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Développement de la technologie des récepteurs couplés à un canal ionique pour des études structure-fonction des récepteurs couplés aux protéines G et du canal Kir6.2

Katarzyna Niescierowicz

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Katarzyna Niescierowicz. Développement de la technologie des récepteurs couplés à un canal ionique pour des études structure-fonction des récepteurs couplés aux protéines G et du canal Kir6.2. Sciences agricoles. Université de Grenoble, 2013. Français. NNT : 2013GRENV056 . tel-01067669

HAL Id: tel-01067669

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UNIVERSITÉ DE GRENOBLE

THÈSE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITÉ DE GRENOBLE

Spécialité : **Biologie Structurale et Nanobiologie**

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préparée au sein du **Laboratoire Canaux**
dans l'**École Doctorale Chimie et Sciences de Vivant**

Developpement de la technologie des Récepteurs Couplés à un Canal Ionique pour des études structure-fonction des Récepteurs Couplés aux Protéines G et du canal Kir6.2.

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Acknowledgments

In the first place I would like to thank Dr. Bernard Mouillac, Dr. Gildas Loussouarn, Dr. Pierre Charnet, as well as Prof. Franck Fieschi for devoting their time in order to evaluate the work presented in the following thesis.

I would like to also thank Prof. Eva-Pebay Peyroula for allowing me to perform my thesis in the Institute of Structural Biology J.P. Ebel.

Big thanks to Dr. Michael Vivaudou, the leader of Channels Group and my thesis Director, for giving me a chance to experience an incredible scientific adventure here in Grenoble; as well as for his good advices and unabated enthusiasm.

I would like to express my special gratitude for Dr. Christophe Moreau, Co-director of my thesis, who demonstrated an angelic patience explaining me repeatedly all the subtleties of the projects we were investigating. Working with him it was a pleasure and it taught me a lot.

Moreover I would like to thank Jean whose help was priceless, including finishing of this thesis report; Maria for her willing, help, and fantastic Italian spirit; Karla for linguistic help and calm; Stephanie for positive attitude and English support for this thesis; thanks to Anne-Claire, Charlotte and Jimmy. Special thanks to Gina for her friendship and support, as well as to Anne M., Takla, Ben, Jonathan, Ali and of course Argerl for scientific (but not only) conversations. Thanks to all the people working with me during these three short years which I was lucky to spend in Grenoble, experiencing undoubtedly amazing time in my life.

I would especially like to thank as well my wonderful family; my parents, Ewa and Krzysztof, and my grandma Albina, for their love, support and encouragement during these long years of my education; my sister Dorota which became mother of my beloved niece Kalinka, for her patience and for long hours spent on our conversations by Skype. Thanks for Marek K., Marek N. and Tomek R. for they crazy ideas and source of positive energy. Dziękuję Wam Kochani!

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List of abbreviations

- ABC** ATP-Binding-Cassette
- ACh** Acetylcholine
- ADP** Adenosine DiPhosphate (MgADP: Magnesium-ADP)
- Amp** Ampicilline
- ATP** Adenosine TriPhosphate (MgATP: Magnesium-ATP)
- AGS** Activators of G protein Signalling
- AP-2** Adaptator Protein 2
- β2** β2-adrenergic receptor
- cAMP** cyclic Adenosine MonoPhosphate
- CCh** Carbachol
- cDNA** complementary Deoxyribonucleic Acid
- C-ter** carboxy-terminus of protein
- COPI** Coat Protein Complex 1
- CTD** cytoplasmic domain
- D2L** Dopamine D₂ long receptor
- DAG** DiAcylGlycerol
- DEND** Delay, Epilepsy and Neonatal Diabetes
- dNTP** deoxynucleotide triphosphate
- ECL** ExtraCellular Loop
- ER** Endoplasmic Reticulum
- ERK** Extracellular-Regulated Kinase
- GABA** γ-AminoButyric Acid
- GDP** Guanosine DiPhosphate
- GAP** GTPase Activating Proteins
- GDP** Guanidine Di-Phosphate
- GIRK** G protein-coupled Inwardly-Rectifying K⁺ channel
- GPCR** G Protein-Coupled Receptor
- GRAFS** Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, Secretin

GRK G-protein Receptor Kinase
GTP Guanidine TriPhosphate
IC₅₀ half maximal inhibitory concentration
ICD IntraCellular Domain
ICCR Ion Channel-Coupled Receptor
i3 loop third intracellular loop
ICL intracellular loop
IP₃ Inositol TriPhosphate
JAK JAnus Kinase
K_{ATP} ATP-sensitive potassium channel
KCO Potassium Channel Opener
Kir Inward Rectifying Potassium Channel
L0 Loop 0
LB Luria-Bertani
MAPK Mitogen Activated Protein Kinase
M2 Muscarinic receptor 2
MβCD Methyl-β-cyclodextrin
mGluR metabotropic Glutamate Receptor
μA microAmpere
μM microMolar
mM miliMolar
mV milivolt
mRNA messenger Ribonucleic Acid
MRP Multidrug Resistance Protein
NBD Nucleotide-Binding Domain
nM nanoMolare
N-ter Extrémité amino-terminale d'une protéine
pA picoAmpere
PCR Polymerase Chain Reaction
PHHI Persistent Hyperinsulinemic-Hypoglycemia of Infancy
PIP₂ Phosphatidyl Inositol 4,5-bis Phosphate

PKA Protein Kinase A

PKC Protein Kinase C

RGS Regulator of G protein Signalling

SUR SulfonylUrea Receptor

T4L T4 phage lysozyme

TEVC Two-Electrode Voltage Clamp

TMD TransMembrane Domain

utr UnTranslated Region

VFTM Venus Fly-Trap Module

wt wild-type

PREAMBLE

I Objectives of the thesis

Ion Channel-Coupled Receptors (ICCRs) are artificial proteins created in our group by a physical and functional coupling of GPCRs with the Kir6.2, an inwardly rectifying potassium channel. It has already been proved with four class A GPCRs (β_2 adrenergic, M2 muscarinic, D_{2L} dopaminergic and rhodopsin receptor) that the channel acts as a direct reporter of the GPCR's conformational changes. The electrical nature of the signal generated by these biosensors allows real-time detection of GPCR agonists and antagonists in single cells as well as in cell-free conditions. This GPCR-channel communication proceeds without any involvement of G proteins and the electrical signal amplitude is correlated with the ligand concentration.

Applications of such biosensors were envisioned in a primary screening of drugs and in *in vitro* diagnostic devices using microelectronic chips. During my thesis, we developed 4 other applications of this technology: 1) functional characterization of modified GPCRs for crystallographic studies; 2) functional assay of cholesterol-dependency of GPCRs; 3) Proof-of-concept of biased ligands detection; and 4) molecular mechanism of the Kir6.2 gating.

Project 1: recently we focused our attention on the development of ICCR for the functional characterization of mutated GPCRs optimized for crystallographic studies that are not able to couple anymore to G proteins. The intrinsic instability of the GPCRs has proved a challenge to obtain high resolution crystallographic structures. A successful approach, introduced in 2007 in the β_2 adrenergic receptor and subsequently applied to other GPCRs, consists in the insertion of the T4 phage lysozyme domain in the 3rd intracellular loop of the receptors. However, this modification abolishes G protein binding and prohibits related functional assays. Current characterization of crystallization-optimized GPCR(T4L) is performed by radiolabeled ligand assays or localized fluorescence spectroscopy. Thus the main objective of my first project was to demonstrate that ICCRs are suitable to easily characterize GPCR with a substituted byT4L (or another fusion protein) ICL3 and usage of this concept in activity screening assay of crystallization-optimized GPCRs. Requiring no biochemical steps, ICCRs could stand as an alternative tool to functionally characterize modified GPCRs that are unable to bind or activate G proteins and not amenable to most GPCR functional assays. We investigated here the validity of this tool with three different GPCRs (M2-muscarinic, β_2 -adrenergic and oxytocin receptors). The final application of this study would be the integration of this technology in the current

crystallographic platforms dedicated to GPCR structure determination or for structure-function studies independent of G protein interaction.

Project 2: Due to the reason that in our group we developed Ion Channel-Coupled Receptor based on the Oxytocin Receptor (OXTR) we decided to investigate the utility of that tool in detection of the receptor's sensitivity to cholesterol. It has been published previously that function and physiological regulation of the OXTR system is strongly cholesterol-dependent as it acts as a positive allosteric modulator of that receptor. Thus, we subsequently examined if our tool allows direct detection of receptor's cholesterol-dependence by comparison of the receptor activities in the cholesterol naturally rich and artificially depleted (with M β cD) cell membrane.

Project 3: Using the same OXTR-ICCR we tested if ICCRs are suitable tool for the primary screening of the new group of GPCRs' ligands called 'biased' agonists.

Project 4: The ICCRs technology allows not only to investigate functional properties of the receptor, but as well the molecular mechanisms of Kir6.2 gating.. Thus, we used the ICCR as a tool for studying the channel regulation by other proteins. Kir6.2 is a pore-forming subunit of the ATP-sensitive potassium channel (K_{ATP}) which is involved in the regulation of the insulin secretion. Dysfunctions of the gating induce physiopathologies. Thus, it has been showed that many gain-of-function mutations in the human Kir6.2 gene directly impaired ATP sensitivity or increased the open frequency of the gates causing severe neurological disorders. The number of gates in Kir channels is still a controversy, the main consensus published by the Nobel Prize of the field being 3. Physiologically, Kir6.2 is physically and functionally associated with another membrane protein: the sulfonylurea receptor (SUR). The molecular mechanisms involved in the channel regulation by the SUR subunit are still unknown. Using the ICCR technology based on the M2 muscarinic receptor, I functionally mapped the gates regulated by a N-terminus interacting membrane protein using a mutagenic approach.

INTRODUCTION

Avant de présenter les résultats obtenus au cours de ces travaux de thèse, une introduction abordera les différents thèmes touchant à notre sujet d'étude et qui sont nécessaires à la compréhension de notre démarche. Nous présenterons des canaux K_{ATP} , des GPCR et des Récepteurs Couplés à un Canal Ionique (ICCRs).

II Ion Channel-Coupled Receptors (ICCRs)

II.1 Composition of ICCRs.

Ion channel-coupled receptors (ICCRs) are artificial ligand-gated channels created by the covalent assembly of a G Protein-Coupled Receptor (GPCR) and an inwardly rectifying potassium channel, Kir6.2 (the pore forming subunit of the K_{ATP} channel). In ICCRs, the cytosolic carboxy-terminus (C-ter) of the GPCR is fused to the amino-terminus (N-ter) of the channel, thus enabling functional ‘communication’ between these two unrelated proteins [Moreau Nat Nanotechnol 2008] (Figure 1). Four GPCR-Kir6.2 monomers co-assemble and create a functional ICCR.

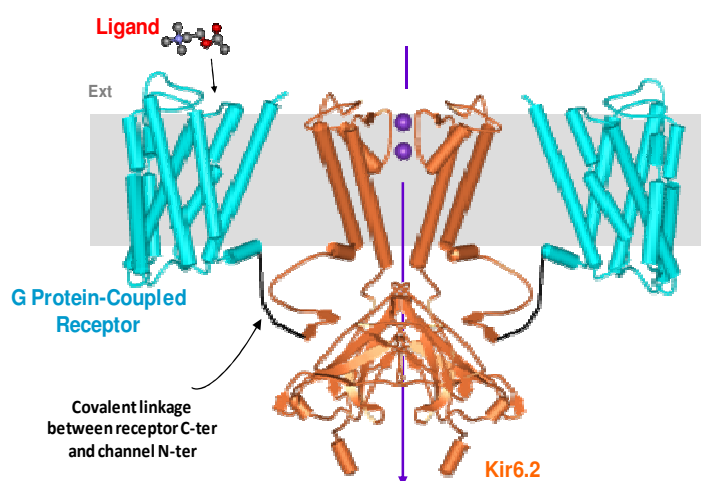


Figure 1. Molecular model of an Ion Channel-Coupled Receptor.

Transmembrane view of a molecular model of a GPCR-Kir6.2 tetramer with front and back monomers removed [Moreau Nat Nanotechnol 2008].

II.2 Principle of artificial-ligand gated channels.

For the ICCR concept, we used the principle of the intermolecular regulation naturally occurring in K_{ATP} channels. Thus, binding of a specific ligand to the target GPCR leads to conformational changes of the receptor that are subsequently transmitted to the channel gating through the covalent link. It results in a change of the K^+ current induced by the GPCR ligand-binding (Figure 2). The amplitude of the signal is correlated with the ligands’ concentration and enables an estimation of apparent affinities by concentration-response measurements. In this way the channel is a direct reporter of the conformational changes of the GPCR, detecting the agonist- and antagonist-bound states of the receptor [Moreau Nat

Nanotechnol 2008]. Until now, this concept has been validated for four class A GPCRs: the human M2 muscarinic, the long D2 dopaminergic, the β 2 adrenergic and the bovine rhodopsin receptor [Moreau Nat Nanotechnol 2008] [Caro PloS ONE 2011] [Caro PloS ONE 2012].

