozymes are widely reported to inhibit GIRK channel and consist of 14 different isoforms that are divided into conventional, novel and atypical. In atrial fibrillation, an imbalance in the expression of novel PKCε has been reported. An increase in PKCε mediated augmentation of GIRK1/4 activity can explain the constitutive activity of this channel.

This study evaluated the effect of novel PKCε on GIRK4 and GIRK2 homomeric channels as well as GIRK1/4 and GIRK1/2 heteromeric channels and the effect of novel PKCδ on GIRK1/2 and GIRK1/4 heteromeric channels expressed in *Xenopus leavis* oocytes and HEK293T cells using electrophysiological techniques. The study showed that PKCε inhibited both basal and dopamine-induced activity via the D2 receptor of GIRK2 homomeric and GIRK1/2 heteromeric channels. PKCδ also inhibited basal activity and diminished inward currents evoked through dopamine stimulation of D2 receptors in both GIRK1/2 and GIRK1/4 channels. In contrast, PKCε augmented both basal and dopamine-induced activity of GIRK4 homomeric and GIRK1/4 heteromeric channels. Furthermore, HTPDQ a small molecule that acts as a specific inhibitor of GIRK1/4 and GIRK1/2 channels was evaluated. This insight into the mechanism of augmented channel activity via novel PKC phosphorylation will assist in the research for allosteric, small-molecule channel inhibitors that balance the cardiac GIRK1/4 channel activity. Also, the extension of this study on GIRK2 channels can contribute in the development of new therapeutic approaches in diseases of the central nervous system, where GIRK2 channels are abundant.

Τίτλος

«Ρύθμιση των GIRK καναλιών από τις πρωτεΐνικες κινάσες C».

Περίληψη

Η κολπική μαρμαρυγή είναι μια καρδιακή αρρυθμία που προσβάλλει 1 στα 10 άτομα άνω των 65 ετών. Το κύριο χαρακτηριστικό της νόσου είναι ο ακανόνιστος καρδιακός ρυθμός, που μπορεί να προκαλέσει θρόμβους στο αίμα, εγκεφαλικό επεισόδιο και καρδιακή ανεπάρκεια. Η αιτιοπαθογένεια της κολπικής μαρμαρυγής δεν έχει προσδιοριστεί πλήρως, ωστόσο έχει εμπλακεί η συνεχής ενεργοποίηση των GIRK1/4 καναλιών (κανάλι καλίου εσωτερικής ανόρθωσης που ενεργοποιείται από G-πρωτεΐνες) στα μυοκύτταρα των κόλπων της καρδιάς. Τα GIRK κανάλια είναι σημαντικά για τη διατήρηση του δυναμικού ηρεμίας και των ανασταλτικών μετά-συναπτικών δυναμικών στο σώμα. Ένας από τους ρυθμιστές των GIRK καναλιών είναι η πρωτεϊνική κινάση C (PKC). Τα ισοένζυμα PKC αναφέρονται ευρέως ότι αναστέλλουν τα κανάλια GIRK και αποτελούνται από 14 διαφορετικές ισομορφές, που χωρίζονται σε κλασσικές, νεοφανείς και άτυπες. Σε κολπικά μυοκύτταρα καρδιάς ασθενών με κολπική μαρμαρυγή, έχει αναφερθεί αυξημένη έκφραση της νεοφανούς PKCε, που μπορεί να εξηγήσει την αυξημένη ενεργοποίηση των GIRK1/4 καναλιών σε ασθενείς με κολπική μαρμαρυγή.

Αυτή η μελέτη αξιολόγησε την επίδραση της νεοφανούς PKCε στα GIRK2 και GIRK4 ομομερή και τα GIRK1/2 και GIRK1/4 ετερομερή κανάλια, όπως και την επίδραση της νεοφανούς PKCδ στα GIRK1/2 και GIRK1/4 ετερομερή κανάλια, που εκφράστηκαν και μελετήθηκαν σε ωοκύτταρα βατράχου *Xenopus leavis* και HEK293T κύτταρα χρησιμοποιώντας ηλεκτροφυσιολογικές τεχνικές. Η μελέτη έδειξε ότι η PKCε αναστέλλει τόσο τη βασική όσο και την επαγόμενη από ντοπαμίνη δραστικότητα των GIRK2 ομομερών και των GIRK1/2 ετερομερών καναλιών. Η PKCδ, επίσης, αναστέλλει τη βασική και επαγόμενη από ντοπαμίνη δραστικότητα των

GIRK1/2 και GIRK1/4 καναλιών. Αντίθετα, η PKCε αυξάνει τόσο τη βασική όσο και την επαγόμενη από ντοπαμίνη δραστικότητα των GIRK4 ομομερών και GIRK1/4 ετερομερών καναλιών. Επιπλέον, αξιολογήθηκε το HTPDQ, ένα μικρό μόριο που δρα ως ειδικός αναστολέας των καναλιών GIRK1/4 και GIRK1/2. Η μελέτη του μηχανισμού που οφείλεται για την αυξημένη ενεργοποίηση των GIRK καναλιών μέσω φωσφορυλίωσης από νεοφανείς PKC, θα συμβάλει στην έρευνα για την ανάπτυξη αλλοστερικών μικρών μορίων, που δρουν ως αναστολείς των GIRK1/4 καναλιών και εξισορροπούν την καρδιακή δραστηριότητα τους. Επίσης, η επέκταση αυτής της μελέτης στα GIRK2 κανάλια μπορεί να συμβάλει στην ανάπτυξη νέων θεραπευτικών προσεγγίσεων σε ασθένειες του κεντρικού νευρικού συστήματος, όπου και εκφράζονται κυρίως τα GIRK2 κανάλια.

Introduction

1. Kir channels

Inwardly rectifying potassium channels (Kir) are a family of K^+ channels that are crucial for the maintenance of the resting membrane potential and inhibitory post-synaptic potentials in the body. These channels prefer to conduct K^+ ions into the cell (inward) rather than out of the cell (outward) giving them the characteristic of "inward rectification". Yet, the direction of the K^+ ions under physiological conditions is outward, serving to hyperpolarize cells and retard excitability. The equilibrium potential (E_K) of Kir channels that refers to the voltage at which the channel conducts no net current in either direction, is very close to the resting membrane potential of the cell. The rectification is due to the cytosolic Mg^{2+} and polyamines, which reduce the outward movement of potassium ions at potentials above E_K (Figure 1A and Yamada *et at.,* 1998). There are 15 Kir subunit genes classified into seven sub-families of inwardly rectifying K^+ channels (Kir1.x to Kir7.x) and four functional groups: a) classical Kir channels (Kir2.x), b) G protein-gated Kir channels (Kir3.x), c) ATP-sensitive K^+-channels (Kir6.x), and d) K^+-transport channels (Kir1.x, Kir4.x, Kir5.x, and Kir7.x) (Figure 1B and Hibino *et al.,* 2010).

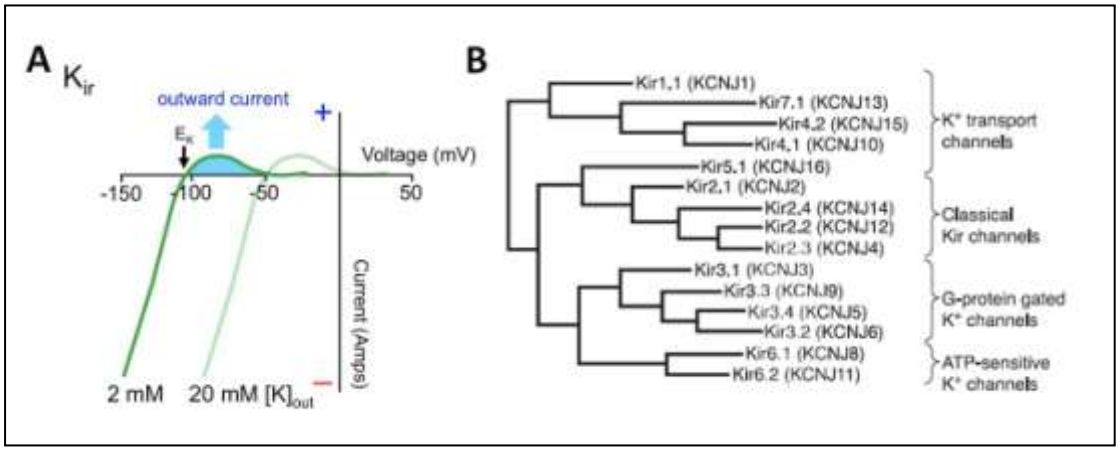


Figure 1: Kir channels function and classification.

A. *Current-voltage relationship shows the property of Kir channels. Cells with a membrane potential that is positive to equilibrium potential (E_K) of the channels will conduct outward potassium current though that will decline substantially at more positive potentials. The gating of the channel shifts with increasing extracellular K^+ concentration.* ***B.*** *The four functional groups of Kir channels: a) classical Kir channels (Kir2.x), b) G protein-gated Kir channels (Kir3.x), c) ATP-sensitive K^+-channels (Kir6.x), and d) K^+-transport channels (Kir1.x, Kir4.x, Kir5.x, and Kir7.x) (Adopted from Lüscher & Slesinger, 2010; Hibino et al., 2010).*

1.1. GIRK channels composition and distribution

The G protein-gated inwardly rectifying potassium (GIRK) channels dysfunction has been linked to a variety of conditions from atrial fibrillation to pancreatic and renal abnormalities as well as epilepsy and drug addiction (Pattnaik *et.al.,* 2012, Rifkin *et al.,* 2018). Mammalian GIRK channels are composed of four, conserved, homologous subunits: GIRK1, GIRK2, GIRK3 and GIRK4 (Luján & Aguado, 2015). These subunits can form homotetrameric or heterotetrameric channels depending on the expression pattern of each tissue. GIRK1 and GIRK3 can only exist as heterotetrameric channels with the other GIRK subunits, in contrast to GIRK2 and GIRK4 that can form both homotetramers and heterotetramers (Logothetis *et al.,* 2015). GIRK channels found in the brain are largely consisting of GIRK1/2 heterotetramers, while in the ventral tegmental area are expressed mainly GIRK2/3 heterotetramers. GIRK2 homotetramers are primarily expressed in the substantia nigra of the brain (Inanobe *et.al.,* 1999), while the GIRK4 subunit is primarily found in cardiac tissue, coupled with GIRK1 to form GIRK1/4 channels (Luscher & Slesinger, 2010). These channels give rise to current known as IK_{ACh} since it is mediated by acetylcholine signaling in the atria. Also, GIRK4 homotetramers are predominantly expressed in atrial tissue in the heart (Corey & Clapham, 1998). Apart from the ability to form homomeric channels, GIRK2 and GIRK4 subunits are also homologous with a sequence identity of almost 69% (Spauschus *et.al.,* 1996). There are four splice variants of GIRK2, termed GIRK2a–d, of that GIRK2a–c are expressed in the brain and GIRK2d in the testes. The brain isoforms differ in the composition and length of their respective carboxy-terminal domains (Inanobe *et al.,* 1999; Isomoto

et al., 1996; Lesage *et al.,* 1994, 1995; Wei *et al.,* 1998; Wickman, Pu, & Clapham, 2002).

1.2. GIRK channel activation

GIRK channels exist in a macromolecular signaling complex with a G-protein coupled receptor (GPCR) and heterotrimeric G-protein (Riven *et al.,* 2006). In 1987, IK_{ACh} channels were the first effector proteins found to be activated by the βγ subunits of G-proteins (Gβγ), rather than the Gα subunits (Logothetis *et al.,* 1987). That conclusion was immediately questioned (Codina *et al.,* 1987), but now Gβγ subunits are accepted as the stimulatory component of GIRK channels (Logothetis *et al.,* 1987; Ito *et al.,* 1992; Logothetis *et al.,* 1988; Reuveny *et al.,* 1994; Wickman *et al.,* 1994). Pertussis toxin-sensitive Gi/o-linked receptors are activated by various neurotransmitters, such as acetylcholine, serotonin, dopamine, opioids, somatostatin, adenosine, GABA and can cause the opening of GIRK channels through their canonical G-protein signaling pathways (Luscher & Slesinger, 2010). After an agonist binds to its GPCR, it promotes the exchange of GDP for GTP on the bound Gi/o protein. This causes the dissociation of G-protein from the receptor and subsequently the dissociation of Gα and Gβγ subunits from each other. Then, the Gβγ interacts with the GIRK channels resulting in channel activation. Hydrolysis of GTP to GDP by GTPase activity of Gα and the rebinding of the Gβγ dimer with the Gα-GDP leads in termination of G-protein signaling and closing of GIRK channels (Figure 2 and Dascal & Kahanovitch, 2015). GIRK channels can also be activated *in vitro* by overexpression of Gβγ released from Gαs coupled receptors, while this does not occur physiologically (Hatcher-Solis et al., 2014, Touhara & MacKinnon, 2018).

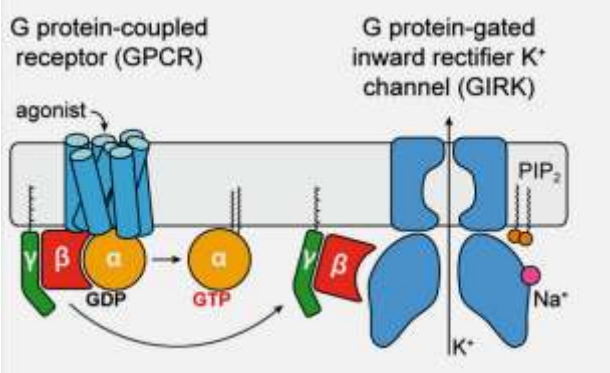


Figure 2: Schematic representation of G-protein coupled receptor (GPCR) activation of GIRK channels.

Agonist binding to a GPCR leads to the exchange of GDP for GTP on the bound Gi/o protein. This promotes the dissociation of G protein from the receptor and subsequently the dissociation of Gα and Gβγ subunits from each other. Then, the Gβγ interacts with the cytoplasmic domain of GIRK channels and in the presence of the phosphoinositide PIP2 results in channel activation. GIRK channels are also activated by elevated levels of intracellular Na^+ ions in the co-presence of PIP2 (Adopted from Whorton & MacKinnon, 2013).