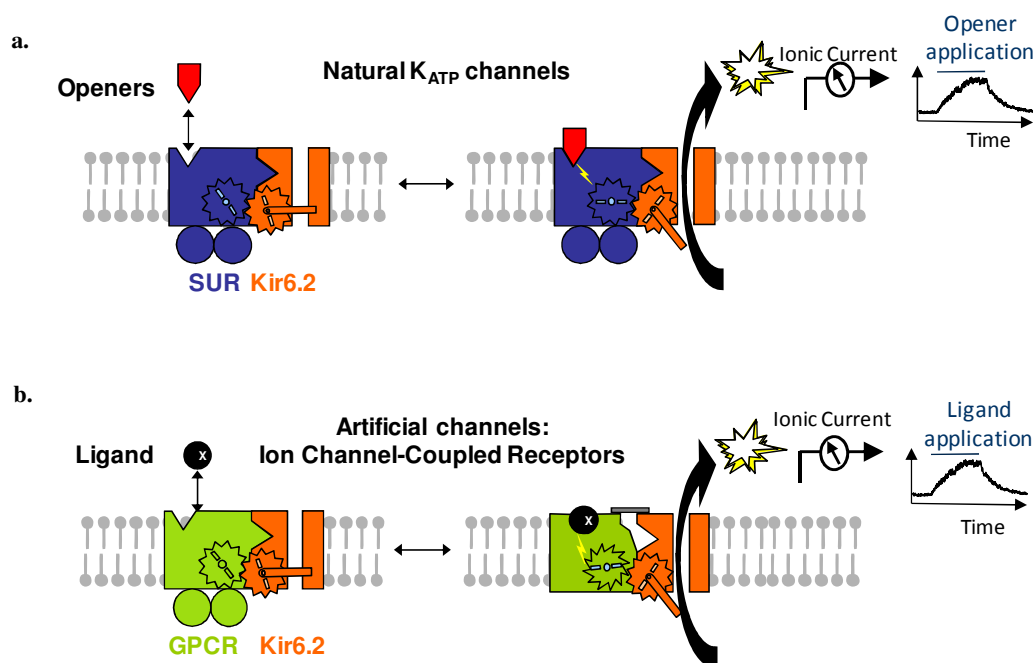


Figure 2. Principle of ion channel-coupled receptors (ICCRs).

a) In naturally occurring K_{ATP} channels, binding of the opener to the regulatory SUR subunits leads to the opening of the Kir6.2 pore-forming subunit of the channel and generation of the K^+ current. b) In ICCRs, GPCR is covalently attached to the ion channel (Kir6.2). When the receptor binds a ligand (agonist) and changes its conformation, this change is directly transmitted to the channel and results in gating alterations and flow of the ionic current through the channel [Channels Group].

Importantly, it has been proved that the receptor fused in the ICCR keeps its physiological functions, as it can activate its cognate G proteins [Moreau Nat Nanotechnol 2008] [Caro PloS ONE 2011].

II.3 Functionality of the coupling between fusion proteins.

In order to obtain a functional ICCR, it is necessary to perform genetic engineering of the linker region. The linkage is modified mostly by deletions in the C-terminal region of the receptor and in the N-terminal region of the channel. It has been found for prototypical M2- and D2-based ICCRs, that truncation of the Kir6.2 first 25 N-terminal residues results in the most efficient coupling between the receptors and the channel [Moreau Nat Nanotechnol 2008]. This finding was confirmed for two subsequently built ICCRs based on β 2-AR and rhodopsin receptor [Caro PloS ONE 2011] [Caro PloS ONE 2012]. On the receptor side, the size of the truncation depends on the length of the C-terminal region. M2-muscarinic and D2-dopaminergic

receptors have rather short C-terminus, thus the truncation of this region was not obligatory. In contrast, β 2-AR and rhodopsin receptor possess longer C-terminus and its truncation was an essential step to obtain functional ICCRs. When Kir6.2 was fused to full length receptors, no response was detected in presence of ligands. The explanation of this phenomenon could be that long C-terminus might preclude proper coupling between receptor and channel.

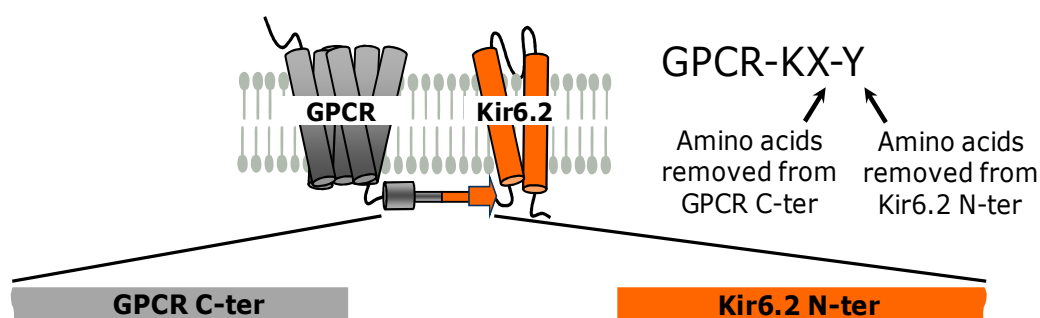


Figure 3. Linker region of ICCRs.

Modifications (short truncations) of GPCR C-terminus and channel N-terminus allow to obtain functional complex, which is then able to detect the binding of different types of ligands ^[Channels Group].

II.4 Applications of the ICCR technology.

Ion channel-coupled receptors were initially designed as electrical biosensors with potential applications in diagnostics and high-throughput drug screenings. These constructs combine the advantages of both partner proteins. The GPCRs recognize a wide range of chemical ligands with high affinity and specificity, thus acting as a molecule receptor; whereas ion channels generate an electrical signal large enough to enable the detection of a single molecule, thus serving as the electrical switch ^[Moreau Nat Nanotechnol 2008]. Undoubtedly, the huge advantage of these artificial proteins is the generation of an electrical signal in response to applied chemical compound, which allows label-free assays with a high signal-to-noise ratio and fast real-time measurements.

III G Protein-Coupled Receptors

III.1 General information.

G protein-coupled receptors represent the largest family of membrane proteins encoded by ~800 genes (approximately 2% of the human genome). These 7-transmembrane

helices receptors recognize many different ligands: from photons to proteins, and are expressed in fungi, plants and animals.

III.1.1 GPCR topology.

GPCRs are polytopic membrane proteins. They are characterized with a very recognizable structure that includes seven membrane-spanning helices (transmembrane domains, TM I-VII), an extracellular amino acid tail (N-terminal segment) and an intracellular amino acid tail (C-terminal segment). The transmembrane helices have been long observed as the most conserved component of GPCRs, harboring some signature motifs important for the functionality. The TM domains are connected by three extracellular loops (ECL1, ECL2, ECL3) and three intracellular loops (ICL1, ICL2, ICL3). The ligand-binding site is either inside the transmembrane domain (rhodopsin-like GPCRs) or involves more largely in other GPCR classes the extracellular (EC) part including the N-terminal domain (which can range from relatively short and unstructured to large and globular) [Lagerström Nat Rev Drug Discov 2008]. On the other side, the intracellular (IC) part is crucial for the interaction of the GPCRs with G proteins, β -arrestins and other downstream effectors. Besides the three intracellular loops, the IC part usually includes a helix VIII as well as a C-terminus carrying signalling sites (for example palmitoylation and phosphorylation) [Tobin Trends Pharmacol Sci 2008][Maeda Proc Natl Acad Sci 2010].

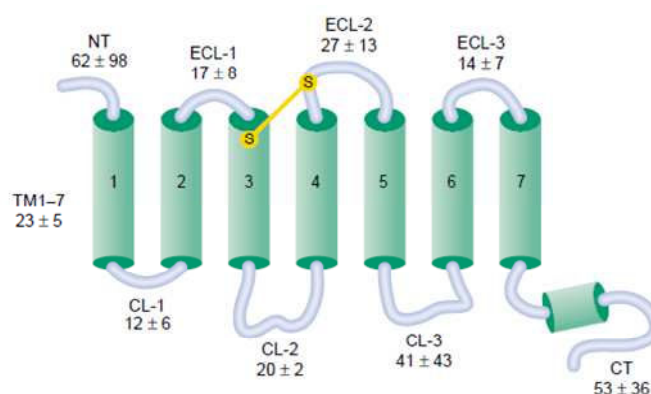


Figure 4. Representation of the secondary structure of G protein-coupled receptors.

The disulfide bond (yellow) that links TM3 and ECL2, is conserved in 91.8% of GPCRs [Karnik Trends Endocrinol Metab 2003].

Despite the similarity of the 7TM topology (Figure 4), the five families of human GPCRs share little sequence identity (SI < 20% in the transmembrane domain) and show remarkable structural diversity. Strikingly, observed diversity concerns not only the loop regions, but also the 7TM helical bundle. Nevertheless, the most variable region among GPCRs is the extracellular ligand-binding site [Katritch Trends Pharmacol Sci 2012].

III.1.2 Physiological relevance

G protein-coupled receptors present in the plasma membrane of the cell receive chemical or physical (photons) signals from the surrounding environment, and thus induce through downstream effector proteins, specific biological response inside the cell. In human, they are expressed in all tissues including: brain, heart, muscles, liver, kidney, bones and blood. As highly versatile membrane sensors, GPCRs have a high specificity of recognition for their ligands. The diversity of ligands is recognized by the different GPCRs' families, ranging from ions, photons, and sensory stimuli (odorant molecules), to lipids, hormones, and neurotransmitters [Lagerström Nat Rev Drug Discov 2008]. Specific responses to these ligands are involved in biologically important processes including vision, smell, mood regulation, and inflammation (Figure 5). These physiological implications make GPCRs a major therapeutic target family that mediates more than 40% of clinically approved drugs [Wise Drug Discov Today 2002].

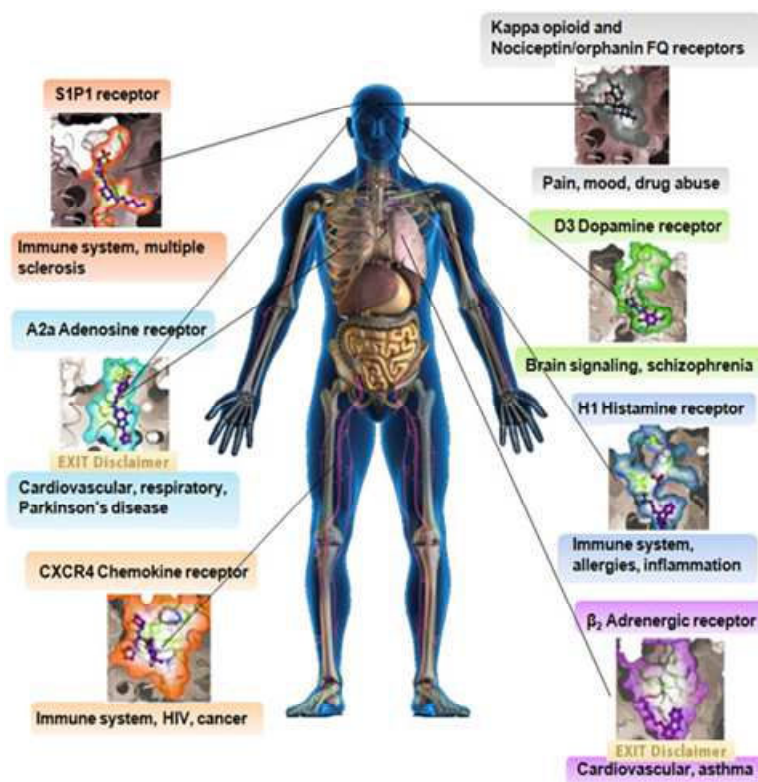


Figure 5. Body distribution and physiological relevance of some representatives of the GPCR superfamily.

III.1.3 Classification of GPCRs.

There are two main requirements for a protein to be classified as a GPCR [Fredriksson Mol Pharmacol 2003].

- the presence of the seven transmembrane α -helices of about 25 to 35 residues, that show a relatively high degree of calculated hydrophobicity. These helices span the plasma membrane in a counter-clockwise manner, thus forming a receptor amenable to bind extracellular ligands and transduce the signal to the intracellular side,
- the ability of the receptor to interact with cognate G proteins,

Numerous classifications, based on different criteria, have been proposed to sort out the GPCR superfamily. One of them divides these receptors into five main families (1-5): Rhodopsin-like, Secretin-like, Metabotropic Glutamate, Fungal Pheromone and cAMP receptors on the basis of ligand binding mode and/or structure of receptors [Bockaert EMBO J 1999]. Nevertheless, one of the most frequently used systems is the division of GPCR family into classes A, B, C, D, E, and F and subclasses assigned with roman number nomenclature [Attwood Protein Eng 1994]. This A-F system allows the covering of all GPCRs, in both vertebrates and invertebrates. It is important to take note that some families included in this classification do not exist in Human (classes D and E).

An alternative system of GPCRs' classification, for the human genome, called GRAFS was recently proposed. This system groups the receptors into clusters, and classifies them based on the phylogenetic relationship into five main families [Fredriksson Mol Pharmacol 2003] (Figure 6a):

- Glutamate (G, with 15 members),
- Rhodopsin (R, 701),
- Adhesion (A, 24),
- Frizzled/taste2 (F, 24),
- Secretin (S, 15),

The rhodopsin family includes the largest number of receptors and for this reason was further subdivided into four main groups (α , β , γ , δ) and 13 branches (Figure 6b).