1.3. Gating of GIRK channels

In 1998, two different publications showed evidence that stimulation of G-protein-sensitive channels are dependent on the phosphoinositide PIP2 (Huang *et al.,* 1998; Sui *et al.,* 1998). PIP2 is found in the inner leaflet of the plasma membrane and together with its precursor, PI(4)P, forms about 0.5-1% of the pool of plasma membrane phospholipids (Logothetis *et al.,* 2015). Like most other ion channels, all channels in the Kir family require interactions with PIP2 for activity. Increase in the membrane PIP2 interactions with Kir channels leads to increase in channel activity (Logothetis *et al.,* 2010).

There are channels like Kir2 constitutively active, because they engage in high affinity interactions with PIP2 and require only PIP2 for channel activity. Although, GIRK channels need for their activation supplementary gating molecules to PIP2, such as Na^+ ions, Mg^{2+} ions, Gβγ, or alcohols. Both the Gβγ subunits and the Na^+ ions were shown to increase the channel affinity for PIP2, proposing that they gated the channel by enhancing channel–PIP2 interactions (Huang *et al.,* 1998; Sui *et al.,* 1996; Sui *et al.,* 1998). Several basic residues of GIRK channels have a crucial role to channel-PIP2 interactions**.** More specifically, distinct positively charged residues on Kir channels form electrostatic interactions with the negatively charged phosphate residues on PIP2 (Logothetis *et al.,* 2015b; Whorton *et al.,* 2011).

GIRK channels are modulated by Gβγ, alcohol, Na^+ ions, protein kinase A (PKA), H2S, and protein kinase C (PKC) to name a few. Gβγ, alcohols (Logothetis *et al.,* 1987,

Reuveny *et al.,* 1994; Kobayashi *et al.,* 1999; Lewohl *et al.,* 1999), PKA (Rusinova *et al.,* 2009) and Na^+ ions (Ho & Murrell-Lagnado, 1999; Zhang *et al.,* 1999) enhance channel-PIP2 interactions resulting in activation of the channel. H2S inhibits GIRK activity by blocking channel-PIP2 interactions through sulfhydration of cytosolic cysteine residues (Ha *et al.,* 2018). PKC also has an inhibitory effect on GIRK activity in a PIP2-dependent manner (Keselman *et al.,* 2007).

It has been shown since the 1970s that the activation of phospholipase C (PLC) through Gq signaling results in hydrolysis of PIP2 into inositol trisphosphate (IP3) and diacylglycerol (DAG). The two hydrolysis products of PIP2 serve as important intracellular signals: IP3 binds and opens ligand-gated Ca^{2+} channels, the IP3R, leading to the release of calcium from endoplasmic reticulum stores, while DAG is responsible for the activation of membrane or membrane-associated proteins, such as protein kinase C (PKC) (Figure 3 and Nishizuka, 1984).

PLC activation by Gq-coupled receptors leads to the inhibition of GIRK channel in two ways: through PIP2 depletion and channel phosphorylation by PKC (Keselman *et al.,* 2007).The modulation of potassium channels by PKC appears to be manifested via channel-PIP2 interactions. The Kir2.1 channel (IRK1) has high affinity for PIP2 and it does not need additional molecules for its activation. Hence, Du *et al.* (2004) showed a minimal modulation of Kir2.1 (IRK1) by PKC due to its strong affinity for PIP2. Although, using a Kir2.1 mutant that decreases channel–PIP2 affinity, they demonstrated a Kir2.1 channel inhibitory effect by PKC modulation. Similarly, Keselman *et al*. (2007) reported that the regulation of GIRK channels by PKC is PIP2-dependent. They demonstrated an augmented inhibition of the channel upon depletion of the membrane PIP2 and a decreased inhibition of the channel upon augmenting PIP2 content using PIP5-K. However, the PKC family includes a variety of isoforms that the previous studies did not assess, thus we should be cautious in generalizing the PKC- mediated inhibitory phenotype prior to addressing the effects of additional PKC isoforms.

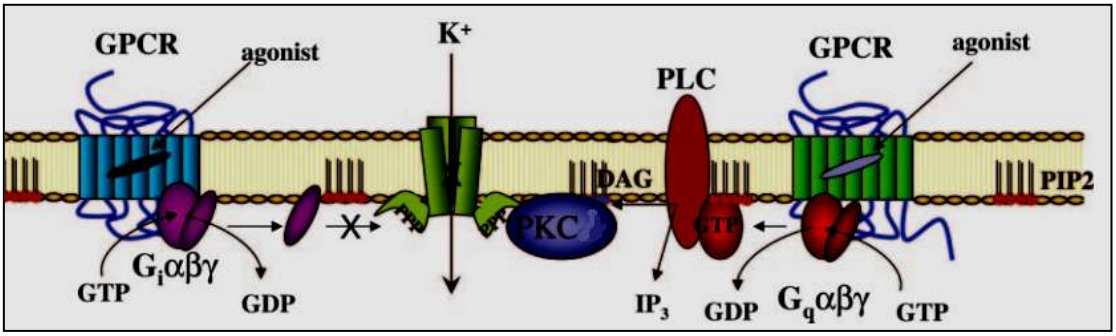


Figure 3: Mechanism for Gq-mediated inhibition of GIRK channels.

The activation of phospholipase C (PLC) through Gq signaling results in hydrolysis of PIP2 into inositol trisphosphate (IP3) and diacylglycerol (DAG). DAG is responsible for the activation of membrane or membrane-associated proteins, such as protein kinase C (PKC). Both the PIP2 depletion as well as PKC mediated channel phosphorylation can diminish channel activity (Adopted by Breitwieser et al., 2005).

1.4. Structure of GIRK channels

The basic building block of all Kir channels consists of a common motif of two putative membrane-spanning domains M1 and M2 linked with an extracellular pore-forming region, known as H5, and cytoplasmic amino- and carboxy- terminal domains. The H5 region serves as the "ion-selectivity filter" that contains the signature sequence T-X-G-Y(F)-G (Heginbotham *et al.,* 1994; Bichet *et al.,* 2003). The transmembrane domain is composed of outer (M1) and inner (M2) membrane spanning helices, with two short additional helical elements, the slide and the pore helices. The channel pore is surrounded by one M2 helix from each of the four Kir subunits. The ion conduction pore can be functionally separated into three distinct zones: the selectivity filter, a water-filled central cavity, and the internal face of the pore made up of the internal bases of the four inner M2 helices. The K^+ channel signature sequence [T-X-G-Y(F)-G] serve as the selectivity filter. This makes a narrow region in the ion conduction pathway that separates the central cavity from the extracellular solution. The central cavity is a 10-A° spherical water-filled space about halfway through the membrane. The four M2 helices one from each subunit, come together to make one more narrow region at the cytoplasmic face of the channel (Hibino *et al.,* 2010).

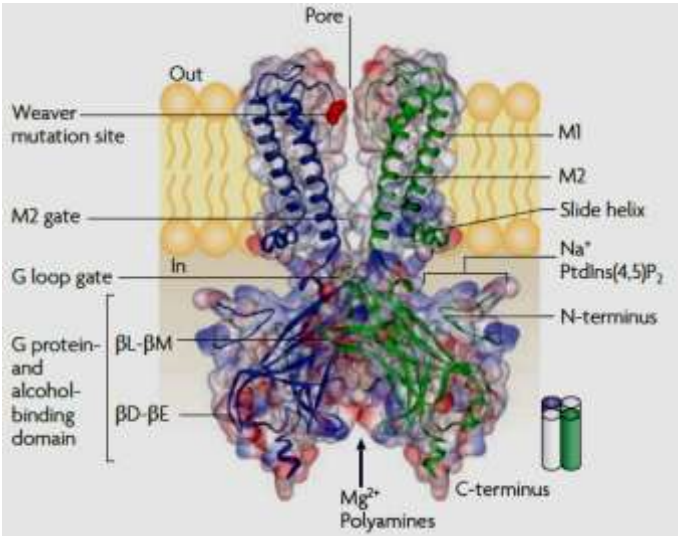


Figure 4: Structural insights of GIRK channels.

*A structure of a chimeric channel comprised of the bacterial inwardly rectifying K^+ channel Kirbac1.3 and the GIRK1**,** shown in the lipid bilayer. This structure contains the cytoplasmic domains (amino- and carboxy- terminal) of GIRK1, and the transmembrane domains (M1 and M2) and pore region of Kirbac1.3. The figure depicts the K^+ selectivity filter (labeled as the weaver mutation site) and the general region implicated in Na^+ and phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2 or PIP2) association. Two channel gates comprising the M2 transmembrane domain and cytoplasmic G-loop form physical barriers to ion permeation forming constrictions referred to as gates. The cylinder cartoon depicts the two opposing units (Adopted from Lüscher & Slesinger, 2010).*

A three dimensional structure of a full-length chimeric channel comprised of the bacterial inwardly rectifying K^+ channel Kirbac1.3 and the GIRK1 was solved in 2007 by Nishida and MacKinnon. This structure contains the cytoplasmic domains (amino- and carboxy- terminal) of GIRK1, the transmembrane domains (M1 and M2) and pore region of Kirbac1.3. Also, the structure reveals the relative positions of the pore (selectivity filter), two conformations of the G-loop gate, the M2 gate, and the cytoplasmic interaction sites (Figure 4 and Nishida *et al.,* 2007).

The GIRK channel has a conserved secondary structure consisting of 14 β-strands and 2 α-helices. These cytoplasmic structures revealed the unique structure in Kir channels, the G-loop, which is formed by the βH–βI sheet (Pegan *et al.,* 2005) and plays an important role in channel gating (Pegan *et al.,* 2005; Chang *et al.,* 2009; Ma *et al.,* 2007). The GIRK channel gating involves the amino-terminal slide helix, the

binding sites for K^+ and polyamines (Nishida *et al.,* 2002; Pegan *et al.,* 2005; Tao *et al.,* 2009; Xu *et al.,* 2009; Peganet *et al.,* 2006), as well as the movement of the M2 and G-loop gates (Tao *et al.,* 2009; Xu *et al.,* 2009; Pegan *et al.,* 2006; Nishida *et al.,* 2007; Kuo *et al.,* 2003; Osawa *et al.,* 2009).

Functional and biochemical studies have identified sites in the amino-terminal and carboxyl-terminal domains of GIRK channels that are involved in Gβγ activation and Gα-binding (Huang *et al.,* 1995; Kunkel et al., 1995; Huang *et al.,* 1997; He *et al.,* 1999; Krapivinsky *et al.,* 1998; Ivanina *et al.,* 2003; Finley *et al.,* 2004; Rubinstein *et al.,* 2009). Specifically, a Leu residue (GIRK1-L333, GIRK2-l344 and GIRK4-l339) in the carboxyl-terminal domain of GIRK channels (βL–βM loop) has a crucial role in the Gβγ-dependent activation of GIRK channels (He *et al.,* 1999; Ivanina *et al.,* 2003; Finley *et al.,* 2004)**.** Additionally, systematic mutagenesis studies have shown that specific amino acids in the M2 transmembrane domain (Yi *et al.,* 2001; Sadja *et al.,* 2001; Jin *et al.,* 2002) and G-loop (Pegan *et al.,* 2005; Chang *et al.,* 2009; Ma *et al.,* 2007) form a barrier to ion conduction in the closed state. These conformational changes triggered in the cytoplasmic domains couple to the channel gates to open the channel.

The high-resolution structures of GIRK channels have also enabled visualization of the amino acids implicated in PIP2 binding, as well as the possible gating mechanisms for modulation by ethanol and Na^+. A group of basic amino acids of the cytoplasmic domain that are positioned close to the plasma membrane showed to interact directly with the negatively charged membrane PIP2 and to couple with the two gates, M2 and G loop (Lopes *et al.,* 2002; Huang *et al.,* 1998; Zhang *et al.,* 1999; Whorton and MacKinnon, 2011, 2013). A hydrophobic pocket formed by the amino-terminal domain and the carboxyl-terminal βD–βE and βL–βM loops has been identified as a site for ethanol-dependent activation of GIRK channels (Aryal *et al.,* 2009)**.** A structural analysis of GIRK2 has also provided a possible mechanism for Na^+-dependent regulation of GIRK channels, indicating that Na^+ promotes PIP2 binding to GIRK channels by breaking a hydrogen bond formed between an Asp residue and an Arg residue in the βC–βD sheet (Inanobe *et al.,* 2007; Rosenhouse-Dantsker *et al.,* 2008).

2. PKC enzymes

2.1. Classification and structure

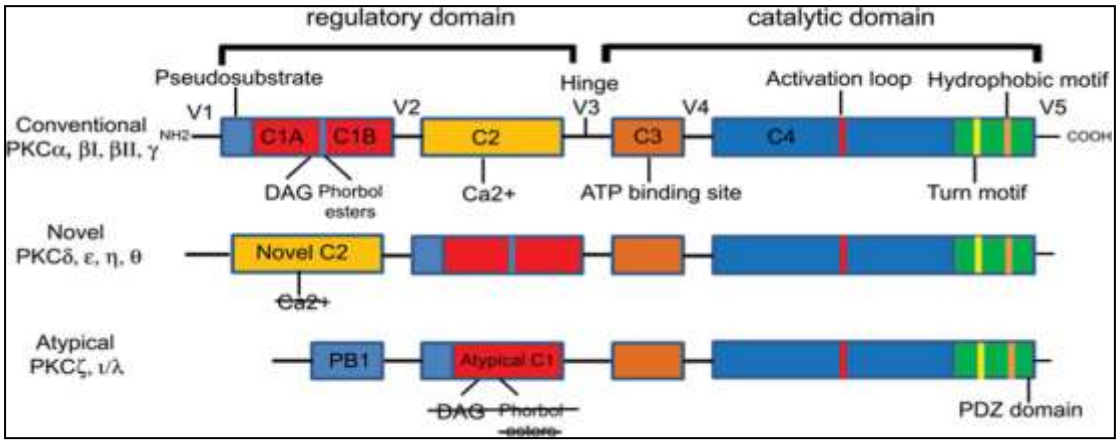


Figure 5: Schematic representation of the enzymatic structure of the three groups of PKC. *PKC isozymes are characterized by conserved regions (C1–C4 domains) spaced out by variable regions (V1–V5) and they are contained by a catalytic (C3-C4) and regulatory region (C1-C2). The regulatory subunit of conventional PKC (α, βI, βII, and γ) contains a C1 region that binds to diacylglycerol (DAG) and a Ca^{2+} sensing, C2 region. The novel PKC (δ, ε, η, and ϑ) do not need Ca^{2+} for activation and unlike conventional PKC, lack the Ca^{2+} sensing ability through the C2 domain. However, novel PKC have a higher affinity for DAG and are activated by the binding of DAG to the conventional-like C1 domain. The atypical PKC* (ι, λ) *are insensitive to DAG and Ca^{2+} and can be activated by phosphatidylserine (Adopted from Poli et al., 2014).*

Protein kinase C (PKC) enzymes were identified about 30 years ago by Nishizuka and collaborators as kinases that are activated by proteolysis (Takai *et al.,* 1977)**.** PKC enzymes belong to a family of serine/threonine kinases that contribute in signal transduction via the second messenger diacylglycerol (DAG) (Azzi *et.al.,* 1993). All of these isoenzymes are characterized by conserved regions (C1–C4 domains) spaced out by variable regions (V1–V5) and they are contained by a catalytic and regulatory region. The catalytic region resides in the carboxy-terminal of PKC and contains a conserved ATP and magnesium-binding site, as well as a binding site for the phosphoacceptor sequence in the substrate proteins. The regulatory region is in the amino-terminal half of the enzyme and composed of C1 and C2 domains. In the

inactive form, the regulatory and catalytic region are bound to each other and that inhibits the activity of the enzyme. The dissociation of these regions from each other results in activation of the enzyme (Figure 5 and Mochly-Rosen *et al.,* 2012).

The greatest homology among the PKC isozymes is in the catalytic region and differ more from each other in the C2 domain of the regulatory domain and intervening (V) domains. Based on their structural and functional properties, the PKC family is divided into conventional, atypical and novel PKC isozymes (Nishizuka, 1995). The regulatory subunit of conventional PKC (α, βI, βII, and γ isoforms) includes a C1 region that binds to DAG and a C2 region that senses Ca^{2+}. Conventional PKC can be activated by DAG, Ca^{2+} and phosphatidylserine (PS). Also, these isozymes are thought to be activated after Ca^{2+} binds to the C2 region and increases its affinity for PS at the cell surface, allowing the binding of DAG for full activation of the enzyme (Kheifets & Mochly-Rosen, 2007). The novel PKC (δ, ε, η, and θ) are activated by the binding of DAG to the conventional-like C1 domain and are not activated through Ca^{2+}, because they lack the Ca^{2+} sensing ability of C2 domain. The atypical PKC (ι, λ) can be activated by phosphatidylserine and are insensitive to DAG and Ca^{2+} and (Figure 5 and Kheifets & Mochly-Rosen, 2007).

2.2. Activation of PKC

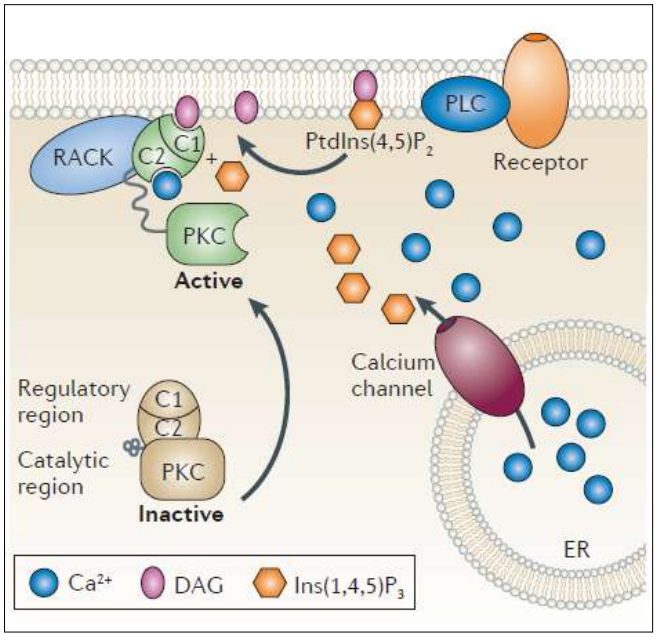


Figure 6: Schematic representation of the activation of the PKC.

Gq-linked receptor activation leads to the activation of phospholipase C (PLCβ1) resulting in hydrolysis of PIP2 into inositol trisphosphate (IP3) and diacylglycerol (DAG). Both novel and conventional PKC isozymes are activated by DAG, but conventional PKCs also require Ca^{2+} for their activation. The Ca^{2+} needed for conventional PKCs is released from the endoplasmic reticulum after binding of IP3 to ligand-gated Ca^{2+} channels on the ER surface. After the activation of PKC, PKC bound to the receptor of activated C-kinase (RACK) phosphorylates several nearby substrates, leading to diverse cellular responses (Adopted from Mochly-Rosen et al., 2012).

PKC isozymes are activated through Gq-linked receptors that can be triggered by a variety of neurotransmitters such as dopamine and endorphin, hormones such as adrenaline and angiotensin as well as growth factors, such as epithermal growth factor and insulin. When these factors bound to their respective receptors, activate phospholipase C (PLCβ1) resulting in hydrolysis of PIP2 into inositol trisphosphate (IP3) and diacylglycerol (DAG), both of which act as second messengers. Moreover, PLCβ2/3 can be activated by the Gβγ subunit of Gi-coupled receptors, leading to the hydrolysis of PIP2. The four novel PKC isozymes are activated by DAG alone, whereas the four conventional isozymes also require Ca^{2+} for their activation, as was mentioned earlier. The Ca^{2+} needed for the activation is released from the endoplasmic reticulum after binding of IP3 to ligand-gated Ca^{2+} channels there. Therefore, a single event, the receptor-dependent PLC activation, leads to the generation of the two second messengers that are required to activate both conventional and novel PKC enzymes (Figure 6 and Mochly-Rosen *et al.,* 2012).

Activation can also occur in the absence of second messengers. High levels of Ca^{2+} can directly activate PLC resulting in PKC activation in the absence of receptor activation. Additionally, many post-translational modifications of PKC lead to the activation of selected PKC isozymes (Persaud *et al.,* 2005). These modifications include activation by proteolysis between the regulatory and catalytic domain, which was noted to occur for PKCδ (Steinberg, 2008). Phosphorylation of various sites plays an important role in the maturation of the newly synthesized enzyme (Newton, 1995) and in activation of mature isozymes: for example, H_2O_2 induces tyrosine phosphorylation of PKCδ (Konishi, H. *et al.,* 1997; 2001). Some other modifications

that have been found to activate PKC are oxidation, acetylation and nitration (Persaud *et al.,* 2005).

Moreover in 1982, it was found that PKC activation is always associated with translocation of the enzyme from the cytosol to the cell particulate fraction that includes the plasma membrane and many other cellular organelles (Mochly-Rosen, 1995). There each isozyme interacts with its anchoring protein, receptor of activated C-kinase (RACK). After the activation of PKC from DAG (and Ca^{2+} for the conventional PKC isozymes), PKC bound to RACK, activates and phosphorylates several nearby substrates, leading to diverse cellular responses (Figure 6 and Mochly-Rosen *et al.,* 2012).

2.3. PKC in disease

Increased activation of selected PKC isozymes has been observed in cancer (Toton *et al.,* 2011; Michie & Nakagawa, 2005), diabetes (Ishii *et al.,* 1996), ischaemic heart disease (Simonis *et al.,* 2003; Bowling *et al.*1999), acute and chronic heart disease (Palaniyandi *et al.,* 2009), heart failure (Bowling *et al.,* 1999; Dempsey *et al.,* 2007) lung (Dempsey *et al.,* 2007) and kidney (Li & Gobe, 2006; Tuttle, 2008) diseases, various dermatological diseases including psoriasis (Maioli *et al.,* 2010), as well as autoimmune diseases (Zanin-Zhorov,2011; Varin *et al.,* 2012). Also, increased PKC activation has been implicated in psychiatric diseases, including bipolar disorder (Manji & Lenox, 2000), and in several neurological indications such as stroke (Bright & Mochly-Rosen, 2005)**,** Parkinson's disease (Burguillos *et al.,* 2004; Zhang *et al.,* 2007)**,** dementia (Sun & Alkon, 2004), Alzheimer's disease (Garrido *et al.,* 2007; Alkon *et al.,* 2007) and pain (Sweitzer *et al.,* 2004)**.** Initially, PKC was implicated in various illnesses owing to abnormal activation or levels of a particular PKC isozyme in the disease state.

Focusing in heart diseases, studies with transgenic mice with cardiac ischemia showed that activation of PKCδ and PKCε has opposing effects on the ischaemic myocardium (Murriel & Mochly-Rosen, 2003). While PKCε activation has a cardioprotective effect (Inagaki *et al.,* 2006), PKCδ activation mediates much of the acute injury induced after transient myocardial ischaemia (Chen *et al.,* 2001;

Churchill *et al.,* 2010). The effect of the opposing outcomes of these two isozymes has been attributed to their regulation of the mitochondrial function. The activation of PKCε protects the proteasome from inactivation leading to fast and selective degradation of the damaging PKCδ at reperfusion. This alters the balance between PKCδ and PKCε in the direction of cardioprotection. Protection of the proteasome also improves cardiomyocyte health by enabling the removal of oxidized and damaged proteins (Churchill *et al.,* 2010)**.**

PKCα is also involved in the progression of heart failure (Braz *et al.,* 2004) and plays a role in cardiac remodeling (Song *et al*., 2015). In atrial myocytes of atrial fibrillation patients a down-regulation of PKCα and an up-regulation of PKCε have been reported (Figure 7 and Voigt *et al.,* 2007). Also in patients with atrial fibrillation an increase in the membrane localization of PKCε has been reported (Makary *et al.,* 2011). The imbalance of PKC isoform expression and localization is thought to be important in atrial fibrillation pathology.

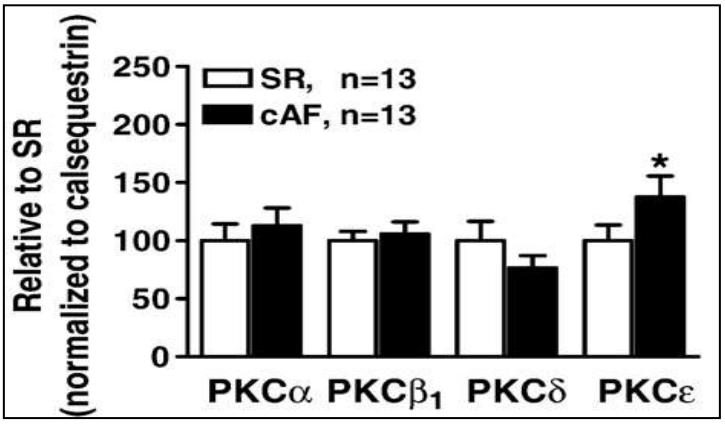


Figure 7: The expression of PKCε is upregulated in the atrial myocytes of a canine model of atrial fibrillation (Adopted from Voigt et al., 2007).

3. Atrial Fibrillation

Atrial fibrillation (AF) is a disorder in which the heart's electrical conduction system is disrupted, leading to a fast and irregular heart rhythm (Lip *et al.,* 2016). One-third of all patients discharged with arrhythmia have AF, affecting 6% of people over 65 years of age, and 10% of those over 80 years of life. It has been associated

with an increased risk of mortality, stroke and peripheral embolism and it might be generated through genetic predisposition, altered regulation by neurohormonal factors like autonomic imbalance and abnormal thyroid function or cardiac remodelling caused by heart disease (Vizzardi *et al.,* 2014). One of the first diagnostic tools used AF is an electrocardiogram (ECG). The cardiac ECG features show complete irregular QRS complexes with absence of P-waves and fine fibrillation waves in the baseline (Lip *et al.,* 2016).

The sinoatrial node is the physiological pacemaker of the heart that produces the normal heart rhythm (sinus rhythm). The normal impulse spreads from the sinoatrial node through the atria traveling through the conducting system formed of Purkinje fibres. The Purkinje fibres transmit the impulse through the atrioventricular node, the space between the atria and the ventricular conducting system to reach the ventricles leading to the cardiac contraction. In AF, the atrial activity is very rapid and irregular. There is no coordinated atrial contraction and the response of the ventricles is irregular and often rapid, depending on the filtering effect of the atrioventricular node (Lip *et al.,* 2016).

The pathobiological mechanisms of AF are unknown, but they are thought to involve cardiac fibrosis and remodeling that change the way electrical impulses are propagated through the heart. This may result in disorganized stimulation of myocardium and subsequent arrhythmic contractions. The uncoordinated activity of the heart can be caused by rapidly discharging foci or by re-entrant activity. AF generally requires a trigger to be initiated, which is typically a focal spontaneous firing. Most commonly arising triggers are generated from pulmonary veins, but there are also non-pulmonary vein triggers (Lip *et al.,* 2016).

The most important triggered activity is a result of 'delayed afterdepolarizations' (DADs) (Heijman *et al.,* 2014). DADs are abnormal spontaneous diastolic depolarizations that take place during phase 4 in cardiomyocytes with the end of normal action potential repolarization. DADs are originated by abnormal diastolic Ca^{2+} release from the sarcoplasmic reticulum via a Ca^{2+} release channel. An alternative mechanism has also been proposed as triggered activity in AF. The early afterdepolarizations (EAD) can generate spontaneous extra-systolic activity. These are particularly likely to occur in the presence of the strong simultaneous discharge

of vagal and sympathetic nerves. Vagal discharge shortens the action potential length and consequently the refractory period, which is the minimum time required for reactivation following the depolarization phases of an action potential when the cell membrane becomes inexcitable. This enhances the probability of spontaneous depolarizations, which is caused by increased Ca^{2+} release from the sarcoplasmic reticulum that depolarize the cell causing an EAD during phase 3 and trigger a spontaneous action potential (Chen *et al.,* 2014; Burashnikov *et al.,* 2003; Patterson *et al.,* 2005).

3.1. Role of GIRK channels in atrial fibrillation

As mentioned earlier, GIRK4 homomers as well as GIRK1/4 heteromers are expressed in atrial tissue of the heart (Stanfield, 2007). GIRK1/GIRK4 channels are activated by acetylcholine stimulation of the muscarinic M2 receptor (IK_{ACh}), resulting in vagal decrease in heart rate (Heijman *et al.,* 2017). The activation of heart GIRK channels mediated via Gβγ dissociation from the Gi-protein heterotrimer. Patients with AF have a constitutive increase in IK_{Ach}, leading to the opening of the channels even in the absence of acetylcholine stimulation. This causes the early repolarization of the myocytes by promoting shorter duration of the action potential (Kovoor *et al.,* 2001; Dobrev *et al.,* 2005).