Introduction

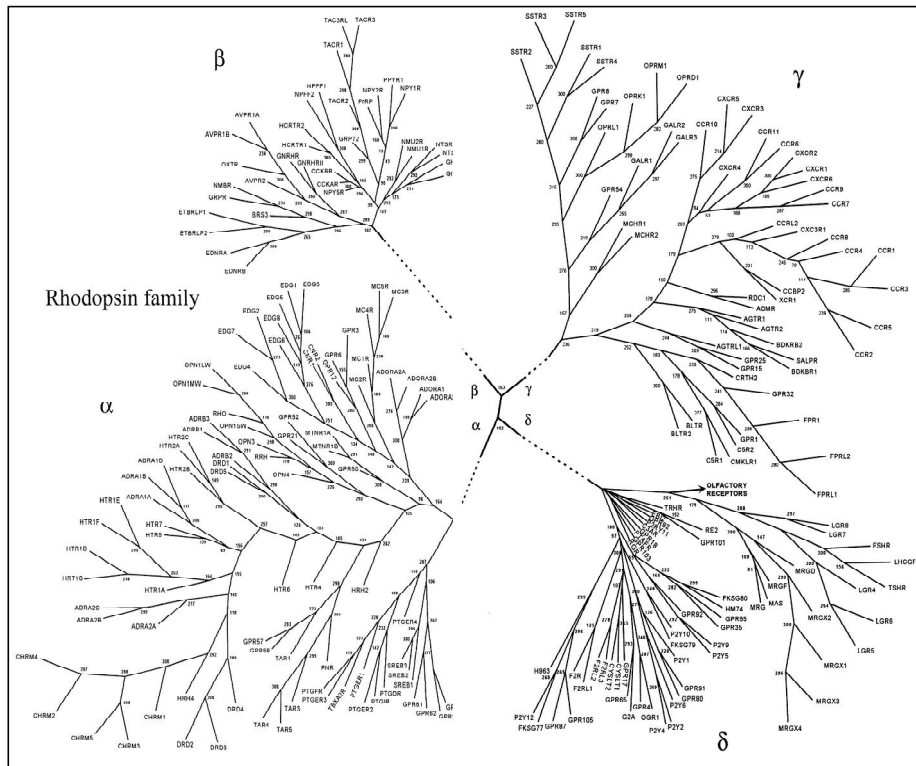
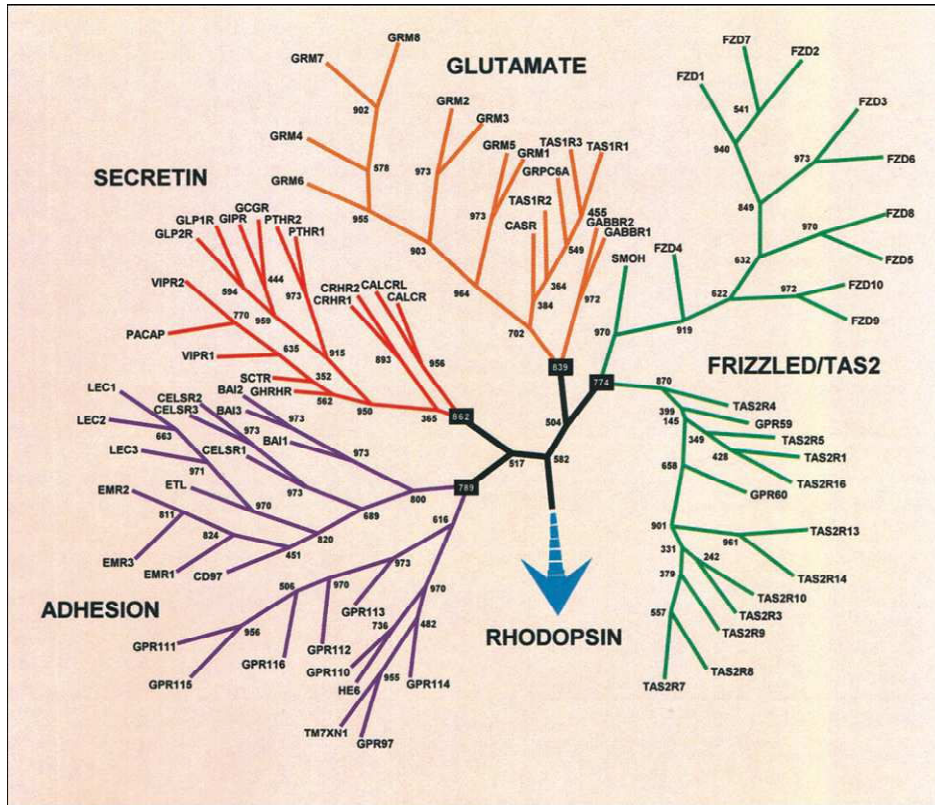


Figure 6. Phylogenetic tree of the GRAFS classification.
a) Overall relationship between the five main families of GPCRs. b) Specific phylogenetic relationship between GPCRs in the human rhodopsin family [Fredriksson Mol Pharmacol 2003]

➤ The rhodopsin family.

The rhodopsin family corresponds to what was previously called the rhodopsin-like receptors (family 1) or class A in the A-F classification, and characterizes with the largest number of receptors. This family of GPCRs includes opsin (light-activated), olfactory receptors (stimulated by odorant molecules); receptors activated either by small molecules (biogenic amines, prostaglandins), peptides (hormones) or proteins (chemokines, glycoprotein hormones). Due to the diversity among this family, rhodopsin receptors are divided into four main groups: α , β , γ , δ . Members of this family possess several characteristic features such as the NSxxNPxxY motif in TM VII and the DRY or D(E)-R-Y(F) motif at the border between TM III and ICL2, which has been shown to play an important role in the receptor stabilization and its coupling with G proteins [Fredriksson Mol Pharmacol 2003][Bockaert EMBO J 1999]. Moreover, in the loops ECL1 and ECL3, two conserved cysteine residues are present and create disulfide bridge, crucial for receptor conformation. Generally, for most of rhodopsin family receptors, the ligand-binding site is localized between TM domains [Baldwin Curr Opin Cell Biol 1994]. Nevertheless, there are some important exceptions to this rule, especially for glycoprotein binding receptors (LH, FSH, TSH, and LG), where the ligand-binding domain is present in the N-terminus of the protein.

➤ The secretin receptor family.

This group of GPCRs corresponds to family 2 and class B in the A-F classification. The name of this family comes from the fact that the secretin receptor was the first to be cloned. The common feature of the members of secretin class is the binding of large peptides (e.g. glucagon, calcitonin, secretin, parathyroid hormone), which characterize with high amino acid sequence identity [Fredriksson Mol Pharmacol 2003]. The N-terminus of these receptors is ~60-80 residues long and contains three conserved disulfide bonds. This domain contains also the ligand-binding sites [Karnik Trends Endocrinol Metab 2003].

➤ The glutamate receptor family.

This family of GPCRs corresponds to family 3 or class C of the receptors in the A-F classification. The glutamate receptor family consists of eight metabotropic glutamate receptors (mGluR), five taste receptors (TAS1), two γ -aminobutyric acid receptors (GABA_{B1/2}) and a single calcium-sensing receptor (CASR) [Fredriksson Mol Pharmacol 2003]. Most of the representatives of this family characterizes by elongated N- and C-terminal regions. A

large extracellular region can be subdivided into two domains: (1) a Venus Flytrap module (VFTM) involved in agonist recognition and binding, and (2) a cysteine-rich domain (CRD) that interconnects the VFTM to the transmembrane core in most glutamate receptor family, except the GABA_B receptors. VFTM has a bilobate structure (Figure 7). It has been shown for mGluR that in the absence of agonist, VFTM stays in the open 'inactive' state, and the binding of glutamate in the cleft between two lobes results in the closed state ^{[Pin Pharmacol Ther 2003] [Pin Biol Cell 2004]}.

➤ The adhesion receptor family.

This is quite new and very particular family of GPCRs which in the A-F classification system was included in class B (family 2), along with secretin receptors ^[Fredriksson Mol Pharmacol 2003]. This family includes receptors with GPCR-like transmembrane-spanning regions fused together with one or several functional domains. Another characteristic feature of these receptors is the long N-terminus domain (200-2800 residues), highly glycosylated and rich in cysteine and proline residues. It contains also one or several adhesion motifs (for example EGF-like repeats) that are likely to participate in cell-to-cell and cell-to-matrix interaction ^[Stacey Trends Biochem Sci 2000]. Moreover, the N-terminus of these GPCRs contains a proteolytic domain very closed to the beginning of the TM I (GPCR proteolysis site: "GPS domain"), that serves as a cleavage site. Natural removal of this domain takes place in the endoplasmic reticulum and in the early compartment of the Golgi apparatus. This process is essential requirement for trafficking and addressing of the receptor to the plasma membrane ^[Krasnoperov J Biol Chem 2002].

➤ The frizzled/taste 2 receptor family.

This family of receptors as referred by its name includes two distinct clusters, the frizzled and the taste 2 (TAS2) receptors. Both groups vary significantly between each other. Nevertheless, both belong to the same family due to specific motifs present in the C-terminus that have not been found in the consensus sequence of the other four families ^{[Karnik Trends Endocrin Met 2003] [Fredriksson Mol Pharmacol 2003]}. The TAS2 receptors are characterized with rather short N-terminus, unlikely to contain a ligand binding domain. The role of TAS2 receptors is not well-known. They are expressed in the tongue, as well as in the palate epithelium, suggesting that they are implicated in the bitter taste sensation. In contrast, frizzled receptors possess

long N-terminus of around 200 residues. Their biological roles include control of cell fate, proliferation, and polarity during metazoan development [Fredriksson Mol Pharmacol 2003]

➤ Other families.

Families D, E and F of GPCRs are not included in the GRAFS classification due to the fact that they are not expressed in humans. Class D contains yeast pheromone receptors, class E cyclic AMP receptors expressed only in *Dictyostelium discoideum*, and class F archaeobacterial opsins [Karnik Trends Endocrin Met 2003].

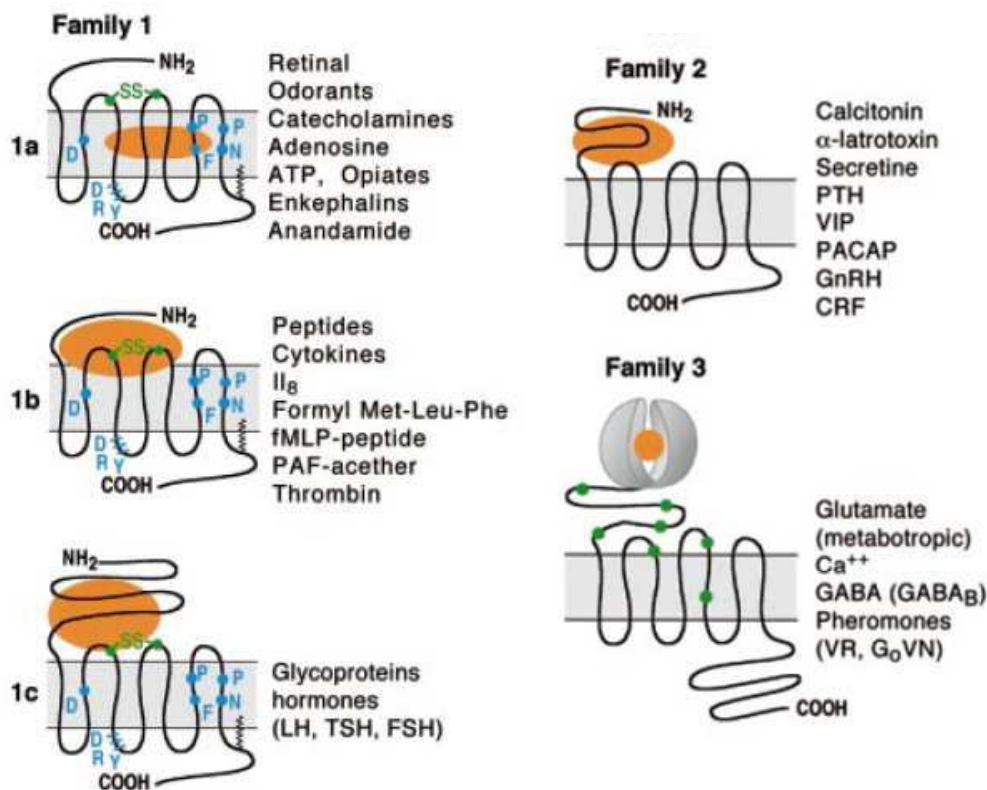


Figure 7. Specific ligand-binding sites found in different GPCRs families [Bockaert EMBO J 1999].

III.1.4 GPCR ligands.

➤ General classification.

Ligands or drugs that interact with GPCRs are classified according to their action when bound to the receptor. These specific ligands are able to stabilize different structural conformations of GPCRs. Based on their biological activity and they operating mode, these ligands can be divided into (Figure 8):

Agonist: a ligand that binds to and activates the receptor, and elicits specific biological effect (stimulation or inhibition)

- full agonist activates and triggers a maximal response of the receptor,
- partial agonist induces or stabilizes less productive conformations of the GPCR in comparison to that elicited by the full agonist,
- inverse agonist binds to the receptor and partially inhibits or fully eliminates (full inverse agonist) the basal/constitutive activity of the receptor (agonist-independent physiological response of the receptor),
- biased agonist very selectively stabilizes a subset of receptor conformational changes induced by natural “unbiased” ligand, thus preferentially activating only certain signaling pathways (through different types of G proteins or through β -arrestins) [Reiter Annu Rev Pharmacol Toxicol 2012],

Neutral antagonist: compound that binds to the receptor and blocks receptor-mediated action of other ligands, while maintaining receptor’s basal activity. Antagonists can interact with GPCRs either through the binding pocket of the agonist (defined as orthosteric site), or by another allosteric site [Kenakin Trends Pharmacol Sci 2004].

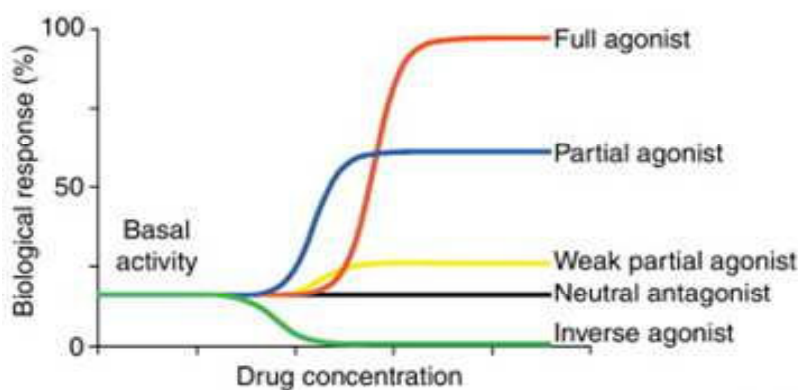


Figure 8. GPCRs ligands-classification.