The enhanced activity of GIRK channels can be explained by both ATP dependent effects and by phosphatase inhibitors (Makary *et al.,* 2011). The phosphatase inhibitors are indicative of a kinase-mediated activity. Makary *et al.* (Figure 8, 2011) demonstrated the effects of different PKC isoforms on cardiac GIRK channel activity. They used inside-out patches of AF atrial myocytes to show that the application of conventional PKC isoforms (PKCα, PKCβI, and PKCβII) inhibits GIRK channel activity. However, GIRK channel activity was enhanced in the presence of novel PKC isoforms (PKCε and PKCδ). The opposite effects of conventional versus novel PKC isoforms on cardiac GIRK channels indicate that the channels are probably phosphorylated by the two PKC isoforms (conventional PKCα and novel PKCε) at different amino acid residues and the mechanism has not been proposed elucidated.

GIRK4 knockout animals cannot form IK_{Ach} (Mesirca *et al.,* 2013) and do not develop AF (Kovoor *et al.,* 2001). Also, GIRK4 or GIRK1 knockout mice have no heart rate variability (HRV) (Bettahi *et al.,* 2002; Lee *et al.,* 2018; Wickman *et al.,* 1998) a crucial characteristic of cardiac health that endows adaptability of each individual and corresponds to high vagal tone. HRV has been shown to be controlled by the GIRK4 homomers and GIRK1/4 heteromers in the atria (Lee *et al.,* 2018).

The balanced activity of GIRK4 homomers and GIRK1/4 heteromers in the atria is critical for both the maintenance of normal heart rate and HRV. If the activity of cardiac GIRK channels fully blocked by IK_{Ach} inhibitors, the AF symptoms would be cured, but that would also decrease HRV and compromise cardiac health. For that reason, the therapeutic goal would be the design of small molecules that reverse the GIRK over-activity.

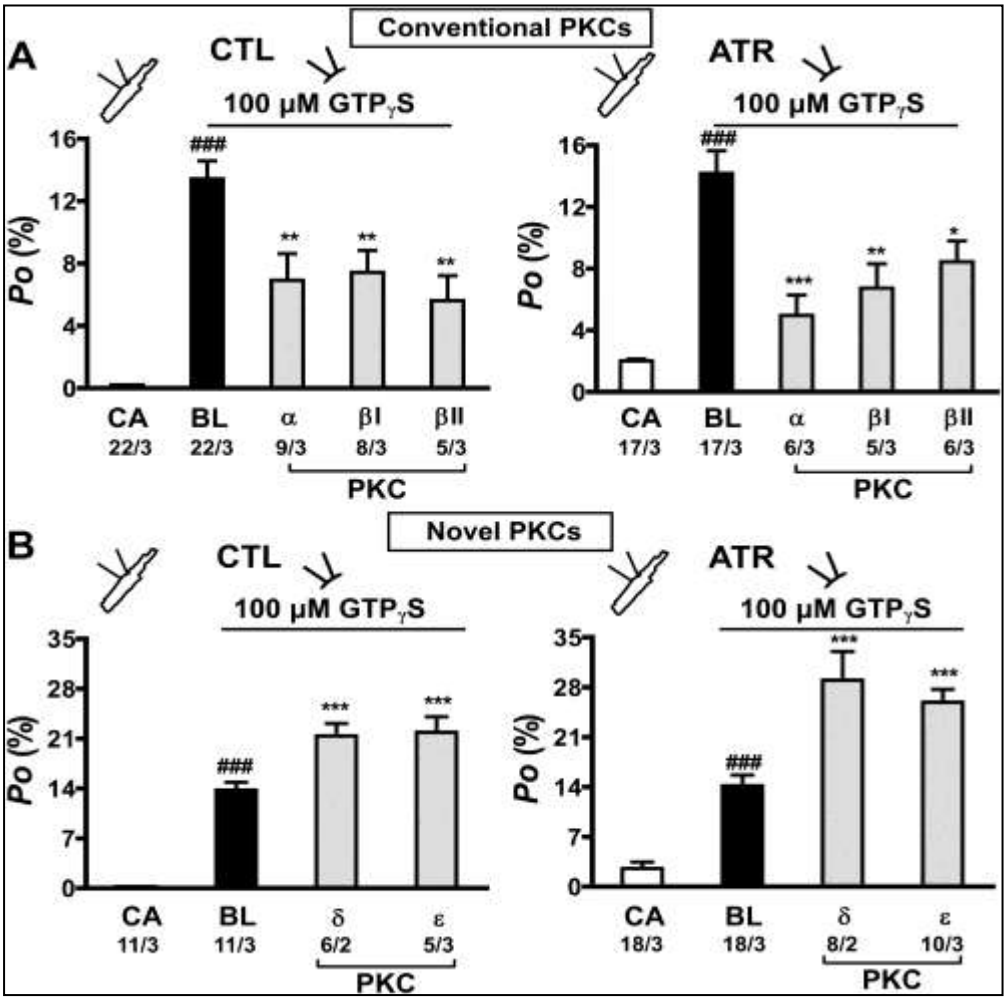


Figure 8: Effect of PKC isoforms on IK_{ACh} current.

The application of conventional PKC isoforms PKCα, PKCβI and PKCβII, to the intracellular side of inside-out, atrial myocyte patches caused an inhibition GIRK channel current in atrial

fibrillation myocytes. Under the same experimental conditions, a significant increase in channel activity was demonstrated in the presence of novel PKC, PKCε and PKCδ (Adopted from Makary et.al., 2011).

3.2. Atrial fibrillation drug development

Antiarrhythmic drugs (AADs) are been used for AF treatment and their goal is the maintenance of normal sinus rhythm (SR) or the control of ventricular rate (El-Haou *et al.,* 2015). Up until now, the class I AADs, which are sodium channel blockers and class III AADs, which are potassium channel blockers have not high efficacy in patients with persistent AF. The main drawback of these drugs is the inhibition of ventricular ion channels, a side effect that leads in high propensity for ventricular pro-arrhythmia, like *torsade de pointes* (TdP) that can be lethal (Waldo *et al.,* 1996; Torp-Pedersen *et al.,* 1999). For this reason the research for new AADs has been focused on targeting K^+ channels that are expressed only in the atria and not in the ventricles. This will help to selectively prolong the atrial effective refractory period (AERP) and cure the AF patients without the risk of QT prolongation or TdP. Also, the target of only atrial K^+ channels will avoid side effects on voltage-gated Na^+ and Ca^{2+} channels promoting the treatment of a broader spectrum of patients with AF that suffer from concomitant heart failure, structural, or ischemic heart disease (El-Haou *et al.,* 2015).

The K^+ channels that have attracted attention as targets for AF are the IK_{Ach} channels, the Kv1.5 K^+ channel that conducts the human atrial ultrarapidly activating delayed rectifier K^+ current (I_{Kur}), more recently the small conductance Ca^{2+}-activated K^+ channels (SK), and the two pore K^+ channels (K_{2P}) (El-Haou *et al.,* 2015). More specifically about the IK_{Ach} channels, there are not selective IK_{Ach} channel inhibitors available for clinical use (Voigt and Dobrev, 2016; Ehrlich *et al.,* 2008). Tertiapin-Q is an oxidation resistant derivative of the bee venom toxin tertiapin and can selectively inhibit Kir channels (i.e. IK_{Ach} and ROMK1 channels) as well as calcium-activated large conductance potassium channels (BK) (Jin and Lu, 1998; Kanjhan *et al.,* 2005), but has no effect of I_{K1} channels (Jin and Lu, 1999). The potential side effects of Tertiapin-Q are the inhibition of BK currents in the central nervous system (Kanjhan

et al., 2005) and the immunogenicity and plasma protein binding in humans (Ehrlich, 2008). The NIP-151 molecule is highly selective and potent IK_{Ach} blocker that works at low micromolar concentrations. It has been tested in both aconitine-induced AF and vagal nerve stimulation-induced AF models and showed efficient termination of AF. Also the molecule prolonged the AERP and had no side effects on ventricular ERP (Hashimoto and Yamashita, 2008).

NTC-801, a substituted benzopyran of undisclosed structure, was the first selective GIRK1/GIRK4 inhibitor to go into clinical studies (Machida *et al.,* 2011). Under vagal nerve stimulation, it prolonged AERP without having any effects on ventricular ERP. NTC-801 was effective to terminate AF in the canine vagal-induced aconitine-induced models of AF and terminated and prevented the induction of AF in an AT-AF dog model of persistent AF (Machida *et al.,* 2011; Yamamoto *et al.,* 2014). Two phase 2 efficacy studies took place for the evaluation of the molecule. The first trial wanted to study the effect of NTC-801 in the maintenance of SR in patients with persistent AF, but it was never completed. The second trial evaluated the effect of NTC-801 on AF burden in paroxysmal AF patients with a permanent pacemaker, but failed to show the desirable result, possibly due to the low dosing for avoiding CNS side effects (El-Haou *et al.,* 2015). AZD2927 is another selective GIRK1/ GIRK4 inhibitor, which passed phase 1 safety and tolerability studies, but in phase 2 trial failed to prolong left atrial effective refractory period in patients with a history of atrial flutter, had preclinical CNS side effects, although it had no side effects on ventricular refractoriness, QTc, ECG, or other cardiovascular parameters (El-Haou *et al.,* 2015).

The HARMONY trial (clintrial.gov identifier: NCT01522651) recently provided evidence that the combination of ranolazine, an inhibitor of late Na^+ channels (late I-Na) and low-doses of dronedarone, an inhibitor of IK_{ACh} channels significantly reduced AF symptoms in paroxysmal AF patients. Although promising, these results need to be verified in a larger study as well as that low-doses of dronedarone are devoid of the side effects of mortality in patients with severe heart failure and permanent AF (Kober *et al.,* 2008; Connolly *et al.,* 2011). OPC-108459 is a new molecule assumed to be an inhibitor of IK_{ACh} or dual IK_{ACh} /I_{Kur}. The two phase 1 evaluations of this molecule are pending and include PK, safety and efficacy studies

to evaluate the dose in patients with AF. The next trial will evaluate the efficacy in 2 separate cohorts of AF patients, one with new onset paroxysmal AF and one with persistent AF and hopefully will provide important information about the two in different AF populations (El-Haou *et al.,* 2015). Therefore, there is a need for developing new inhibitors, including one that selectively inhibit partially the IK_{ACh} channels.

3.3. HTPDQ: a new anti-arrhythmic candidate?

HTPDQ is a tricyclic benzopyran derivative compound with full name (3R,4S)-7(hydroxymethyl)-2,2,9-trimethyl-4-(2-phenylethylamino)3,4dihydropyrano[2,3-g] quinolin-3-ol. HTPDQ is a selective inhibitor of IK_{ACh} channels. Due to its inhibitory effect on IK_{ACh} channel activity, HTPDQ prolongs the refractory period only in the atrial cardiomyocytes without having any affects on the ventricular of the heart, making this a potential anti-arrhythmic treatment [patent: US 20080004262 A1]. Meng Cui et al. (unpublished data) delineated the molecular mechanism of HTPDQ on IK_{ACh} channels showing its specificity on GIRK1/4 and GIRK1/2 channels over the homomeric GIRK and IRK channels. As it has been shown before using ML297, the activator of these channels, the F137 residue in the pore helix of GIRK1 was also critical for the selective response of HTPDQ on GIRK1/4 and GIRK1/2 channels. Using computational approaches, the group predicted the HTPDQ pore blocking mechanism and its interactions via hydrogen bonds with the E141 and D173 residues in GIRK1 and they validated these predictions by mutating these residues and abolishing the HTPDQ inhibitory activity. Finally, using an *in vivo* animal model, they confirmed HTPDQ as a blocker of cardiac IK_{ACh} channels.

Aim

Constitutive active GIRK1/4 channels in atrial myocytes have been implicated in the etiopathogenesis of atrial fibrillation. The cardiac GIRK channel augmented activity leads to premature termination of atrial action potentials and decreased action potential duration resulting in arrhythmias. The constitutive activity of GIRK1/4 channel can be explained by up-regulation in PKCε mediated enhance activity of the channel. The aim of this study is to examine the effect of novel PKCε on GIRK4 and GIRK2 homomeric channels as well as GIRK1/4 and GIRK1/2 heteromeric channels, the effect of novel PKCδ on GIRK1/2 and GIRK1/4 heteromeric channels and evaluate HTPDQ, a small molecule that acts as specific inhibitor of GIRK1/4 and GIRK1/2 channels. This insight into the mechanism of augmented channel activity via novel PKC phosphorylation will assist in the research for allosteric, small-molecule channel inhibitors that balance the cardiac GIRK1/4 channel activity. Also, the extension of this study on GIRK2 channels can contribute in the development of new therapeutic approaches in diseases of the central nervous system, where GIRK2 channels are abundant.

Materials and Methods

1. Molecular Biology

The following cDNAs used in the *Xenopus* oocyte heterologous expression system:

- hGIRK1 was subcloned into the pGEMHE vector (see section 5).
- mGIRK2* (E152D), hGIRK4* (S143T), mGIRK2a, hGIRK4, mD2R, mPKCε-CAT were subcloned into the pXOOM vector (see section 5).

The cRNA for the different channels, receptor and kinases were made by linearization of the DNA plasmids with restriction enzymes and *in vitro* mRNA transcription with T7 RNA Polymerase (T7 RNA transcription kit of Thermo Fisher Scientific).

The homomeric GIRK channels give low currents in heterologous expression systems like in *Xenopus leavis* oocytes. In order to make reliable measurements, GIRK4 (S143T) (Vivaudou *et al.,* 1997) and GIRK2 (E152D) (Yi *et al.,* 2001) activity-boosting mutants were used for the recordings of homomeric GIRK channels. These point mutants (also referred to as GIRK2* and GIRK4*) are activated similarly as their wild-type counterparts carrying larger inwardly rectifying currents under the same stimuli.

The following cDNAs used in the HEK293T cells heterologous expression system:

- mGIRK2a and hGIRK4 were subcloned into the pXOOM vector (see section 5).
- hGIRK1 and hD2R-long-mTFP1 were subcloned into pcDNA (see section 5).
- mPKCε-CAT was subcloned into the pHACE vector (see section 5).
- mPKCδ-CAT was subcloned into the pMax (-) vector (see section 5).

cDNA constructs for channels, receptor and kinases were made by extracting plasmid DNA from *E.coli* bacterial after overnight cultures (Pure Link PCR Purification kit of Thermo Fisher Scientific).

To bypass the need for activation of protein kinase C (PKC) via Gq receptors, catalytic subunits of PKCε and PKCδ were used in both two-electrode voltage-clamp

and whole-cell patch-clamp experiments to compare channel activity with and without the kinase, avoiding this way the confounding effect of PIP2 hydrolysis and decreased channel activity.