Schematic representation of the functional consequences of the specific binding of ligands on the receptor’s activity [Tate Trends Biochem Sci 2012].

III.1.5 Therapeutic potential of biased agonists.

The concept of biased agonism (also referred to collateral efficacy, functional selectivity or stimulus trafficking) is related to the fact that the ‘efficacy’ of G protein-coupled

receptors (classically considered as linear) is pluridimensional [Rajagopal Nat Rev Drug Discov 2010]. Thus, GPCRs are able to activate diverse signalling pathways; through different heterotrimeric G proteins or through β -arrestins, and can adopt multiple active conformations upon agonist binding. As it was mentioned these biased agonists stabilize only a very selective subset of the receptor's conformational changes (Figure 9) [Reiter Annu Rev Pharmacol Toxicol 2012]. Due to their high selectivity and their precision of action, the biased ligands can target beneficial signalling pathways and block unwanted pathways, decreasing side effects, toxicity, and intolerance. Over the last decade, a diversity of biased ligands for GPCRs has been identified that selectively activate G proteins or β -arrestins. Interestingly, in the case of G protein signalling, biased ligands can preferentially activate signalling through one family of G proteins, often not considered as the canonical partner for a given GPCR. Thus, many of the biased ligands demonstrate distinct functional consequences compared to traditional ligands with a more linear efficacy [Whalen Trends Mol Med 2011].

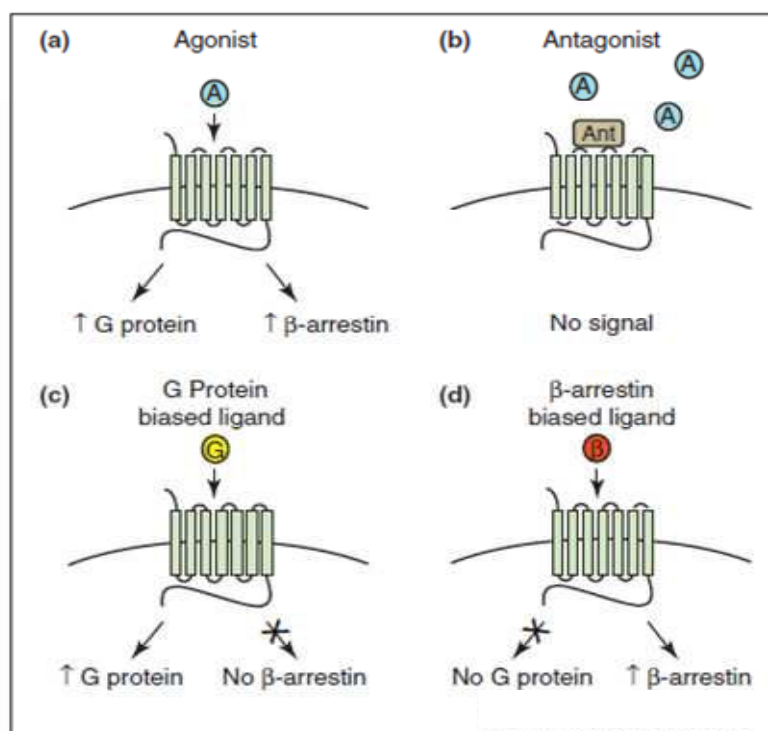


Figure 9. Differential GPCR-stimulated G protein- and β -arrestin-mediated signalling.

a) Traditional 'unbiased' agonist characterizes with a balanced activity for signalling through G protein and β -arrestins pathways. b) Neutral antagonist blocks all GPCRs' signalling pathways. c) G protein biased ligand promotes G protein signalling in the absence of β -arrestin activation. d) β -arrestin biased ligand promotes β -arrestin signalling and GPCR sequestration in the absence of G protein activation [Whalen Trends Mol Med 2011].

Due to their restrictive way of action, biased ligands seem to be promising therapeutic agents. There is a growing number of drugs showing pharmacological usefulness due to their biased agonist properties.

III.1.6 Project #1a: Pre-screening of potential biased agonists.

Due to the reason that biased agonists activate specific signal transduction pathways, most of them was not identified as agonists by classical G protein-based assays, and subsequently classified as neutral antagonists. It is likely that some drugs classified as neutral antagonists are actually biased agonists. The ability of a compound to behave as an antagonist for one signalling pathway and as an agonist for another requires currently an intensive screening of all known pathways, including the different families of G proteins and the β -arrestins. Nowadays, drug screening assays based on cell-lines are designed to detect signalling downstream of GPCRs (accumulation of second messenger molecules). Moreover, to test all the compounds one by one using standard cell assays is very laborious and time-consuming. The ideal solution would be to perform a primary screening that detects “hits” independently of downstream signalling processes.

Ion channel-coupled receptors (ICCRs) are able to detect the ligand-evoked conformational changes independently of G protein binding and activation. We therefore decided to investigate if this technology would be able to detect the binding of biased ligands. To test this possible application, we used the oxytocin receptor and its well-known biased agonist atosiban, as a model.

Human oxytocin receptor, activated by the neurohormone oxytocin, has been shown to activate three types of $G\alpha$ proteins: $G\alpha_q$, $G\alpha_i$, and, to a minor extent $G\alpha_s$, triggering diverse cellular responses. Atosiban is an oxytocin derivative, commonly used as a drug against preterm labor. Its binding to GPCR selectively stabilizes the conformation of the oxytocin receptor that activates only G_i protein and simultaneously blocks the G_q pathway (responsible for increased myometrial cells contractility) [Reversi J Biol Chem 2005]. Obtained by us results demonstrating potential of ICCRs in biased ligands detection are presented in the “Results” chapter.

III.1.7 Models of GPCR activation

Until now, several theoretical models have been proposed in order to explain the mechanisms of GPCR activation (Figure 10).

One of the oldest is a simple linear **Katz two-state model**, in which binding of the specific agonist (A) to an inactive receptor (R) results in the formation of the activated agonist-receptor (AR*) complex that triggers activation of the downstream effector proteins (cellular response) [Rajagopal Nat Rev Drug Discov 2010]. This model subsequently evolved into **an initial ternary complex model** proposed by Lefkowitz and his co-workers De Lean and Stadel in 1980.

The ternary complex model proposed that the activation of the receptor was followed by topographically distinct binding of the active-state receptor to the G proteins. Thus, the ternary complex including the extracellular ligand (agonist), transmembrane GPCR and intracellular G protein, serves as an activated signalling unit (AR*G) [De Lean J Biol Chem 1980].

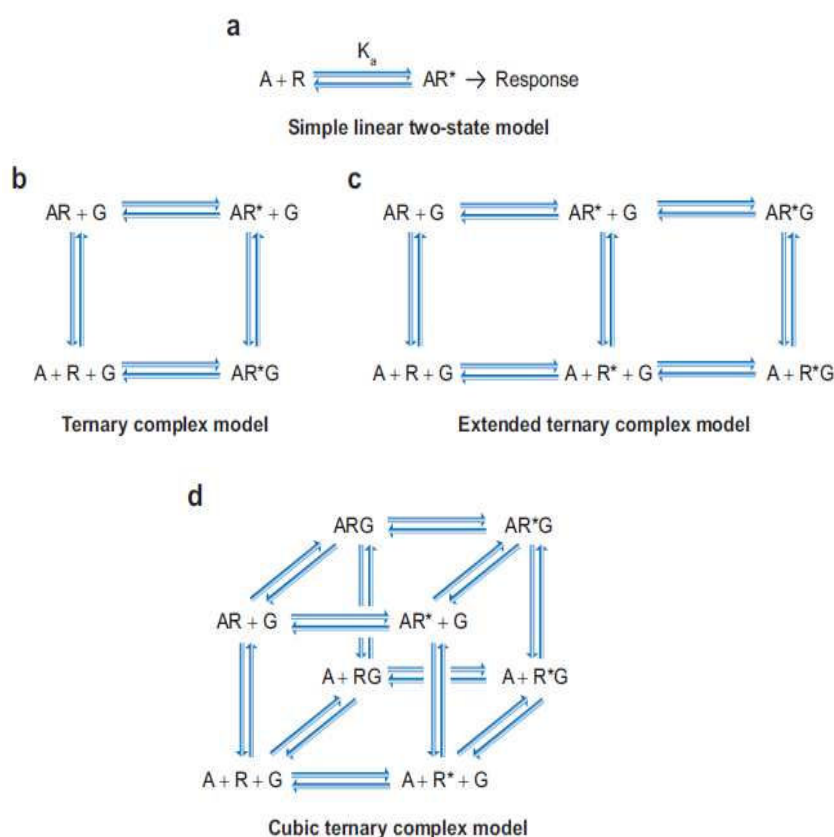


Figure 10. Different models of the signalling pathways of G protein-coupled receptors [Bridges ACS Chem Biol 2008]

GPCRs can be constitutively active. This shows spontaneous coupling of the receptor with G proteins in which the receptors are activated even in the absence of ligands. This observation made it clear that the classic model of receptor-G protein interaction was not sufficiently comprehensive. This resulted in the development of **the extended ternary complex model (seven-sided model)** proposed by Samama et al. This model proposes that the receptor exists in equilibrium of two functionally distinct states: the inactive (R) and the active (R*) state. In the absence of agonists, the level of the receptor activity is determined by equilibrium between these two states. Thus, receptor can spontaneously adopt an active conformation to interact and activate G proteins (R*G) in an agonist-independent manner. Moreover, the extended ternary complex model accounts for the effects of different classes of ligands on receptor signalling. The efficacy of ligands is thought to be the reflection of their ability to alter the equilibrium between these two states. Agonists (activating ligands) preferentially bind to the R* state of receptor, thus shifting the equilibrium on the side of the active state of the receptor. On the other hand non-activating ligands, like inverse agonists act inversely, by binding and stabilizing the inactive state of the receptor (R). Finally antagonists are recognized as the intermediate ligands demonstrating the same affinity for the receptor in R and R* states, and thus not inducing any change in equilibrium between both conformational states [Samama J Biol Chem 1993] [Gether J Biol Chem 1998].

Weiss et al. proposed a new equilibrium model of interaction between receptors, ligands and G proteins, called **the cubic ternary complex model (CTC)**. The CTC model is a generalization of the extended ternary complex model of Samama et al. It incorporates all the features of this model, but in contrast to it allows G proteins to bind to inactive receptors (R) [Weiss J Theor Biol 1996].

III.2 GPCRs-mediated signalling

III.2.1 Signaling through G Protein-Coupled Receptors

- Heterotrimeric G proteins.

The paradigm of GPCR signaling involves an activation of heterotrimeric guanine-nucleotide binding proteins (G proteins, $G\alpha\beta\gamma$). In the conventional model of G proteins activation, the binding of the specific ligand to the GPCR induces conformational changes not only in the structure of the receptor, but also in the structure of the cognate G proteins. This

results in a signalling cascade (Figure 11). An agonist-stimulated receptor activates as many as several hundred G proteins, what results in an amplification of the induced response [Lambright Nature 1996]. Moreover, the high amount of the second messenger produced by G protein-activated effector protein additionally enhanced cellular response [Robshaw Curr Opin Cell Biol 2004].

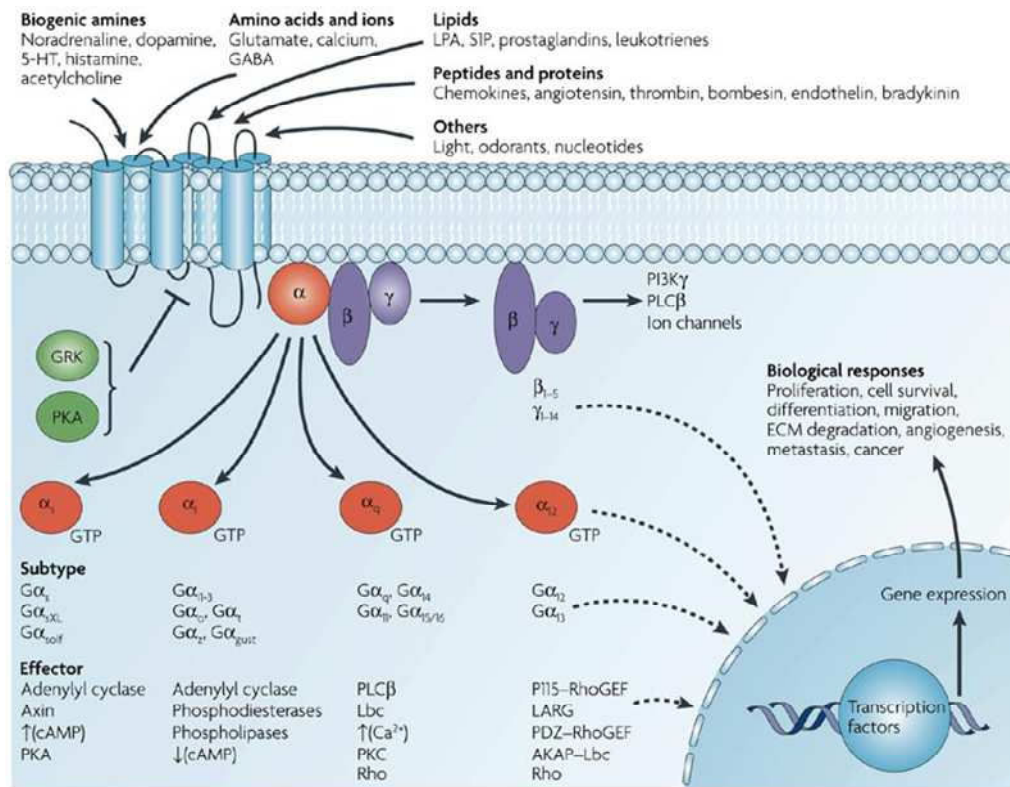


Figure 11. G protein-mediated signalling network of GPCRs [Dorsam Nat Rev Cancer 2007].