2. Two-electrode voltage-clamp experiments

2.1. Oocyte preparation and injection

For all two-electrode voltage-clamp experiments, oocytes were surgically isolated from female *Xenopus leavis* frogs (African clawed frog). Briefly, *Xenopus* were anesthetized with a buffered Tricaine solution and ovarian tissue was excised unilaterally. Excised oocytes were defolliculated using a collagenase solution following standard protocols (Logothetis, Movahedi *et al.* 1992). Oocytes were then washed and incubated at 18^{o} C in OR2 solution (Table 1). Stage V or VI oocytes were microinjected with mRNA according to Table 3 and each oocyte was microinjected with mRNA in 50nL of RNAase-free water. After 36-48hr of incubation at 18^{o} C, the oocytes were ready for whole-oocyte recording using two-electrode voltage clamp.

2.2. Two-electrode voltage clamp

Micropipettes were created by Borosilicate glass tubing after it was pulled using a Flaming-Brown micropipette puller. The micropipettes were then back filled with a 3M potassium chloride in 1% agarose solution. Acceptable resistances for the micropipettes were between ~0.2-1 MΩ. Two-electrode voltage clamp whole-cell current recordings were performed using a GeneClamp 500 amplifier (Axon Laboratories). A voltage ramp protocol was used to monitor inward current, from -80 mV to +80 mV from a holding potential of 0 mV.

At the start of each two-electrode voltage-clamp experiment, oocytes were perfused with a low potassium (Low K^{+}) solution (2mM KCl) (Table 1) prior to measuring the basal activity of the channel using a high potassium (High K^{+}) solution (96mM KCl) (Table 1). Currents in High K^{+} were allowed to stabilize and the agonist was dissolved in High K^{+} and perfused into the recording chamber prior to applying

3mM $BaCl_2$ to establish barium-sensitive GIRK activity. Current amplitudes were measured at -80mV.

Basal GIRK activity, defined as channel activity in the absence of agonist-induced Gi-coupled receptor stimulation is typically assessed using an extracellular solution containing 96mM K^+, aiming for a reversal potential $E_K \approx 0$. The voltage ramp from -80mV to +80mV is used to produce a current-voltage relationship, reversing at 0 mV. The agonist-induced activity of GIRK channels was measured by co-expressing Gi-coupled receptors together with the channels in the heterologous expression systems and using their respective agonists during the experiment. For these experiments, the Gi-coupled dopamine receptor D2R was used and stimulated by dopamine to activate GIRK channels through Gβγ dissociation.

Salt	OR2 1X Buffer	Salt	LOW K+ (2mM KCl) 10X Buffer	HIGH K+ (96 mM KCl) 10X Buffer
NaCl	85 mM	**NaCl**	96 mM	-
HEPES	5 mM	**HEPES**	5 mM	10 mM
KCl	5 mM	**KCl**	2 mM	96 mM
NaOH	5 mM	**CaCl2**	1.8 mM	1.8 mM
MgCl2.6H20	1 mM	**MgCl2.6H20**	1 mM	1 mM
CaCl2	1.8 mM			
Pen/ Strep	1%			

Table 1: OR2 1X Buffer, Low K^+ 10X Buffer, and High K^+ 10X Buffer.

3. Whole-cell patch-clamp experiments

3.1. Cell Transfection

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin at 37°C in a 5% CO_2 humidified atmosphere. Cells were trypsinized and placed in droplets on a glass coverslip in a 35mm dish with DMEM supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin. The

next day, the cells were transiently transfected with the amounts of DNA shown in Table 4 using the transfection reagent polyethylenimine (PEI, (1mg/mL) 4µl per µg DNA). Cells were used for experiments after 24-48 hours of transfection.

3.2. Whole-cell patch clamp

Whole-cell patch clamp current recordings were performed with Patch Clamp L/M-EPC7, Axopatch 200A, and Axopatch 200B amplifiers and Axon 8.1 software (Axon Instruments, Union City, CA). Electrophysiology experiments were performed at room temperature using pipettes with resistances ranging from 2-5MΩ and filled with internal solution (Table 2). Cell capacitances ranged from 3-10pF. Cells were co-transfected with hD2R-mTFP and fluorescent hD2R-mTFP-expressing cells were selected for patch clamp. Currents were recorded at -80mV in whole-cell mode, using a ramp protocol from -80mV to +80mV.

At the start of each whole-cell patch-clamp experiment, the HEK293T cells were perfused with a low potassium (Low K^+) solution (Table 2) prior to assessing the basal activity of the channel using a high potassium (High K^+) solution (Table 2). Currents in High K^+ were allowed to stabilize and the agonist was dissolved in High K^+ was perfused into the recording chamber prior to applying 5mM $BaCl_2$ to establish barium-sensitive GIRK channel activity. Current values were divided by the membrane capacitance (pF) to normalize for the size of each cell.

Salt	LOW K^+ (External Solution)	HIGH K^+ (External Solution)	Salt	Internal Solution
NaCl	135 mM	5 mM	**KCl**	140 mM
KCl	5 mM	135 mM	**MgCl2.6H20**	2 mM
MgCl2.6H20	1.2 mM	1.2 mM	**EGTA**	1 mM
CaCl2	1.5 mM	1.5 mM	**Na2ATP**	5 mM
Glucose	8 mM	8 mM	**Na-GTP**	0.1 mM
HEPES	10 mM	10 mM	**HEPES**	5 mM

Table 2: Low K^+ 10X Buffer, High K^+ 10X Buffer, and internal solution.

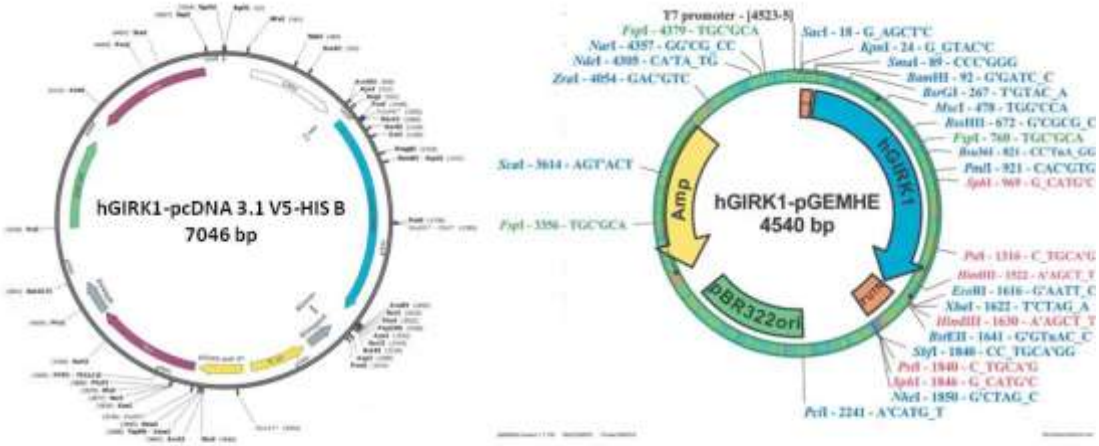
3.3. Dose-response curves

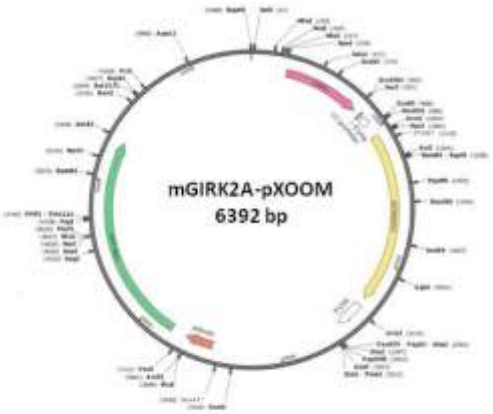
Dose-response curves of HTPDQ, (3R,4S)-7-(hydroxymethyl)-2,2,9-trimethyl-4-(2-phenylethylamino)-3,4-dihydropyrano[2,3-g]quinolin-3-ol, molecule on GIRK1/4 and GIRK1/2 channels were performed separately. The cells were transiently transfected with the amounts of DNA shown in Table 5 and were selected by GFP expression for patch clamp. Low K^+, High K^+, 0.001µM of HPTDQ, 0.01 µM of HPTDQ, 0.1µM of HPTDQ, 1µM of HPTDQ, 10µM of HPTDQ and 5mM $BaCl_2$ were applied sequentially. For data analysis, the remaining current after applying 5mM $BaCl_2$ was subtracted and was normalized against the High K^+ current.

4. Statistical analysis

Error bars in each figure represent standard error of the mean (SEM). In both oocytes and HEK293T cell experiments, statistical significance between 2 groups was assessed using an unpaired student t-test with the statistical analysis program, Graph Prism. In two-electrode voltage-clamp experiments were performed in at least 5 recordings from oocytes from 3 different *Xenopus leavis* frogs, in each group. In whole-cell patch clamp at least 5 recordings were performed from HEK293T cells at different passages, in each group. Statistical significance was set at $p < 0.05$, 0.01, or 0.001, denoted in figures by *, **, or *** asterisks, respectively.

5. Maps of the plasmid were used in the experiments

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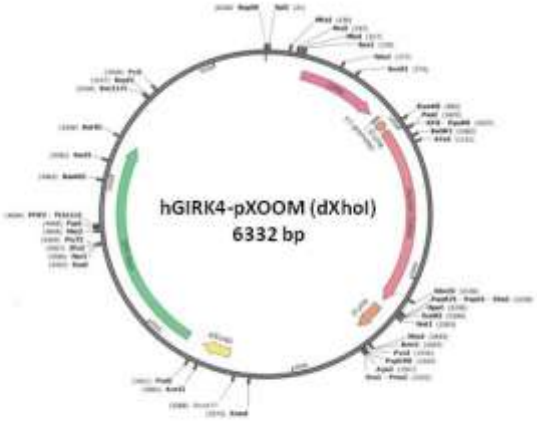
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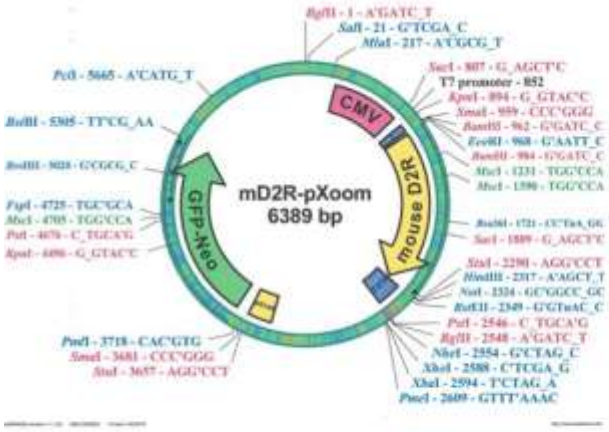
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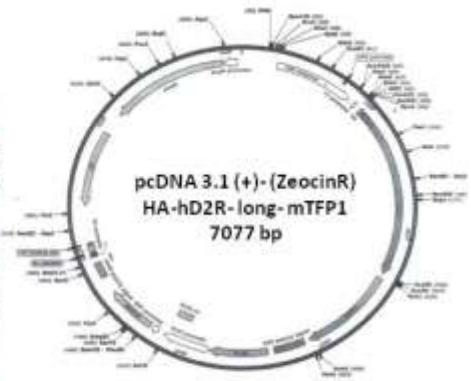
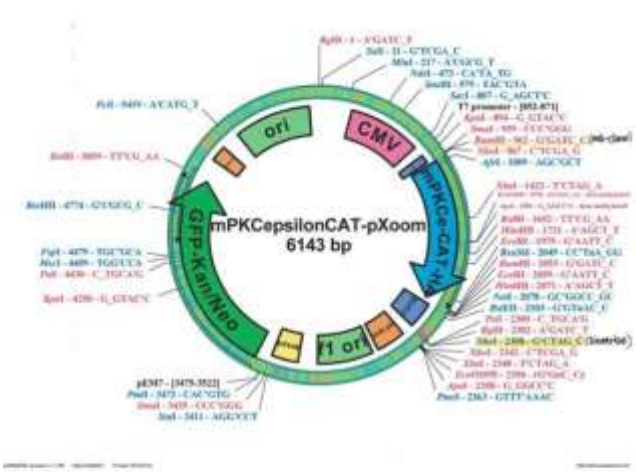
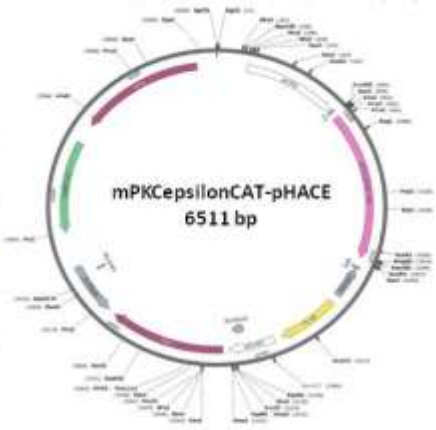
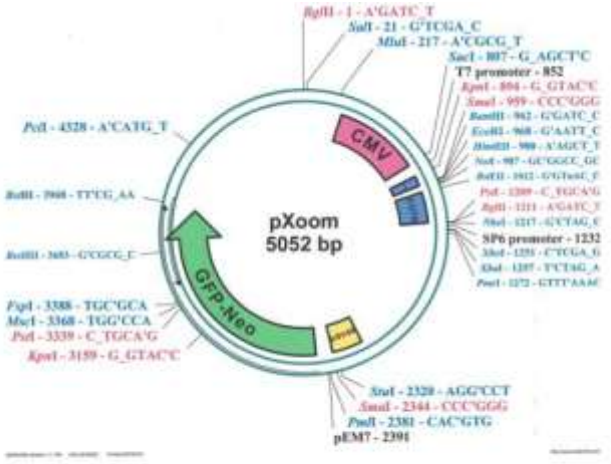
mouse D2R
GFP-Neo


pcDNA 3.1 (+)-(ZeocinR)
HA-hD2R-long-mTFP1
7077 bp


mPKCepsilonCAT-pXoom
6143 bp
ori
CMV
GFP-Kan/Neo
f1 ori


mPKCepsilonCAT-pHACE
6511 bp


pXoom
5052 bp
CMV
GFP-Neo
T7 promoter - 852
SP6 promoter - 1232
pEM7 - 2391

Condition 1a	Amount of mRNA per oocyte			Amount of H2O
mGIRK2* + mD2R	mGIRK2*	mD2R		50 nL
	1 ng	1 ng		

Condition 1b	Amount of mRNA per oocyte			Amount of H2O
mGIRK2* + mD2R + mPKCε-CAT	mGIRK2*	mD2R	mPKCε-CAT	50 nL
	1 ng	1 ng	2ng	

Condition 2a	Amount of mRNA per oocyte			Amount of H2O
hGIRK4* + mD2R	hGIRK4*	mD2R		50 nL
	1 ng	1 ng		

Condition 2b	Amount of mRNA per oocyte			Amount of H2O
hGIRK4* + mD2R + mPKCε-CAT	hGIRK4*	mD2R	mPKCε-CAT	50 nL
	1 ng	1 ng	2ng	

Condition 3a	Amount of mRNA per oocyte				Amount of H2O
hGIRK1 + mGIRK2 + mD2R	hGIRK1	mGIRK2	mD2R		50 nL
	1 ng	1 ng	1 ng		

Condition 3b	Amount of mRNA per oocyte				Amount of H2O
hGIRK1 + mGIRK2a + mD2R + mPKCε-CAT	hGIRK1	mGIRK2	mD2R	mPKCε-CAT	50 nL
	1 ng	1 ng	1 ng	2ng	

Condition 4a	Amount of mRNA per oocyte				Amount of H2O
hGIRK1 + hGIRK4 + mD2R	hGIRK1	hGIRK4	mD2R		50 nL
	1 ng	1 ng	1 ng		

Condition 4b	Amount of mRNA per oocyte				Amount of H2O
hGIRK1 + hGIRK4 + mD2R + mPKCε-CAT	hGIRK1	hGIRK4	mD2R	mPKCε-CAT	50 nL
	1 ng	1 ng	1 ng	2ng	

Table 3: Amount of mRNA used for microinjection of oocytes.