The inactive G proteins are composed of two tightly associated elements (high-affinity ~nM), GDP-bound Gα monomer (Gα·GDP) and an obligatory Gβγ heterodimer. In this complex Gβγ sequesters the switch II element on Gα in such a way that it is unable to interact with the second messenger effector proteins. However, agonist activation of the GPCR induces a conformational change, which subsequently catalyzes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) in Gα subunit. This way, GPCRs serve as guanine nucleotide exchange factors (GEFs) for Gα·GDP/Gβγ complexes. The release of GDP and subsequent binding of GTP induces conformational changes in the three flexible “switch regions” of Gα resulting in the disruption of the G protein-GPCR complex, and dissociation of Gβγ heterodimer from Gα·GTP. Subsequently liberated Gα·GTP and Gβγ

subunits relay signals to several downstream effectors (ion channels, adenylyl cyclases, phospholipases), which in turn generate second messenger molecules (ions, cAMP, IP₃, DAG), directly involved in physiological processes.

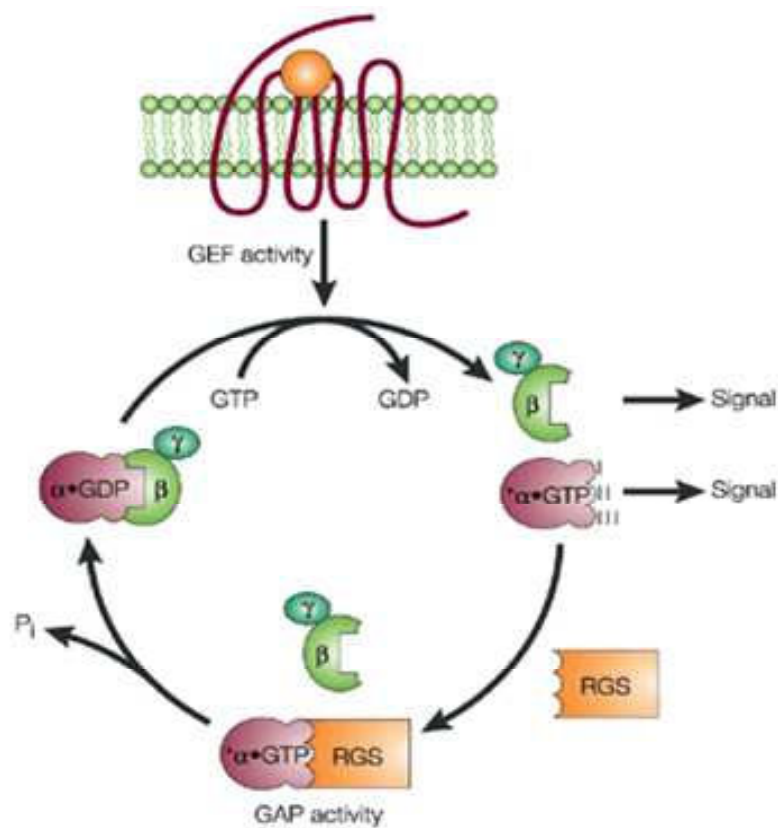


Figure 12. The guanine nucleotide cycle of heterotrimeric G proteins (Gαβγ).

Activation (by GEF activity and subunits dissociation) and deactivation (by GTP hydrolysis and subunits reassociation) is the reason why G proteins act as the “on/off” switch of GPCR signalling [Neubig Nat Rev Drug Discov 2002]

Deactivation of G protein-mediated signalling follows the intrinsic hydrolysis of GTP to GDP, which is catalyzed by the Gα subunit. This process can significantly be enhanced by the action of members of the superfamily called “regulators of G protein signaling” (RGS) proteins, which serve as GTPase-accelerating proteins (GAPs) [Siderovski Curr Biol 1996] [Johnston Mol Pharamcol 2007]. The inactivated, GDP-bound Gα subunit reveals low affinity for effectors, but high affinity for Gβγ what results in the reassociation of the Gαβγ heterotrimer capable once again of interacting with the activated receptor (Figure 12).

Though early models of GPCR signalling postulated that the effector activation occurred through a linear pathway (GPCR-> G protein-> effector), new models indicate the existence of numerous receptor- and G protein-binding partners able to modulate the signalling at each

level. The above-mentioned regulators of G protein signaling (> 30 family members, divided into six subfamilies) are among the most prominent of these newly appreciated binding partners [Siderovski Int J Biol Sci 2005] [Hendriks-Balk Eur J Pharmacol 2008]. RGS proteins directly bind to an activated $G\alpha$ ·GTP subunit to enhance its intrinsic GTPase activity with a minimum of 40-fold over basal levels, thus acting as the modulators of G protein signalling [Hollinger Pharmacol Rev 2002]. Interestingly, it has been shown that RGS proteins are, not only able to discriminate between specific $G\alpha$ subfamilies, but to discriminate as well between GPCRs coupled to the same $G\alpha$ subfamily [Bernstein J Biol Chem 2004] [Hague J Biol Chem 2005]. The N-terminal region of RGS proteins has been proved to be engaged in the specific interaction with the ICL3 or the C-terminal tail of the receptor [Hague J Biol Chem 2005]. This particular feature of RGS proteins allows, for the highly selective regulation of the receptor-mediated signalling.

➤ The α subunit of G proteins.

The $G\alpha$ subunit of G proteins contains three distinct structural components: (1) a Ras-like GTPase domain ($G\alpha$ Ras) consisting of six-stranded β -sheet surrounded by six helices (α 1- α 5 and α G), (2) a globular, α -helical domain ($G\alpha$ AH) consisting of a long central helix (α A) surrounded by five short helices (α B- α F), and (3) an N-terminal helix (α N) projecting away from the ‘core’ of $G\alpha$ (Figure 13) [Lambright Nature 1996]. The Ras-like GTPase domain contains most of the catalytic residues implicated in GTP hydrolysis as well as regions crucial for the interaction with $G\beta\gamma$ heterodimer and effector proteins [Sprang Annu Rev Biochem 1997]. The most extensive interface between $G\alpha$ and $G\beta\gamma$ heterodimer involves residues in the switch I and II regions of $G\alpha$ that interacts with the residues from the loops present in the top $G\beta$ domain. The second interface is between the N-terminus of $G\alpha$ and the side of the $G\beta$. In contrast to the strong interactions with $G\beta$, no direct interactions have been found for $G\alpha$ and $G\gamma$ [Lambright Nature 1996]. The guanine nucleotide tightly binds in a deep cleft between domains of the $G\alpha$ Ras and $G\alpha$ AH. The GDP- and GTP-bound states of $G\alpha$ characterizes with different structures in the both mentioned region, which are referred as ‘switch regions’. The $G\alpha$ subunit interacts with the intracellular regions of the receptor, including ICL2 and ICL3, and the C-terminus [Wess FASEB J 1997] [Gether Endocr Rev 2000]. This interaction predominantly involves the N- and C-terminus α 4- β 6 loops of $G\alpha$ [Oldham Nat Rev Mol Cell Biol 2008] [Chung Nature 2011]. Moreover, the C-terminus of the $G\alpha$ subunits penetrates into the cytoplasmic core of the transmembrane bundle of GPCRs. Upon activation, the ligand-bound receptor perturbs the structure of the amino-terminal region of the $G\alpha$ subunit and consequently alters the ‘P-loop’, (in the

nucleotide-binding pocket), that binds the β -phosphate of GDP. The stabilization of the 'P-loop', as well as, the coordination of the β -phosphate are key determinants of GDP binding affinity. Thus, structural changes evoked by the agonist-bound receptor and 'transmitted' through the N- and C-terminus of $G\alpha$ to the 'P-loop' region results in the release of the nucleotide [Chung Nature 2011].

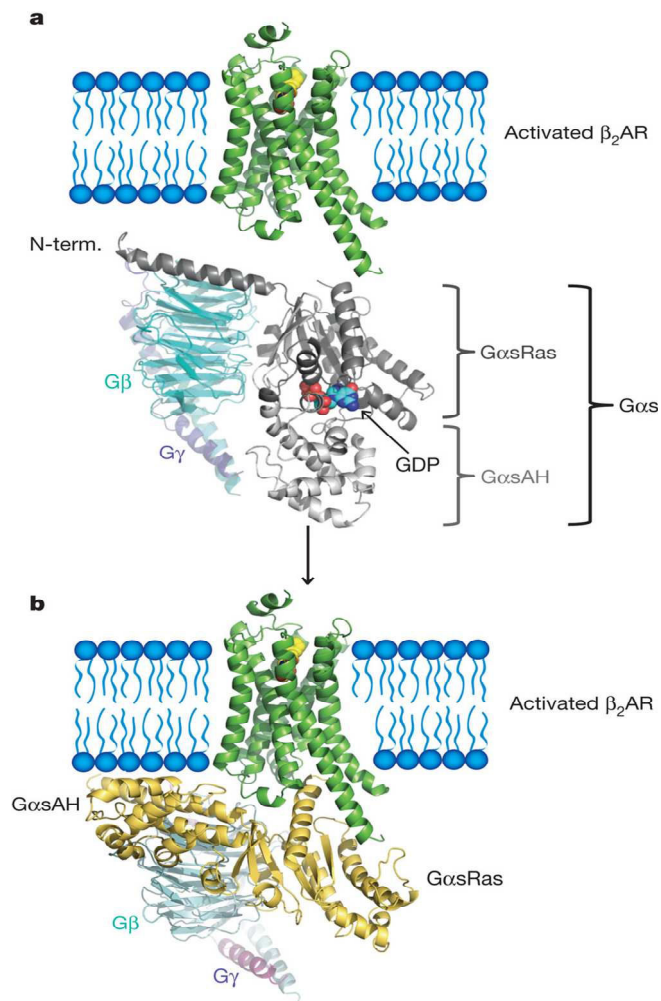


Figure 13. Structure of G protein heterotrimer (G $\alpha\beta\gamma$) alone and in association with β_2 -AR.

a) The nucleotide-binding G α subunit (shown in grey) is composed of Ras-homology domain (G α Ras) and α -helical domain (G α AH). The interface of these two G α subdomains forms the nucleotide-binding pocket. b) The crystal structure of an agonist-bound β_2 -AR with G α s protein reveals large domain movement in the α -subunit (yellow), associated with nucleotide release [Chung Nature 2011].

Based on their sequence homology and differential regulation of effectors, 23 subtypes of G α subunits (Table 1), encoded in human by 16 genes, were grouped into four main classes: G α_s , G $\alpha_{i/o}$, G α_q and G $\alpha_{12/13}$ [Hamm J Biol Chem 1998]. Their molecular weight varies between 39 and 46 kDa. All the G α subunits, with the exception of G α_t , possess palmitoylated cysteine

residue at its N-terminus (reversible modification). Moreover, the N-terminus of $G\alpha_i$ subunits is myristoylated (an irreversible modification) [Smotrys Annu Rev Biochem 2004].

The Gas family activates adenylyl cyclase, which in turn produces cyclic AMP (cAMP). The increase in cAMP level triggers the activation of cAMP-dependent protein kinases (Protein Kinase A, PKA), which through phosphorylation regulates the activity of other proteins. GTPase activity of α_s , α_t , and α_{Olf} is inhibited by the cholera toxin through the ADP-ribosylation mechanism.

The Gai/o family includes α_i , α_o , α_t and α_z subunits which can, among others, inhibit adenylyl cyclase (thus inducing a decrease in the level of cellular cAMP), or modulate in a negative or positive manner ion channels. ADP-rybosilation catalyzed by pertussis toxin results in the disruption of the coupling between G protein and receptor.

The Gq/11 family members trigger cellular response through the activation of phospholipases C- β (PLC) that hydrolyse PIP_2 (phosphatidylinositol-4,5-bisphosphate) to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 in turn triggers the release of calcium from intracellular stocks, whereas DAG activates the Protein Kinase C (PKC).

The G $\alpha_{12/13}$ family constitutes the smallest family among the $G\alpha$ proteins. Members of this family activate phospholipase A2 (generating arachidonic acid as a signalling molecule), c-Jun Kinase and the L-type (long-lasting) voltage-gated Ca^{2+} channels.

Type	Subtype	Effector protein
α_s	α_{SL1} , α_{SL2} , α_{SS1} , α_{SS2} , α_{Olf}	Adenylyl cyclase (+)
$\alpha_{i/o}$	α_{i1} α_{i2} , α_{i3}	Adenylyl cyclase (-), Cl^- and K^+ channels (+), Phospholipases C and A2 (+)
	α_{t1} , α_{t2} , α_{Gus}	GMPC Phosphodiesterase (+)
	α_{o1} , α_{o2}	Voltage-depenent Ca^{2+} channels (-), K^+ channels (+)
	α_z	Adenylyl cyclase (-)
$\alpha_{q/11}$	α_q , α_{11} , α_{14} , α_{15} , α_{16}	Phospholipase C (+)
$\alpha_{12/13}$	α_{12} , α_{13}	Phospholipase A2 (+), c-Jun Kinase (+), Ca^{2+} chnnels (L) (+)

Table 1. Subunits of $G\alpha$ proteins and corresponding effector proteins. (+) activation of the effector, (-): inhibition.

➤ The $\beta\gamma$ subunit of G proteins.

There are at least 5 genes encoding the $G\beta$ subunits and 12 genes for the $G\gamma$ -subunits [Cabrera-Vera Endocr Rev 2003]. The $G\beta$ subunit has an N-terminal helix followed by a repeating module of seven β -sheets (each with four antiparallel strands). Concerning the $G\gamma$ subunit, it contains two helices and does not show any tertiary structure. The N-terminal helix of $G\gamma$ interacts with the N-terminal helix of $G\beta$ (forming coiled-coil), whereas the other domains of the $G\gamma$ extensively interacts with the β -sheets of the $G\beta$ [Lambright Nature 1996]. The $G\beta\gamma$ subunit does not participate in the catalytic activity of G proteins, but can modulate the activity of numerous downstream effectors. Dissociated $G\beta\gamma$ subunits target a range of signalling pathways involving activation of ion channels, mitogen-activated protein kinases (MAPKs), phosphatidylinositol-3-kinase (PI3K), PLC β , adenylyl cyclase and small GTP-binding proteins [Cabrera-Vera Endocr Rev 2003][Schwindinger Oncogene 2001]. In addition, released $G\beta\gamma$ subunits recruit proteins involved in GPCR desensitization and downregulation [Desai J Pharmacol Exp Ther 2006].

Type	Subtype	Effector protein
β	β_1 - β_6	Phospholipase C and A2 (+)
γ	γ_1 - γ_{12}	Adenylyl cyclases: I (-), II and IV (+), GIRK channels (+), Na ⁺ channels (+), Ca ²⁺ channels (-) MAPK (+), PI3 kinase (+), c-Jun kinase (+) Protein Kinases C and D (PKC, PKD) (+)

Table 2. Subunits of $G\beta\gamma$ heterodimer and their effectors. (+) activation of the effector, (-): inhibition.

III.2.2 β -arrestin mediated signalling.

III.2.2.1 Arrestins: adaptors and transducers in GPCR signalling.

In mammalian cells, there are at least four functional members of the arrestin family: two visual arrestins (in rod and cone cells), present almost exclusively in the retina; and β -arrestin 1 (referred to arrestin 2) and β -arrestin 2 (arrestin 3), expressed in a wide variety of tissues [Luttrell J Cell Sci 2002]. The crystal structures of visual arrestin and subsequently obtained β -

arrestins revealed that these proteins are composed of two major domains, N and C domains, each of which is structured with a seven stranded β -sandwich (Figure 14) [Graznin Nature 1998][Hirsch Cell 1999]. The mutagenesis studies performed on visual arrestin suggested that the N domain is important for the recognition of light-activated receptor, whereas the C domain contains the secondary receptor-binding region [Gurevich J Biol Chem 1995]. Between these two domains, in the fulcrum of the arrestin molecule, a polar core is embedded. This core contains charged residues and has been shown to act as a phosphate sensor. In an inactive state, extended C-terminal tail of arrestins interacts with the sensor region, stabilizing this conformation of proteins [Hirsch Cell 1999][Nobles J Biol Chem 2007]. The interaction of arrestin with the phosphorylated form of the receptor ‘opens’ the protein. That open conformation of arrestins may provide an additional docking site for other proteins implicated in the regulation and signalling of GPCRs [Miller Curr Op Cell Biol 2001].

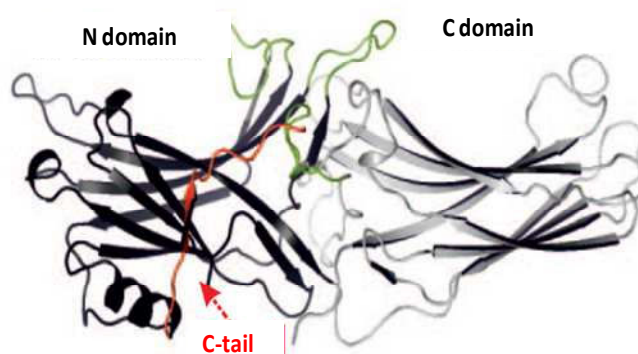


Figure 14. Structure of arrestin-1 (visual arrestin) in its basal (inactive) state (PDB entry 1CF1, molecule D) [Hirsch Cell 1999].

All members of the family can specifically bind to light- or agonist-activated GPCRs, previously phosphorylated by GRKs (G protein-coupled receptor kinases). The phosphorylation of the receptors by GRKs, subsequently followed by the recruitment of arrestins to an agonist-occupied receptors, often terminates signalling by steric block of the interaction between the receptor, and the G proteins [Koenig Trends Pharmacol Sci 1997][Barak J Biol Chem 1997].

However, it has been proved that arrestins activated by phosphorylated receptor, can also mediate G protein-independent signalling through recruitment of additional signalling molecules (Figure 15). Thus, they appear to be involved in termination and in initiation of different GPCR signalling events [Miller Curr Op Cell Biol 2001].

The proteins interacting with β -arrestins connect GPCRs to two major categories of signalling pathways. First, the interaction of β -arrestins with molecules such as clathrin, AP-2 (adapter protein 2) and NSF (N-ethylmaleimide-sensitive fusion protein) directs clathrin-mediated internalization of G protein-coupled receptors. The process of GPCR sequestration is important not only for attenuating of the signalization, but also for receptor resensitization and downregulation. On the second hand, it has also been shown that β -arrestins can act as a GPCR signal transducers. They constitute a second group of GPCR-activated signalling proteins and modulate the activity of downstream signalling networks.

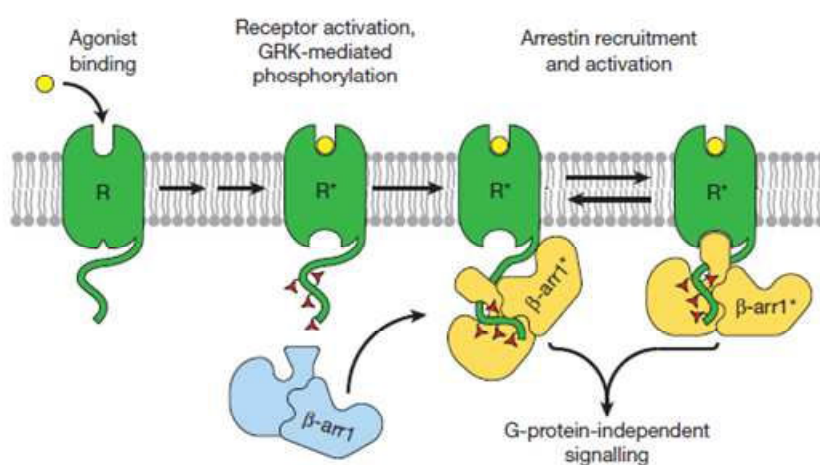


Figure 15. β -arrestin recruitment by agonist-activated GPCR.

Binding of the specific ligand induces phosphorylation of activated GPCR, leading to the binding of arrestins. Interaction between phosphorylated receptor and β -arrestin results in turn in its activation and subsequent blockage of G protein signalling and initiation of β -arrestin mediated signalling ^[Shukla Nature 2013].

Arrestins have been shown to be implicated in the regulation of mitogen-activated protein kinases (MAPKs) cascades through their direct interaction with molecules such as Src, ERK1/2, ASK1 and JNK3 ^{[Miller Curr Op Cell Biol 2001][Luttrell J Cell Sci 2002]}. The activation of the JNK (the c-Jun N-terminal kinases) pathway is critical for the stress response. The physiological importance of GPCR-mediated JNK activation is not clear. Nevertheless, it is known that cardiac hypertrophy, related to chronic GPCR (β 2-AR) stimulation leads to the activation of several JNK pathways. The second important β -arrestin mediated signalling pathway relates to the activation of the Extracellular-Regulated Kinases (ERKs). Modulation of this MAPK pathway typically involves the formation of multiprotein complexes which mediate the response to receptor stimulation. Recent studies have shown that several components of the ERKs pathway (mostly members of the Src kinase family) form complexes with β -arrestin,

via direct protein-protein interactions. This complex is recruited to the receptor in an agonist-dependent manner. The general importance of the β -arrestin mediated recruitment of Src family kinases is not well known, however it has been found that this direct interaction might play a crucial role in the signalling of numerous GPCRs (β 2-AR, neurokinin NK1 receptor or CXCR1) [Luttrell Science 1996][DeFea Proc Natl Acad Sci 2000] [Barlic Nat Immunol 2000]. Moreover, β -arrestins can also regulate the transcription process, either indirectly, through the regulation of signalling pathways that control transcription factors, or directly through β -arrestin activity in the nucleus [Rajagopal Nat Drug Discov 2010].

Thus, the range of β -arrestin-mediated activity is very wide, from its classical function in receptor desensitization, internalization and trafficking, to signalling via the control of different kinases signalling pathways, receptor transactivation and transcriptional regulation. Signalling mediated by β -arrestin proteins remarkably broadens the repertoire of GPCRs effectors in addition to these activated throughout the canonical pathways mediated by G proteins.

III.2.2.2 Structural insight into arrestin activation.

Despite the central role of arrestins in the regulation and signalling of GPCRs, a structural understanding of their activation and interaction with GPCRs was missing. Although it is known that the displacement of the C-tail of arrestins occurs when bound to the phosphorylated receptor, the molecular mechanisms of this process remained unknown. Recent structures of bovine arrestin-1 in a 'pre-activated' state and the β -arrestin 1 (arrestin-2), bound to a fully phosphorylated 20-aa C-ter peptide from the human V2 vasopresin receptor, provides detailed insights into the general molecular mechanism of the activation of these versatile proteins [Kim Nature 2013] [Shukla Nature 2013]. The comparison of these two structures with the structure of inactive (basal) state arrestin reveals significant differences.

In both arrestins structures it has been observed the significant breakage of the central core inducing rearrangements of key-receptor binding loops, including finger loop, middle loop 139 and gate loop, and rotation of $\sim 20^\circ$ for N and C domains relative to each other (Figure 16) [Shukla Nature 2013] [Kim Nature 2013]. Verification of these conformational changes with side-directed fluorescence spectroscopy allowed to put forward the mechanism for arrestin activation [Kim Nature 2013]. According to this mechanism, the displacement of the C-tail enables the transition of the critical polar core loops from restricted to extended conformations, thus allowing the interaction with the activated GPCR. Simultaneously, a significant increase in

the flexibility between the N and C domain facilitates the proper fitting of arrestin to the phosphorylated receptor and possibly expose the interaction interfaces with other binding partners of arrestins [Kim Nature 2013] [Shukla Nature 2013]

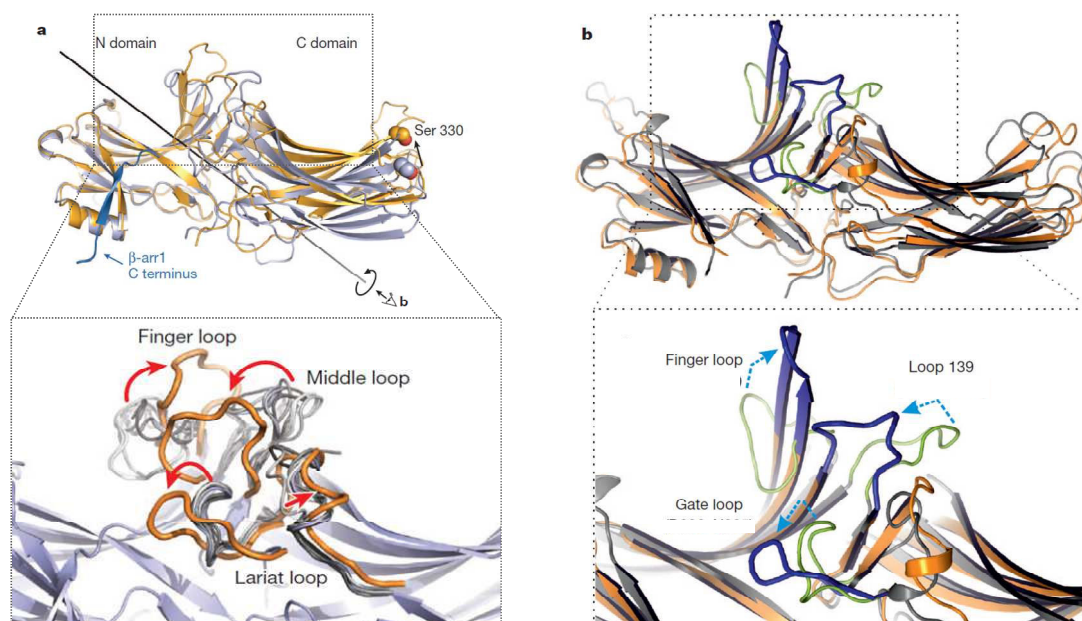


Figure 16. Structural comparison between the structure of the basal state arrestin and the pre-activated and activated state of the protein.

a) Superposition of inactive (grey) and active (gold) structures of β -arrestin 1, with the C-terminus highlighted in blue. In zoom: comparison of the inactive (grey) and active (orange) conformation of key-receptor binding loops in β -arrestin 1 [Shukla Nature 2013]. b) Superposition of basal arrestin-1 and pre-activated arrestin p44. The N and C domains of basal arrestin-1 are shown in grey and important loops in the polar core are green. Arrestin p44 is colored in orange with key loops highlighted in blue [Kim Nature 2013][Shukla Nature 2013]

III.2.3 The trafficking of GPCR: from the endoplasmic reticulum to the plasma membrane

The life cycle of GPCRs starts in the endoplasmic reticulum (ER), where they are synthesized, folded and assembled. Properly folded receptors that pass the quality control checkpoints are subsequently recruited and packaged into COPII-coated vesicles. These ER-derived vesicles, which carry the cargo receptors migrate from the ER and pass through the ER-Golgi intermediate complex (ERGIC), the Golgi apparatus and the trans-Golgi network (TGN). During trafficking, receptors undergo post-translational modifications (e.g. glycosylation). From the TGN, mature GPCRs get their place of destination: the cell's plasma membrane [Dong Biochim Biophys Acta 2007]

III.2.3.1 Motifs involved in the GPCR export from the ER and the Golgi apparatus

GPCRs possess conserved sequences/motifs essential for their exit from the ER and Golgi apparatus. These signals were mostly found in the C- and N-terminal parts of the receptor [Dong Biochim Biophys Acta 2007].