Condition 1a	Amount of DNA					Amount of PEI
hGIRK1 + mGIRK2 + hD2R-mTFP + pZoom	hGIRK1	mGIRK2	hD2R-mTFP	pZoom	TOTAL	15 µl
	0.75 µg	0.75 µg	0.75 µg	1.5 µg	3.75 µg	

Condition 1b	Amount of DNA					Amount of PEI
hGIRK1 + mGIRK2 + hD2R-mTFP + mPKCε-CAT	hGIRK1	mGIRK2	hD2R-mTFP	mPKCε-CAT	TOTAL	15 µl
	0.75 µg	0.75 µg	0.75 µg	1.5 µg	3.75 µg	

Condition 2a	Amount of DNA					Amount of PEI
hGIRK1 + hGIRK4 + hD2R-mTFP + pZoom	hGIRK1	hGIRK4	hD2R-mTFP	pZoom	TOTAL	15 µl
	0.75 µg	0.75 µg	0.75 µg	1.5 µg	3.75 µg	

Condition 2b	Amount of DNA					Amount of PEI
hGIRK1 + hGIRK4 + hD2R-mTFP + mPKCε-CAT	hGIRK1	hGIRK4	hD2R-mTFP	mPKCε-CAT	TOTAL	15 µl
	0.75 µg	0.75 µg	0.75 µg	1.5 µg	3.75 µg	

PKCε

Condition 3a	Amount of DNA					Amount of PEI
hGIRK1 + mGIRK2 + hD2R-mTFP + pZoom	hGIRK1	mGIRK2	hD2R-mTFP	pZoom	TOTAL	15 µl
	0.75 µg	0.75 µg	0.75 µg	1.5 µg	3.75 µg	

Condition 3b	Amount of DNA					Amount of PEI
hGIRK1 + mGIRK2 + hD2R-mTFP + PKCδ-CAT	hGIRK1	mGIRK2	hD2R-mTFP	mPKCδ-CAT	TOTAL	15 µl
	0.75 µg	0.75 µg	0.75 µg	1.5 µg	3.75 µg	

Condition 4a	Amount of DNA					Amount of PEI
hGIRK1 + hGIRK4 + hD2R-mTFP + pZoom	hGIRK1	hGIRK4	hD2R-mTFP	pZoom	TOTAL	15 µl
	0.75 µg	0.75 µg	0.75 µg	1.5 µg	3.75 µg	

Condition 4b	Amount of DNA					Amount of PEI
hGIRK1 + hGIRK4 + hD2R-mTFP + PKCδ-CAT	hGIRK1	hGIRK4	hD2R-mTFP	mPKCδ-CAT	TOTAL	15 µl
	0.75 µg	0.75 µg	0.75 µg	1.5 µg	3.75 µg	

PKCδ

Table 4: Amount of DNA used for transiently transfection of HEK293T cells.

Condition 1	Amount of DNA				Amount of PEI
hGIRK1 + mGIRK2 + GFP	hGIRK1	mGIRK2	GFP	TOTAL	10 µl
	1 µg	1 µg	0.5 µg	2.5 µg	

Condition 2	Amount of DNA				Amount of PEI
hGIRK1 + hGIRK4 + GFP	hGIRK1	hGIRK4	GFP	TOTAL	10 µl
	1 µg	1 µg	0.5 µg	2.5 µg	

Table 5: Amount of DNA used for transiently transfection of HEK293T cells.

Results

The effect of PKCε was evaluated on GIRK2 homomers and GIRK1/2 heteromers by co-expressing the catalytic domain of the novel PKC isoform, PKCε-CAT, with GIRK2* and GIRK1/2 channels as well as the Gi-coupled dopamine receptor D2R, in two-electrode voltage-clamp experiments. PKCε-CAT significantly decreased both basal (High K^+-induced activity of the channel) and agonist-induced (activity in response to 10μM dopamine in High K^+) activity of GIRK2* (Figure 1) and GIRK1/2 (Figure 2) channels in the *Xenopus leavis* oocyte expression system. 3mM $BaCl_2$ in High K^+ was used to block channel activity confirming the activation of GIRK channels in these experiments. In order to examine, whether the effect of PKCε is independent of the expression system, whole-cell patch-clamp experiments with HEK293T cells expressing GIRK1/2 channel, D2R and PKCε-CAT were performed. Both the basal and agonist-induced activities of GIRK1/2 channels were significantly decreased in cells expressing PKCε-CAT (Figure 3). These experiments indicated inhibition of GIRK1/2 currents by PKCε-CAT, which was independent from the expression system used.

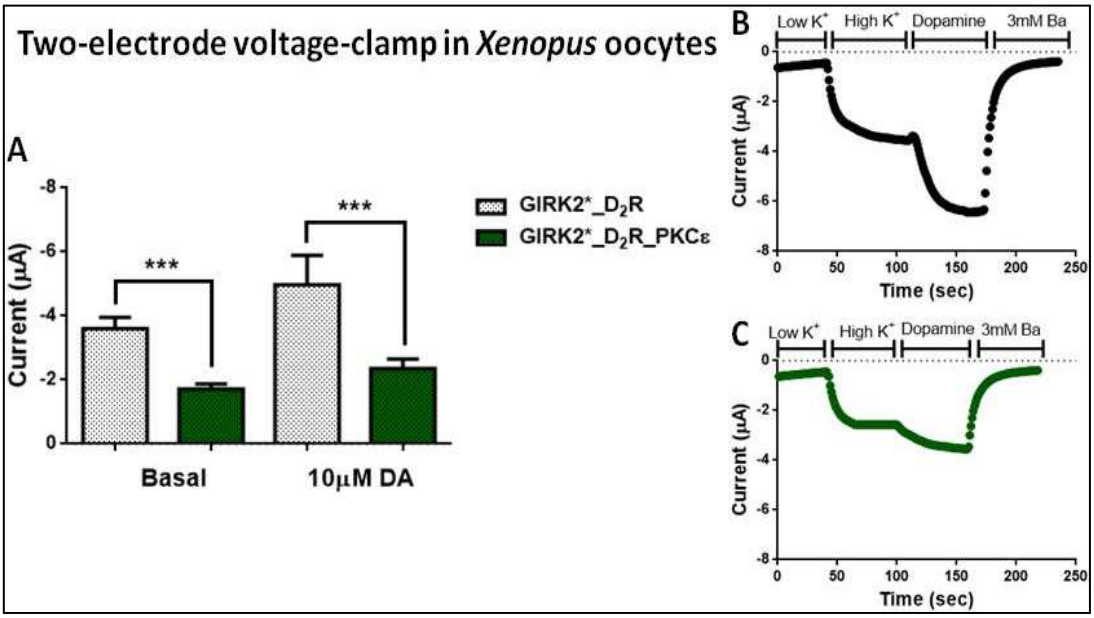


Figure 1: PKCε inhibits the activity of GIRK2* containing currents. (A) *The catalytic subunit of PKCε inhibits both the basal (activity in response to High K^+ - 96mM K^+) and agonist-induced activity (10μM dopamine in High K^+) of GIRK2* channels. Data represent current amplitudes recorded from Xenopus oocytes injected with channel, kinase and receptor mRNA using two-electrode voltage clamp and are shown as averages +/- S.E.M. (at least 5 recordings from oocytes from 3 different Xenopus leavis frogs, in each group).* ***(B)*** *Representative trace of current recording of GIRK2* channels after application of Low K^+, High K^+, 10μM dopamine and 3mM $BaCl_2$.* ***(C)*** *Representative trace of current recording of GIRK2* channels in the presence of PKCε after application of Low K^+, High K^+, 10μM dopamine and 3mM $BaCl_2$.*

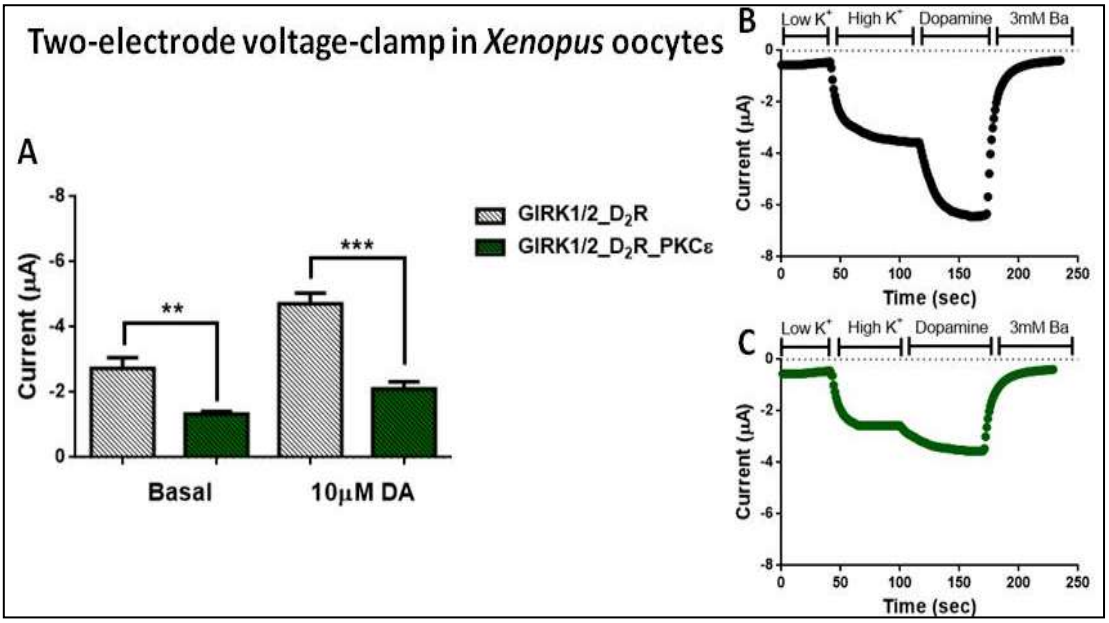


Figure 2: PKCε inhibits the activity of GIRK1/2 containing currents. (A) *The catalytic subunit of PKCε inhibits both the basal (activity in response to High K^+ - 96mM K^+) and agonist-induced activity (10μM dopamine in High K^+) of GIRK1/2 channels. Data represent current amplitudes recorded from Xenopus oocytes injected with channel, kinase and receptor mRNA using two-electrode voltage clamp and are shown as averages +/- S.E.M. (at least 5 recordings from oocytes from 3 different Xenopus leavis frogs, in each group).* ***(B)*** *Representative trace of current recording of GIRK1/2 channels after application of Low K^+, High K^+, 10μM dopamine and 3mM $BaCl_2$.* ***(C)*** *Representative trace of current recording of GIRK1/2 channels in the presence of PKCε after application of Low K^+, High K^+, 10μM dopamine and 3mM $BaCl_2$.*

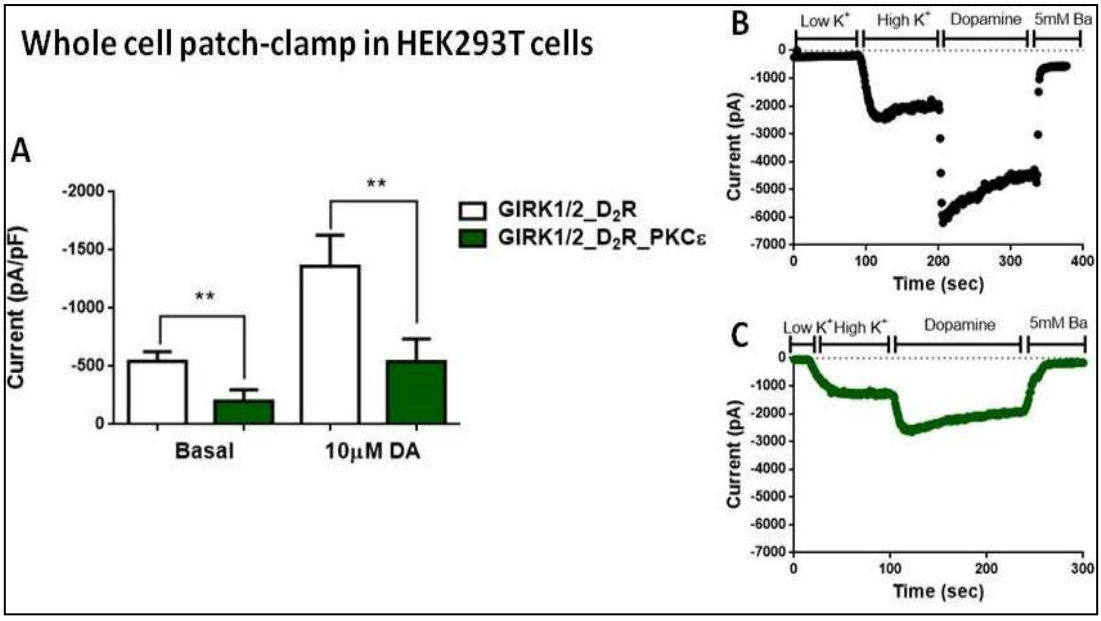


Figure 3: PKCε inhibits the activity of GIRK1/2 containing currents. (A) *The catalytic subunit of PKCε inhibits both the basal (activity in response to High K^+) and agonist-induced activity (10μM dopamine in High K^+) of GIRK1/2 channels. Data represent current amplitudes recorded from HEK293T cells using whole-cell patch clamp and are shown as averages +/- S.E.M. (at least 5 recordings from HEK293T cell in different passage, in each group).* ***(B)*** *Representative trace of current recording of GIRK1/2 channels after application of Low K^+, High K^+, 10μM dopamine and 5mM $BaCl_2$.* ***(C)*** *Representative trace of current recording of GIRK1/2 in the presence of PKCε after application of Low K^+, High K^+, 10μM dopamine and 5mM $BaCl_2$.*

Similar sets of experiments were tested in GIRK4 homomers and GIRK1/4 heteromers. In contrast with GIRK2* and GIRK1/2 channels, a significant increase in basal as well as 10μM dopamine-induced of GIRK4* (Figure 4) and GIRK1/4 (Figure 5) channel activity was observed in oocytes co-expressing PKCε-CAT, in two-electrode voltage-clamp experiments. GIRK1/4 channels activity, in response to PKCε-CAT, was also measured in whole-cell patch-clamp experiments with HEK293T cells. PKCε-CAT significantly increased both basal and agonist-induced activity of GIRK1/4 channels (Figure 6), consistent with the data obtained from TEVC experiments in oocytes.

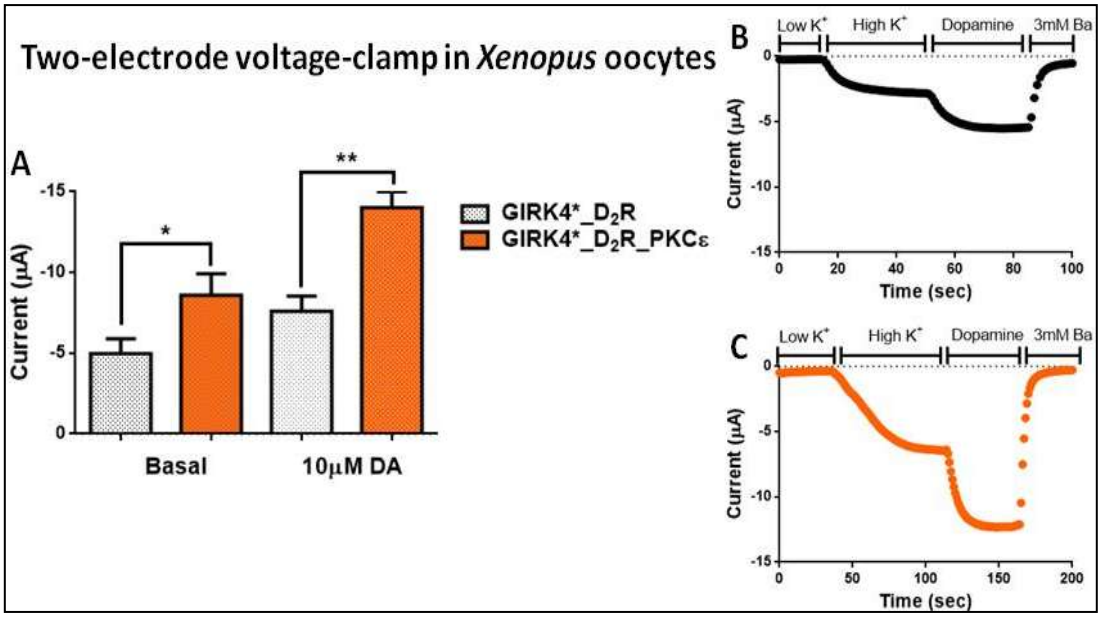


Figure 4: PKCε augments the activity of GIRK4* containing currents. (A) *The catalytic subunit of PKCε inhibits both the basal (activity in response to High K^+ - 96mM K^+) and agonist-induced activity (10μM dopamine in High K^+) of GIRK4* channels. Data represent current amplitudes recorded from Xenopus oocytes injected with channel, kinase and receptor mRNA using two-electrode voltage clamp and are shown as averages +/- S.E.M. (at least 5 recordings from oocytes from 3 different Xenopus leavis frogs, in each group).* ***(B)*** *Representative trace of current recording of GIRK4* channels after application of Low K^+, High K^+, 10μM dopamine, and 3mM $BaCl_2$.* ***(C)*** *Representative trace of current recording of GIRK4* channels in the presence of PKCε after application of Low K^+, High K^+, 10μM dopamine and 3mM $BaCl_2$.*

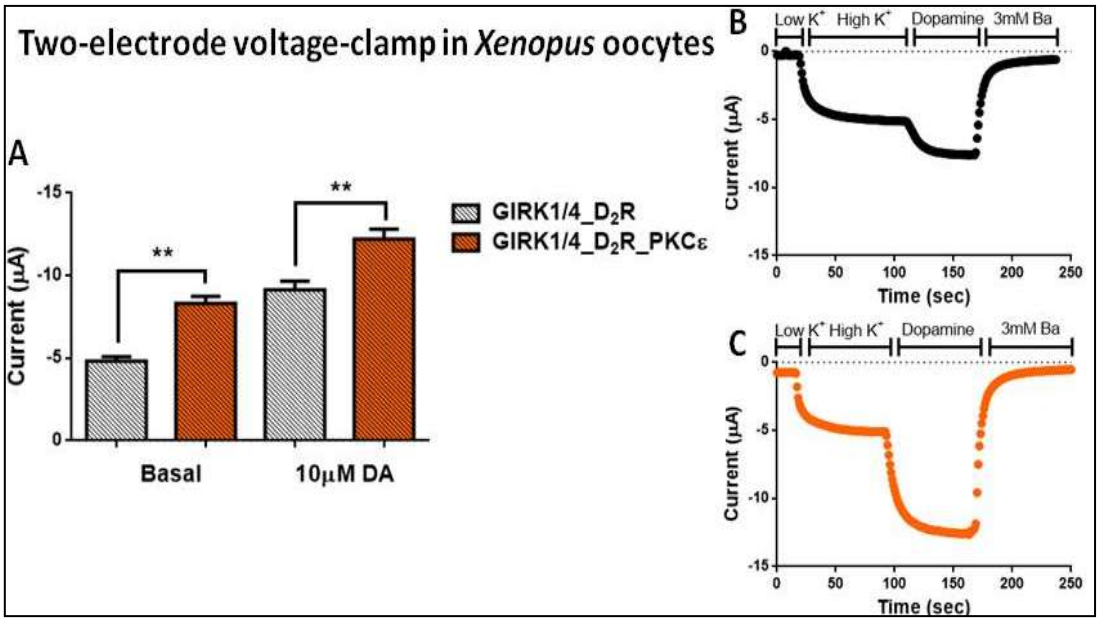


Figure 5: PKCε augments the activity of GIRK1/4 containing currents. (A) *The catalytic subunit of PKCε inhibits both the basal (activity in response to High K^+ - 96mM K^+) and agonist-induced activity (10μM dopamine in High K^+) of GIRK1/4 channels. Data represent current amplitudes recorded from Xenopus oocytes injected with channel, kinase and receptor mRNA using two-electrode voltage clamp and are shown as averages +/- S.E.M. (at least 5 recordings from oocytes from 3 different Xenopus leavis frogs, in each group).* ***(B)*** *Representative trace of current recording of GIRK1/4 channels after application of Low K^+, High K^+, 10μM dopamine and 3mM $BaCl_2$.* ***(C)*** *Representative trace of current recording of GIRK1/4 channels in the presence of PKCε after application of Low K^+, High K^+, 10μM dopamine and 3mM $BaCl_2$.*

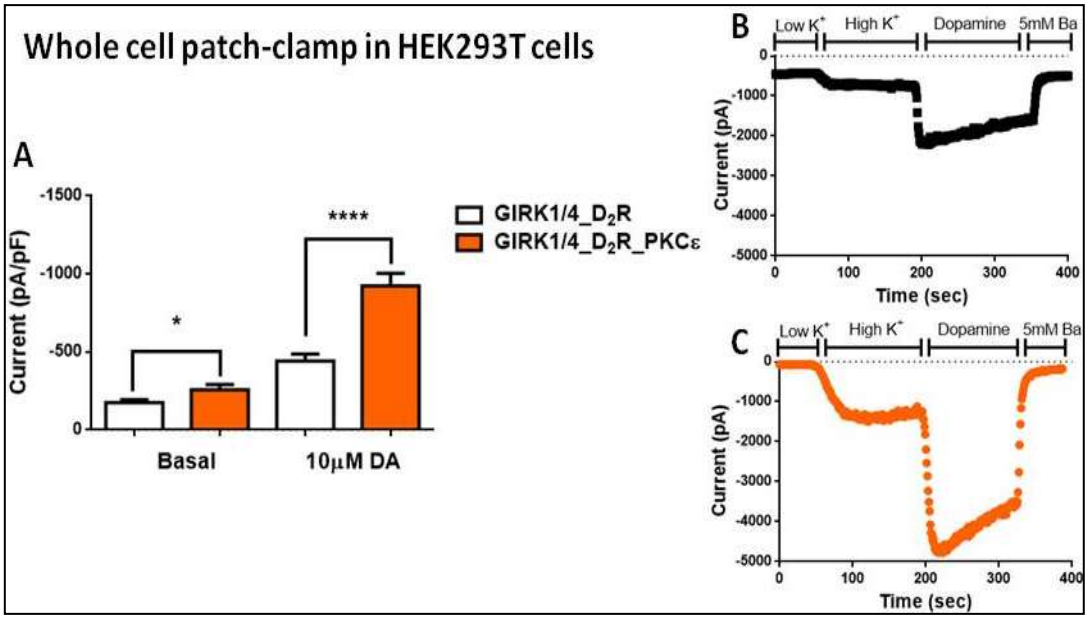


Figure 6: PKCε augments the activity of GIRK1/4 containing currents. (A) *The catalytic subunit of PKCε inhibits both the basal (activity in response to High K^+) and agonist-induced activity (10μM dopamine in High K^+) of GIRK1/4 channels. Data represent current amplitudes recorded from HEK293T cells using whole-cell patch clamp and are shown as averages +/- S.E.M. (at least 5 recordings from HEK293T cell in different passage, in each group).* ***(B)*** *Representative trace of current recording of GIRK1/4 channels after application of Low K^+, High K^+, 10μM dopamine and 5mM $BaCl_2$.* ***(C)*** *Representative trace of current recording of GIRK1/4 in the presence of PKCε after application of Low K^+, High K^+, 10μM dopamine and 5mM $BaCl_2$.*

After the effect of PKCε was established as stimulatory for the cardiac (GIRK4 and GIRK1/4) and inhibitory for the neuronal (GIRK2 and GIRK1/2) channels and it was shown that it is independent of the expression system, the effect of PKCδ-CAT was tested. Thus, similar studies were performed with PKCδ-CAT in GIRK1/2 and GIRK1/4 channels in individual whole-cell patch-clamp experiments using HEK293T cells. Cells were co-expressed with GIRK1/2 or GIRK1/4 channel, D2R and PKCδ-CAT. A significant decrease in both basal and 10μM dopamine-induced activity of GIRK1/2 (Figure 7) and GIRK1/4 (Figure 8) channels was observed, in response to PKCδ-CAT.

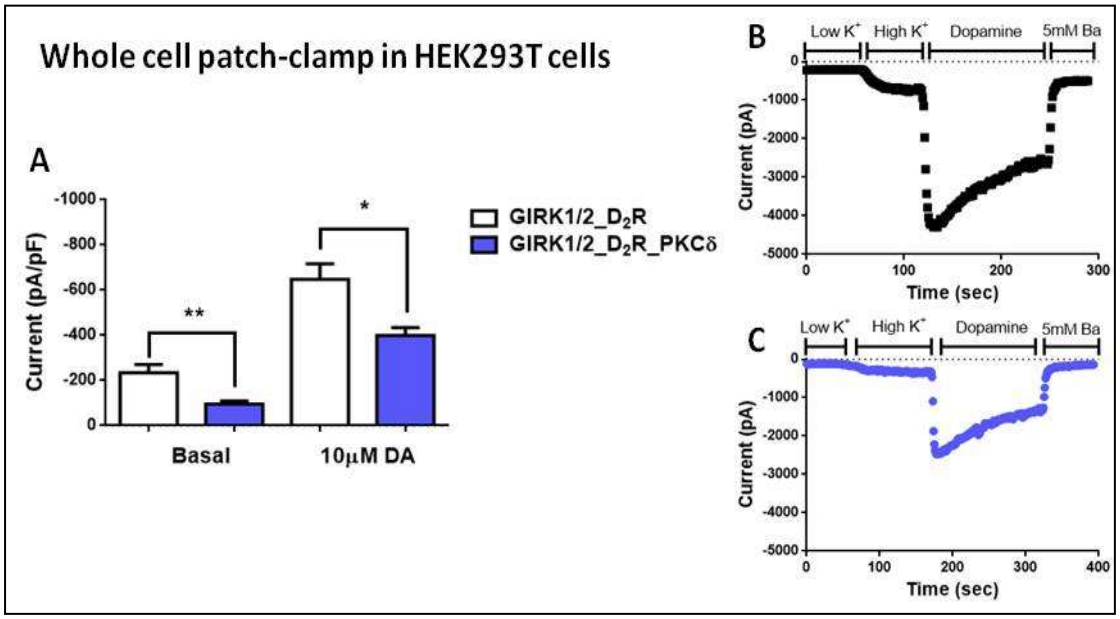


Figure 7: PKCδ inhibits the activity of GIRK1/2 containing currents. (A) *The catalytic subunit of PKCδ inhibits both the basal (activity in response to High K^+) and agonist-induced activity (10μM dopamine in High K^+) of GIRK1/2 channels. Data represent current amplitudes recorded from HEK293T cells using whole-cell patch clamp and are shown as averages +/- S.E.M. (at least 5 recordings from HEK293T cell in different passage, in each group).* ***(B)*** *Representative trace of current recording of GIRK1/2 channels after application of Low K^+, High K^+, 10μM dopamine and 5mM $BaCl_2$.* ***(C)*** *Representative trace of current recording of GIRK1/2 in the presence of PKCδ after application of Low K^+, High K^+, 10μM dopamine and 5mM $BaCl_2$.*