C-terminal motifs The implication of the C-terminal region of GPCRs, particularly its membrane-proximal part, has been demonstrated for numerous GPCRs. Mutagenesis studies of the C-termini have led to the identification of several motifs that play an essential role in the export of the GPCR from the ER [Dong Biochim Biophys Acta 2007]. One of them is the E(X)₃LL motif with two adjacent leucins and an upstream glutamate residue. These have been shown to be critical for cell-surface expression of the vasopresin V2 receptor (V2R) [Shulein Mol Pharmacol 1998]. Moreover, in the C-terminal part of two other human vasopresin receptors: V1b/V3, another motif consisting of dileucine and surrounding hydrophobic residues, FN(X)₂LL(X)₃L has been identified, that constitutes an ER export signal [Robert J Biol Chem 2005]. Another motif enabling GPCR departure from the ER, is a triple phenylalanine motif F(X)₃F(X)₃F in the C-terminus of the dopaminergic D1 receptor (D1R) [Bermak Nat Cell Biol 2001]. The last known ER export motif consisting of a phenylalanine and double leucine spaced by six residues, F(X)₆LL, has been shown to be more ubiquitous and involved in the ER export of a few GPCRs including: β 2-, α 2B- and α 1B-adrenergic receptors as well as angiotensin II type 1 receptor (AGT1R) [Duvernay J Biol Chem 2004].

N-terminal motifs The role of GPCRs N-termini in the regulation of their export from the ER has been much less investigated and is still quite controversial. The deletion of the N-terminal region of the receptors can trigger two opposite effects. For instance, it has been shown for endothelin B and α 2B-adrenergic receptors that cleavage of the N-terminal part reduces surface expression of those GPCRs [Dong Biochim Biophys Acta 2007]. In contrast to this observation, removal of the N-terminus facilitates transport to the cell surface of the α 1D-adrenergic receptors [Hague J Pharmacol Exp Ther 2004]. Strikingly, in the N-terminus of α 2-AR a first motif (consisting of tyrosine and serine, YS), which is crucial for export from the Golgi compartment has been identified [Dong Biochim Biophys Acta 2007]. Protein export from the Golgi to the plasma membrane is conventionally considered as a constitutive process. Nevertheless, some studies have proposed that this transport may be as well mediated, like in the case of the ER, through highly specific signals.

III.2.3.2 Homo- and heterodimerization in GPCR biosynthesis and maturation

The first piece of evidence strongly supporting the idea that dimerization of GPCRs can be an important prerequisite to pass the quality control checkpoints in the biosynthetic pathway was provided by studies of the metabotropic GABA_B receptor (family 3 of GPCRs). It has been shown that early heterodimerization between GABA_{B1} and GABA_{B2} isoforms is an obligatory step during biosynthesis for the expression at the cell-surface of a functional receptor [Marshall Trends Pharmacol Sci 1999]. When expressed alone, GABA_{B1} is retained in the ER. Heterodimerization of GABA_B receptor subunits masks a RXR-based ER retention signal present in the C-terminus of GABA_{B1}, thus allowing its export from the ER [Bulenger Trends Pharmacol Sci 2005]. The requirement of heterodimerization for proper cell-surface expression has been as well demonstrated for two related receptors from family 1: α 1D- and α 1B-adrenoreceptors. Only in this condition α 1D-AR can reach the surface of the plasma membrane [Hague J Biol Chem 2004]. Finally, it has been observed that the co-expression of the taste receptors (TR) T1R2 and T1R3, or T1R1 and T1R3 is indispensable to generate functional proteins, what strongly suggests that heterodimerization is as well mandatory for the biogenesis of these sensory receptors [Bulenger Trends Pharmacol Sci 2005]. Moreover it has been shown that the homodimerization of many GPCRs (e.g. melatonin, δ -opioid, CXCR4 and CCR5 receptors) is constitutive (receptor activation is not required) and occurs early in the biosynthetic process [Ayoub J Biol Chem 2002] [Issafras J Biol Chem 2002] [Babcock J Biol Chem 2003]. Thus, dimerization of GPCRs ('obligatory' heterodimerization, as well as homodimerization) appears to occur in the ER and might be a common requirement for GPCRs to pass the quality-checkpoints along their biosynthetic pathway (Figure 17).

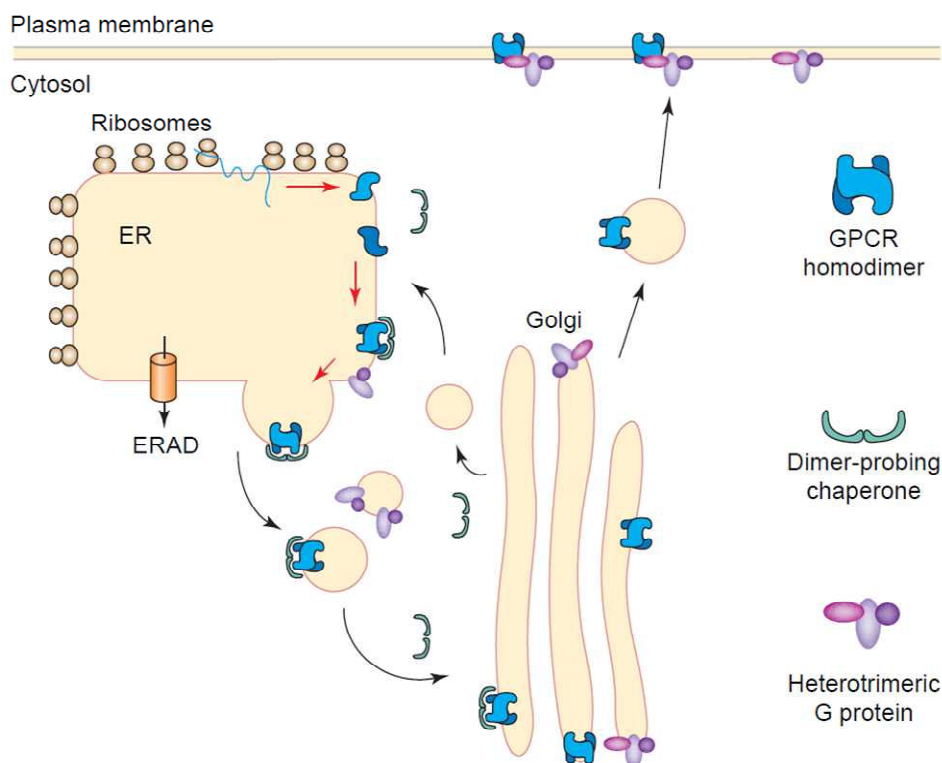


Figure 17. A model proposing that GPCR dimerization occurs during biosynthetic pathway.

According to this model dimerization is an essential step to pass the quality-control checkpoints. Nascent GPCR folding, dimerize in the ER and interact with cytosolic chaperone proteins. Unfolded or monomeric GPCRs are degraded by the ER-associated degradation pathway (ERAD) ^[Bulenger Trends Pharmacol Sci 2005].

III.2.3.3 Chaperone proteins

The interaction of newly synthesized GPCRs with chaperone proteins (e.g. calnexin, calreticulin, and BiP) stands as a critical step in the ER quality control and ERAD (endoplasmic reticulum-associated protein degradation) for the biosynthesis of GPCRs. This interaction modulates the availability of fully functional receptors in the plasma membrane ^[Kleizen Curr Opin Cell Biol 2004]. These proteins fulfill a dual function; they not only promote proper folding of the immature receptors, but also prevent the transport of misfolded GPCRs from the ER to the Golgi.

III.2.4 GPCR Oligomers in signal transduction

G protein-coupled receptors have classically been perceived as receptors that do not 'need' to dimerize. At the bottom of that assumption lies a paradigm that their heptahelical

structure enables a variety of movements and rearrangements that allow an efficient transfer of the activation signals across the plasma membrane of the cell. Moreover tightly packaged structure of these receptors seems to ensure ligand specificity as well as docking of cognate G proteins to the intracellular surface of activated GPCRs, thus providing suitable transduction mechanisms [Lohse *Curr Opin Pharmacol* 2010]. Indeed, many functional studies corroborate that monomeric signalling of GPCRs is possible and occurs with the physiological kinetics. It has been shown for β 2-adrenergic as well as for rhodopsin, and μ -opioid receptors (reconstituted in small lipid vesicles) that in a monomeric form they can efficiently couple to their respective G proteins [Whorton *Prot Natl Acad Sci* 2007] [Whorton *J Biol Chem* 2008] [Kuszak *J Biol Chem* 2009].

Nevertheless, during the last two decades, there has been a growing body of evidence that GPCRs can exist as dimers or higher oligomers which seem to play an important role in G proteins activation (signal transduction). The dimerization of 7TM receptors is clearly an essential mechanism of receptor activation for the glutamate family of GPCRs. These receptors, including the metabotropic mGluRs and Ca-sensing receptor, are homodimers linked to each other by disulfide bond at the level of their large extracellular domain [Bockeart *EMBO J* 1999]. It has been proved by crystallography that ligand binding induces changes in the dimer interface of the N-terminal ligand-binding domain [Kobilka *Biochim Biophys Acta* 2007]. Although members of the rhodopsin family of GPCRs can effectively transduce signals as monomers, numerous studies have suggested the existence of homo- and heterodimers, and higher oligomers in many GPCRs. For example, formation of receptor homodimers have been reported for the β 2-adrenergic receptor, the δ -opioid receptor, the dopamine D1, D2 and D3 receptors, and the chemokine receptors CCR2b, CCR4 and CCR5 [Gether *Endocr Rev* 2000]. In addition, it has been demonstrated that in the case of the purified leukotriene B(4) receptor, a pentameric complex is created when GPCR monomers were reconstituted with purified G_i proteins. This finding suggests that receptor homodimers are needed to form the complex with heterotrimeric G proteins [Baneres *J Biol Chem* 2003]. On the other hand, an intriguing example which indicates a functional relevance of heterodimerization between GPCRs subtypes has been reported for opioid receptors. It has been shown that heterodimers between two fully functional opioid receptors, δ and κ , result in a new receptor that displays distinct binding and functional properties compared to these of either of the receptors [Jordan *Nature* 1999].

Multiple studies exploiting a wide diversity of techniques (biochemistry, FRET, BRET, FRAP) have been pursued to investigate GPCRs dimerization/oligomerization events in the cell. Many contradictory results have been provided. Nevertheless, it seems that

dimers/oligomers of GPCRs, created either during the biosynthetic pathway or in the cell's surface promoted by ligand binding, appear to be very important for the activity of these receptors.

III.2.5 Cholesterol regulation of GPCRs' function

III.2.5.1 Cholesterol in biological membranes

Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in the membrane's organization, dynamics, function and sorting. Eighty-nine per cent of whole-cell cholesterol is contained within the plasma membrane, where it constitutes 20-25% of all lipid molecules [Chini J Mol Endocrinol 2009]. Cholesterol influences the functional properties of the plasma membrane by interacting with other resident lipids and proteins [Chini J Mol Endocrinol 2009]. It has been reported to modulate the conformation and hence, the function of integral membrane proteins either through 1) a specific and localized molecular interaction, or 2) due to alterations in the plasma membrane physical properties induced by its presence, or 3) a combination of both [Pucadyil Prog Lip Res 2006].

III.2.5.2 Few examples of GPCRs modulated by cholesterol.

The functional properties of many GPCRs have been shown to be regulated by the cholesterol molecules present in the plasma membrane. One of the best characterized receptors which function is strongly cholesterol-dependent is the oxytocin receptor (OXTR). It has been shown for human OXTR that high-affinity agonist binding highly depends on the cholesterol content in the plasma membrane [Klein Biochemistry 1995]. The specificity of cholesterol was determined by means of structure-activity analyses of a variety of steroids replacing cholesterol. Interestingly, only a few of them were able to restore the oxytocin receptor to its high-affinity binding state for an agonist in cholesterol-depleted membranes. This finding proved that the interaction of cholesterol with OXTR is of high specificity and not due to changes of membrane fluidity [Gimpl Biochemistry 1997]. Modulation of ligand binding affinity has also been shown for few others GPCRs including: galanin (GAL2), metabotropic glutamate (mGlu1), δ and κ opioid receptors [Pang Biochem 1999] [Eroglu Proc Natl Acad Sci 2003] [Huang Biochem Pharmacol 2007] [Xu J Pharmacol Exp Ther 2006].

Another GPCR which functional properties have been proved to be modulated by cholesterol molecules is bovine hippocampal serotonin_{1A} receptor (5-HT_{1A}). Interestingly, in

the case of this particular GPCR, it has been shown that the cholesterol modulates not only its ligand-binding activity, but also the receptor-G protein interaction. Depletion of the cholesterol induced concentration-dependent decrease in the specific binding of the agonist to 5-HT_{1A} receptor. Moreover, in cholesterol-poor membranes, the receptor displayed ~2.5-fold lower sensitivity to GTP- γ -S (non-hydrolyzable analogue of GTP) indicating a reduced extent of receptor/G protein interaction [Pucadyil Biochim Biophys Acta 2004].

Another mechanism of cholesterol effect on ligand-binding function has been demonstrated for the cholecystokinin receptor (CCK₂). Depletion of cholesterol from the plasma membrane resulted in a decreased binding of cholecystokinin. However, in contrast to the oxytocin receptor, agonist binding to CCK₂ receptor was re-established by each of the tested steroids. These results revealed that the ligand-binding of the CCK₂ receptor strongly depends on the membrane's fluidity, and not on the specific molecular interaction with cholesterol molecules [Gimpl Biochemistry 1997].

Moreover, another piece of evidence indicating the essential role of cholesterol was provided by crystallographic structure of β 2-adrenergic receptor revealing a specific cholesterol binding site for this receptor [Hanson Structure 2008].

III.2.5.3 Project #2: Cholesterol-dependence for GPCR function.