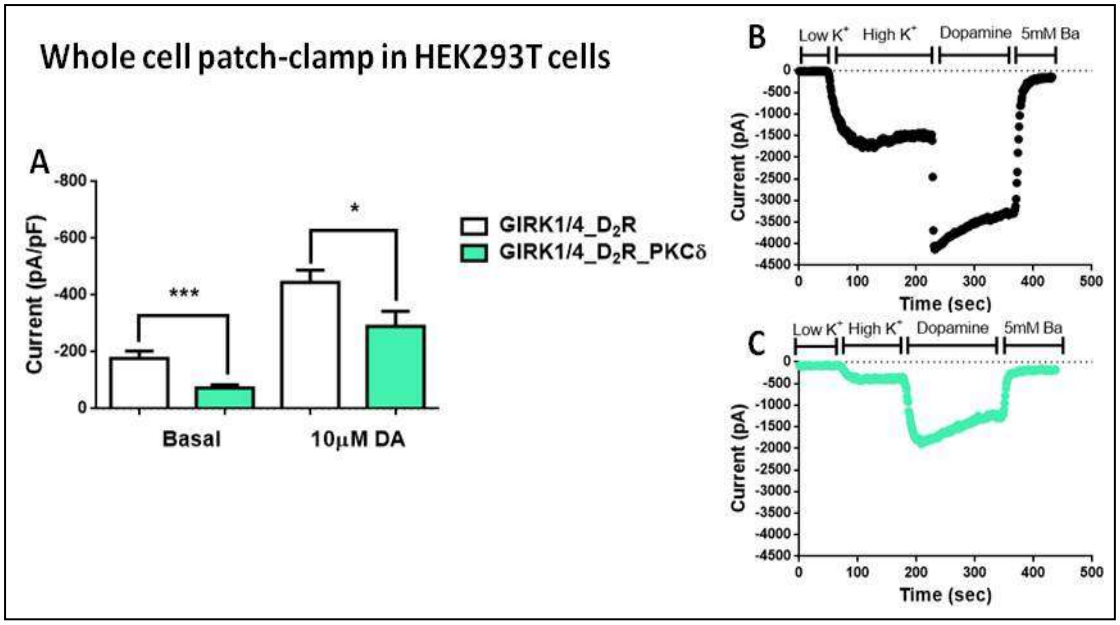


Figure 8: PKCδ inhibits the activity of GIRK1/4 containing currents. (A) *The catalytic subunit of PKCδ inhibits both the basal (activity in response to High K^+) and agonist-induced activity (10μM dopamine in High K^+) of GIRK1/4 channels. Data represent current amplitudes recorded from HEK293T cells using whole-cell patch clamp and are shown as averages +/- S.E.M. (at least 5 recordings from HEK293T cell in different passage, in each group).* ***(B)*** *Representative trace of current recording of GIRK1/4 channels after application of Low K^+, High K^+, 10μM dopamine and 5mM $BaCl_2$.* ***(C)*** *Representative trace of current recording of GIRK1/4 in the presence of PKCδ after application of Low K^+, High K^+, 10μM dopamine and 5mM $BaCl_2$.*

Next, the effects of the benzopyran derivative HTPDQ were examined in cardiac (GIRK1/4) and neuronal (GIRK1/2) channels in whole-cell patch-clamp experiments using HEK293T cells. For that reason, dose response curves of HTPDQ molecule were performed in cells that were expressed with GIRK1/4 and GIRK1/2 channels in individual experiments. A dose response manner inhibition was obtained in both GIRK1/4 and GIRK1/2 channels with IC_{50} of HTPDQ in GIRK1/4 channels to be 10.52nM and in GIRK1/2 to be 32.19nM (Figure 9).

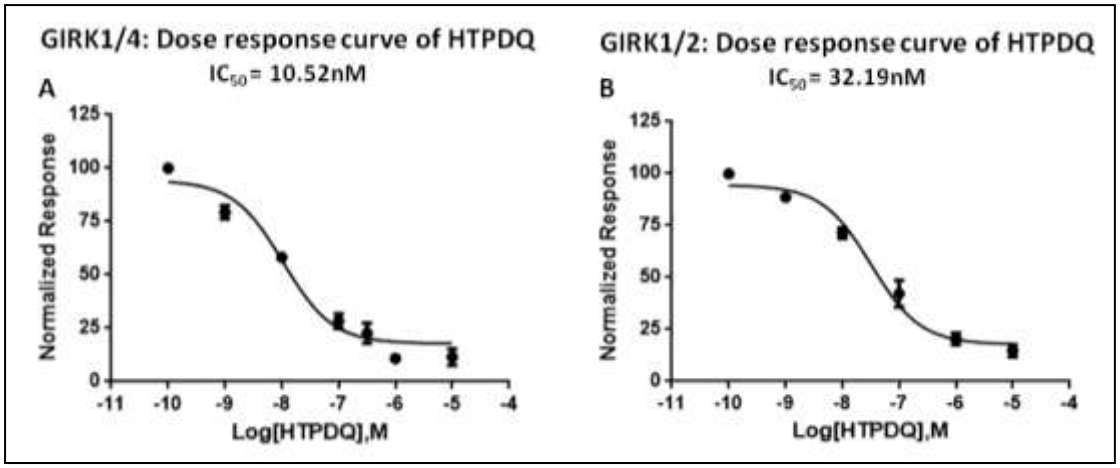


Figure 9: Dose response curves of HTPDQ molecule on GIRK1/4 and GIRK1/2 channels. *Data are current amplitudes recorded from HEK293T cells using whole-cell patch clamp. The current was normalized based on High K^+ current.* ***(A)*** *The IC_{50} of HTPDQ on GIRK1/4 channels is 10.52 nM and* ***(B)*** *the IC_{50} of HTPDQ on GIRK1/2 channels is 32.19 nM.*

Discussion

G-protein-gated inwardly rectifying potassium (GIRK) channel dysfunction has been linked to a variety of conditions from atrial fibrillation to drug addiction as well as anxiety and depression (Pattnaik *et al.,* 2012, Rifkin *et al.,* 2018). GIRK channels contribute to the control of the resting membrane potential and inhibitory post-synaptic potentials in the body (Hibino *et al.,* 2010). One of the several modulators of GIRK channel activity is protein kinase C (PKC) (Keselman *et al.,* 2007). PKC isozymes are reported to mainly inhibit GIRK channels and consist of 14 different isoforms that are divided into conventional, novel and atypical (Nishizuka, 1995).

GIRK4 homomers as well as GIRK1/4 heteromers are expressed in atrial tissue (Stanfield, 2007). The cardiac GIRK channels are activated by acetylcholine stimulation of the muscarinic M2 receptor, resulting in vagal decrease in heart rate (Heijman *et al.,* 2017). Constitutive active GIRK1/4 channels in atrial myocytes have been implicated in the etiopathogenesis of atrial fibrillation (AF). Augmentation of the cardiac GIRK channel activity leads to premature termination of the atrial action potential and decreased action potential duration resulting in arrhythmias (Kovoor *et al.,* 2001; Dobrev *et al.,* 2005). In AF, up-regulation in the expression of the novel PKCε has also been reported (Voigt *et al.,* 2007). Therefore, an increase in PKCε mediated augmentation of GIRK1/4 activity could explain the constitutive activity of this channel.

The present study has shown that the novel PKCε isoform increases both basal and agonist-induced activity through D2 receptor of GIRK4 homomeric and GIRK1/4 heteromeric channels. This was shown in electrophysiological experiments in two distinct heterologous expression systems, such as *Xenopus leavis* oocytes and HEK293T cells. These data agree with the data of Makary *et al.* (2011) that showed that novel PKC isoform PKCε caused a significant increase in GIRK channel current in inside-out patches of atrial AF myocytes. Since, Makary *et al.,* (2011) had reported similar responses for PKCδ in atrial myocytes, the effect of PKCδ was evaluated on GIRK1/4 channels. In contrast to the Makary *et al.,* (2011) results, PKCδ inhibited

basal and dopamine-induced currents of GIRK1/4 channels, when HEK293T cells were used as the expression system. Thus, the stimulation of cardiac GIRK1/4 channel activity is uniquely attributed to the novel PKCε isoform.

The effects of PKCε and PKCδ on GIRK2 homomers and GIRK1/2 heteromer was also evaluated using electrophysiological techniques in the heterologous systems of *Xenopus leavis* oocytes and HEK293T cells. PKCε and PKCδ inhibited both basal and agonist-induced activity through D2 receptor of GIRK2 homomeric and GIRK1/2 heteromeric channels. The inhibition of GIRK2 homotetramers and GIRK1/2 heterotetramers that are expressed in the brain (Logothetis *et al.,* 2015) have been linked with many disorders, such as epilepsy (Signorini *et al.,* 1997). They, also, shape many of the behavioral effects of drugs of abuse, including opioids, psychostimulants (cocaine) and ethanol. Specifically, studies in GIRK2 knockout mice indicated that mice consumed enhanced amounts of ethanol (Blednov *et al.,* 2001). Also, variations in the Girk2 gene in humans is associated with decreased therapeutic efficacy of opioid analgesics and pain sensitivity (Nockemann *et al.,* 2013; Bruehl *et al.,* 2013) and GIRK1 and GIRK2 knockout mice have decreased sensitivity to analgesia (Mitrovic *et al.,*2003; Marker *et al.,* 2004; Blednov *et al.,* 2003; Marker *et al.,* 2005; Cruz *et al.,*2008). Therefore, the inhibitory effects of novel PKC phosphorylation on GIRK2 channel could contribute similar problems and warrant development of new therapeutic approaches, leading to such disorders.

A recent study of Abney *et al.,* (2019) demonstrated that VU0466551 GIRK activator has analgesic effects, when dosed alone or in combination with effective doses of morphine. Our laboratory published recently that GAT1508, a highly specific, potent and efficacious activator of brain GIRK1/2 channels (Xu Yu *et al.,* 2020) was effective in fear condition models of post-traumatic stress disorder (PTSD). GAT1508 showed no other behavioral and cardiac GIRK1/4 side effects suggesting its potential for use in pharmacotherapy for analgesia, drugs of abuse, epileptic seizures, post-traumatic stress disorder and a variety of conditions caused by brain GIRK1/2 channel inhibition.

Based on a cohort study with AF patients in Sweden (Wändell *et al.,* 2016) and a cross-sectional study with AF patients (Akintade *et al.,* 2015), patients with AF show increased depression and anxiety. Augmentation of brain GIRK channel activity has

been linked with depression and increased anxiety (Pravetoni and Wickman, 2008; Blednov *et al.,* 2001). The basis for these associations (AF and increased anxiety and depression) is not understood. HTPDQ is a tricyclic benzopyran derivative compound that has been characterized in our lab as novel inhibitor of GIRK1/4 and GIRK1/2 channel activity with IC_{50} in GIRK1/4 channels 10.52nM and in GIRK1/2 32.19nM. These results highlighted that HTPDQ is slightly more potent for cardiac than brain GIRK channels. HTPDQ is a candidate molecule that could be used for treatment of AF patients with depression or anxiety, due to the fact that could inhibit the excess activity of cardiac GIRK channels. However, the fact that this inhibitor is not specific, limits it utility for AF treatment, as a result of the possible side effects in the central nervous system. Moreover, HTPDQ may inhibit too much of the heart rate variability (HRV) to be clinically useful.

Our laboratory has also designed a GIRK1/4 specific inhibitor, GAT1578 that has no effects on the GIRK1/2 channel. GAT1578 acts through the GIRK1 channel, causes inhibition in the GIRK1/4 heterotetramer and could be studied as a potential therapeutic in AF (not shown). Yet, another novel small molecule, SPEC20, acts as a GIRK4 specific activator that has no effect on GIRK1/2 and GIRK1/4 heteromeric channels (not shown). As mentioned before, GIRK4 homomers and GIRK1/4 heteromers in the atrial play an important role in the control of HRV, a crucial characteristic of cardiac health that endows adaptability of each individual corresponding to high vagal tone (Lee *et al.,* 2018). GIRK4 knockout animals do not develop AF (Kovoor *et al.,* 2001) and GIRK4 or GIRK1 knockout mice show no HRV (Bettahi *et al.,* 2002; Lee *et al.,* 2018; Wickman *et al.,* 1998). Therefore, the balanced activity of GIRK4 homomers and GIRK1/4 heteromers in the atria is critical for both the control of normal heart rate and HRV. If the activity of cardiac GIRK channels is fully blocked by IK_{Ach} inhibitors, the AF symptoms would be cured, but that would also decrease HRV and compromise cardiac health. A combination of a GIRK1/4 specific inhibitor, like GAT1578, and GIRK4 specific activator, like SPEC20, might decrease pathological GIRK activity, while keeping the baseline activity.

A future direction of this work can be the evaluation of oxidative stress on the interaction between PKC and GIRK channels. The incidence of AF increases with advancing age and oxidative stress is inducted during aging. One of our

collaborators, Dr. Noujaim at the University of South Florida, has shown that oxidative stress causes the translocation of PKCε from the cytoplasm to the membrane, where it can phosphorylate its targets, like GIRK channels. Furthermore, Kirin Gada, a PhD student in the lab, has already identified the PKCε phosphorylation sites on the GIRK4 subunit. It would be also very important to identify the PKCε phosphorylation sites on GIRK1 and GIRK2 channels that could provide us with insights useful for the development of molecules that act specifically on the different GIRK2 channel homo- and heterotetramers.

In conclusion, in the last decade several studies and clinical trials have been conducted for the development of specific and potent antiarrhythmic drugs (AADs) for AF treatment. Most AADs have serious side effects, mainly due to the inhibition of ventricular ion channels, a side effect that leads in high propensity for ventricular pro-arrhythmia that can be lethal (Waldo *et al.,* 1996; Torp-Pedersen *et al.,* 1999). For this reason, research for new AADs has been focused on targeting atrial specific ion channels, like GIRK4 homomeric and GIRK1/4 heteromeric channels that are not expressed in the ventricles. Insight into the mechanism of augmented channel activity via the novel PKCε phosphorylation would assist in the search for allosteric, small-molecule channel inhibitors able to balance the cardiac GIRK1/4 channel over-activity. Extension of this study on GIRK2 channels under conditions of increased PKC activity can contribute to the development of new therapeutic approaches in diseases of the central nervous system, where GIRK2 channels are abundant and critical for pre-synaptic neurotransmitters release and post-synaptic neuronal activity.

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