There is a growing number of GPCRs which functional properties have been proved to be modulated by the level of cholesterol in the plasma membrane. Today, the investigation of potential modulatory effects of cholesterol on GPCR functional properties is performed mostly with radioligand binding and saturation assays. These assays contain some complex steps, including membrane preparation and solubilisation, and subsequent usage of hazardous radioligands. Thus, there is a demand for simpler assay allowing direct detection of cholesterol effect. Due to this reason, we have decided to examine if Ion Channel–Coupled Receptors are able to detect cholesterol dependence of GPCRs. In this project, we used ICCR based on the oxytocin receptor, one of the most extensively studied GPCR in terms of cholesterol regulation. We tested the function of OXTR fused in ICCR in depleted and enriched in cholesterol environments using methyl- β -cyclodextrin (M β CD), a cholesterol complexing agent. The results are presented in the “Results” chapter.

III.3 Structural coverage of the GPCR superfamily.

Signal transduction by G protein-coupled receptors is fundamental for most physiological processes, from vision, smell, and taste to neurological, cardiovascular, endocrine and reproductive functions. This makes GPCRs major pharmaceutical targets. Thus, intensified trials have been pursued for many years in order to obtain atomic resolution structures for the representatives of this potent superfamily. The determination of GPCRs' structures constitutes a precious source for understanding the molecular mechanism of the receptor's activation, and it is important not only in fundamental biology, but holds as well great potential to enhance human health, by rational drug screening. Currently, modulation of the GPCRs' activity with drugs is used in the treatment of several diseases, including asthma, migraines and cardiac malfunction ^[Venkatakrishnan Nature 2013]. Nevertheless, the huge diversity of these receptors remains a challenge for the development of highly specific drugs involved in the treatment of neurological disorders, inflammatory diseases, or cancers. This is exemplified by the recent computational and experimental drug screening of subtype specific muscarinic ligands ^[Kruse Mol Pharmacol 2013].

III.3.1 Breakthrough in GPCRs crystallographic studies.

Until 2007, information about structural features of GPCRs was limited to the crystal structures of bovine rhodopsin and to structures of the extracellular domains of the Secretin (class B) and Glutamate (class C) families of these receptors. However, since 2007 there has been significant progress in crystallographic studies of GPCRs. By June 2013, structures of 19 different class A GPCRs have been determined in a complex with various ligands, antibodies and G proteins. Moreover, this year structures of GPCRs from other classes have been revealed such as the human smoothed receptor (SMO) which represents the Frizzled/Taste 2 family of GPCRs, as well as the glucagon receptor (GCGR) and the corticotropin-releasing factor receptor (CRF1R) that belongs to the Secretin family (class B) ^{[Wang Nature 2013][Siu Nature 2013][Hollenstein Nature 2013]}.

The breakthrough in GPCR crystallographic studies was initiated by the introduction of several innovative protein engineering techniques and crystallographic methods which in turn resulted in an almost exponential growth in the number of solved structures ^[Venkatakrishnan Nature 2013] (Figure 18). These protein engineering methods include:

Introduction

- co-crystallization with monoclonal antibody fragments from either mouse or camelids [Rasmussen Nature 2007] [Hino Nature 2012] [Rasmussen Nature 2011]
- thermostabilization of GPCRs throughout systematic scanning mutagenesis or by engineering disulphide bonds [Warne Nature 2008] [Lebon Nature 2011] [Standfuss Nature 2011]
- creation of the receptor-T4L lysozyme or receptor-apocytochrome chimeras which yielded the highest number of new GPCRs structures [Cherezov Science 2007] [Rosenbaum Science 2007] [Thompson Nature 2012] [Chun Structure 2012]

Moreover, required receptor stability was enhanced by the deletion of the flexible regions of GPCRs that perturb the crystallogenesis process and through the usage of the high-affinity/low off-rate ligands. Additionally, optimization of lipidic cubic phase and new detergents improved the quality of crystals [Caffrey Annu Rev Biophys 2009].

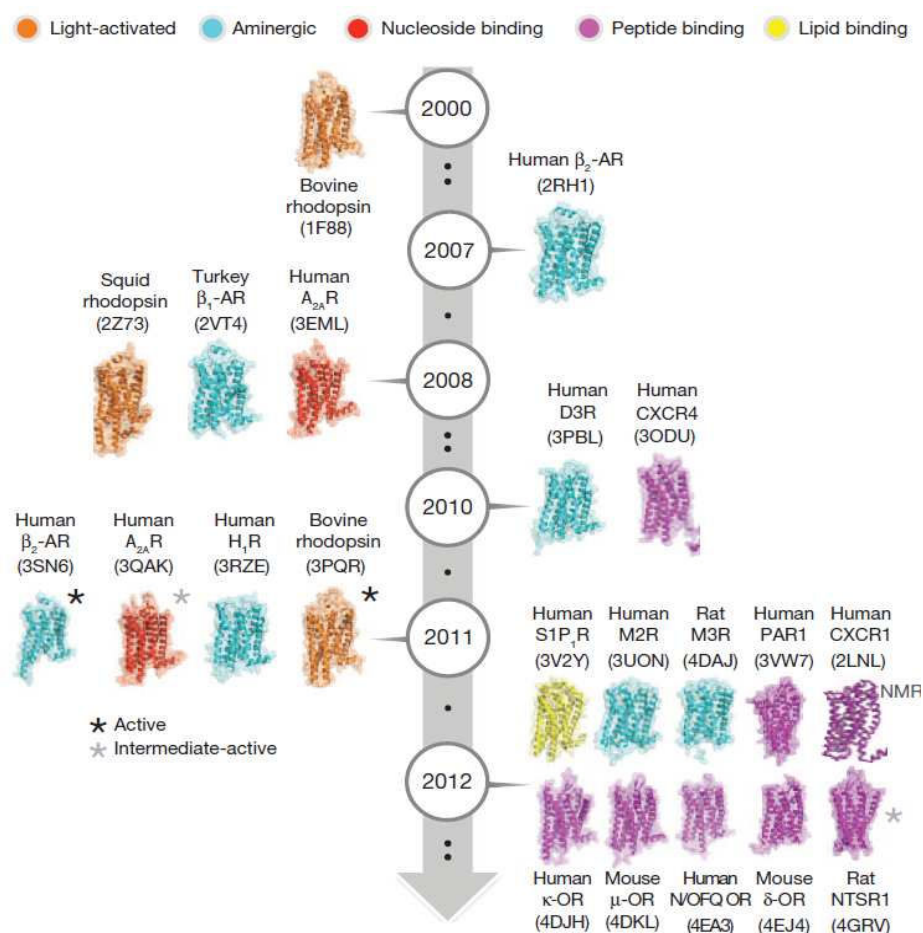


Figure 18. Time-line of GPCR structures.

Time line shows representative crystal structures of GPCRs and year of publication [Venkatakrisnan Nature 2013].

Approximately half of the solved GPCR's structures are aminergic receptors (class A, α -group). These GPCRs, that bind monoamine neurotransmitters and acetylcholine, have been shown to play a key role in the pharmacology and drug development [Katritch Annu Rev Pharmacol Toxicol 2013]. This group includes: the β -adrenergic receptors (human β 2- and avian β 1-AR), the muscarinic acetylcholine receptors (human M2R and rat M3R), the histamine H₁ receptor (human H₁R), the dopamine D₃ receptor (human D₃R) and two serotonin/5-hydroxytryptamine receptors (human 5-HT_{1B} and 5-HT_{2B}). Other available structures include bovine and squid rhodopsins, the human nucleoside-binding adenosine A_{2A} receptor (A_{2A}R), a lipid-binding human sphingosine-1 phosphate (S1P₁) receptor, and several members of the peptide-binding GPCRs: the human CXCR4 chemokine receptor, opioid receptors (human nociceptin receptor and κ -OR and mouse μ -OR and δ -OR), the rat neurotensin receptor (NTSR1) and the human protease-activated receptor (PAR1) [Venkatakrishnan Nature 2013] [Katritch Annu Rev Pharmacol Toxicol 2013]. The first structure of a non-class A GPCR was obtained with the human smoothed receptor (SMO) [Wang Nature 2013]. Moreover the human CXCR1 chemokine receptor is the first GPCR structure that was determined with NMR spectroscopy [Park Nature 2012]. The crystal structures of all the mentioned GPCRs (excluding NTSR1, 5-HT_{1B} and 5-HT_{2B} receptors) have been obtained in an inactive state in which receptors bound either the inverse agonist (reducing basal activity of GPCR) or neutral antagonist (maintaining the basal activity). Importantly, conformational differences between the multiple inactive-state structures of the same receptor were found to be minor and most key-features were preserved. This reproducibility is very important and allows establishing of a baseline for structural comparison between different GPCRs and between different functional states [Venkatakrishnan Nature 2013]. Moreover, the rat NTSR1, the bovine rhodopsin, the avian β 1-AR and the human: β 2-AR, A_{2A}R, 5-HT_{1B}R and 5-HT_{2B}R were crystallized with an agonist which induced an increase of the biological activity resulting in active (or intermediate-active) states of these receptors [Katritch Annu Rev Pharmacol Toxicol 2013].

III.3.2 Structural similarities and differences between GPCRs subfamilies and subtypes.

- The extracellular region and ligand binding pocket.

Sequence alignment of GPCRs demonstrates the very high diversity in the length and amino acid composition of the extracellular loops and N-terminus of GPCRs.

Crystallographic data corroborated this diversity in the extracellular region in terms of secondary structures and disulfide crosslinking patterns. Importantly, such variations in the extracellular loops (ECLs) and the proximal part of the 7TM helical bundle, create a variety of sizes, shapes, and electrostatic properties of the ligand-binding pockets in different GPCRs. Class A GPCRs structures revealed two distinct types of extracellular region: either these regions occlude the ligand-binding pocket or they leave it water-accessible. The rhodopsin and S1P₁ receptors characterize with the first type of extracellular region. They have occluded binding pockets, probably due to the reason that they bind hydrophobic ligands, which can easily enter into the receptor, from the lipid bilayer. A very crucial role in ligand-binding is attributed to ECL2, as in the case of the rhodopsin and S1P₁ receptor, where it has been shown to occlude the entry of the binding pocket together with the N-terminus domain [Venkatakrishnan Nature 2013]. In the receptors that bind soluble ligands, ECL2 can differ structurally but in all of them, it drives the route for the ligand entry into the binding-pocket. Molecular dynamics simulations suggest that ECL2 could be involved in the first steps of the ligand's recognition and selectivity in β 2ARs [Dror Proc Natl Acad Sci 2011]. In contrast to ECL2, ECL1 and ECL3 are relatively short and tend to lack secondary structures [Unal Trends Pharmacol Sci 2012].

A unique feature of the extracellular region is the presence of disulphide bridges implicated in the receptor's stability. There are several subfamily-specific disulphide bridges, but the one is crucial between the two cysteines in TM3 and ECL2, as it has been shown to be conserved in most GPCRs structures (except S1P₁). It has been proposed, that it anchors the EC site of TM3 near the ligand-binding site and restricts the range of conformational changes of this region upon GPCR activation [Venkatakrishnan Nature 2013].

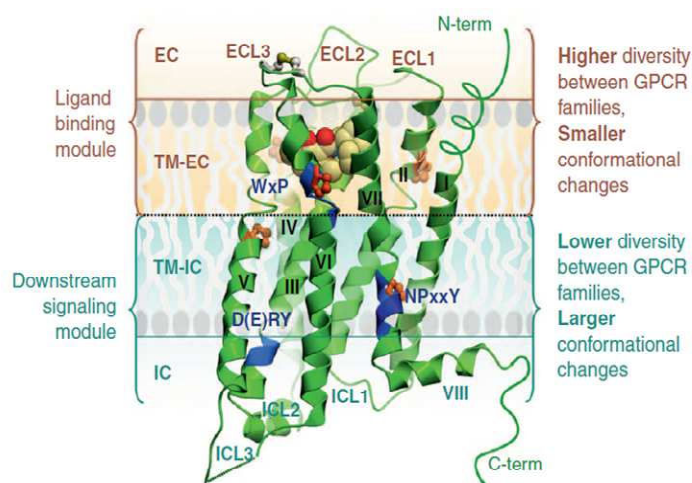


Figure 19. General architecture and modularity of GPCRs.

Numerous proline-dependent kinks in the structure of the 7TM core divide the receptor into two main modules. The EC module (ECLs and TM-EC regions) characterizes with higher structural diversity and is implicated in ligand binding. The IC module (ICLs and IC-TM regions) is more conserved among GPCRs and undergoes large conformational motions crucial for the activation of the downstream effectors (e.g. G proteins, β -arrestins) [Katrictch Trends Pharmacol Sci 2012].

- The conserved structural scaffold in the TM region.

The transmembrane helices bundle serves as a link between the extracellular ligand-binding site and the intracellular G protein-coupling region. GPCRs share very similar architecture of the seven TM helices which are held together by tertiary contacts. Nevertheless, the sequences of these helices vary among the GPCRs. The analysis of the different GPCRs' structure (in inactive, and active state) revealed a consensus network of 24 inter-transmembrane contacts mediated by 36 topologically equivalent residues [Venkatakrishnan Nature 2013]. The importance of these positions is highlighted by the fact that mutations in 14 out of 36 have been proved to induce either an increase or a loss of receptor activity [Madabushi J Biol Chem 2004]. Spatially, these inter-TM tertiary contacts are positioned mostly in the central and cytoplasmic part of the TM core and are clustered at the interfaces of TM1-TM2, TM3-TM4, TM3-TM5 and TM3-TM6-TM7.

- The structural and functional importance of the intracellular region.

Residues present in the intracellular region and C-terminus of GPCRs have been shown to directly interact with the downstream effectors, including the G proteins, β -arrestins and GRKs. In the currently available structures, ICL1 and ICL2 are rather short. Interestingly,

structures of muscarinic receptors and all opioid receptors revealed the presence of conserved Arg in the ICL2 (FRY motif) that form a salt bridge with the Asp residue present in TM3. This interaction allows tethering of the ICL2 to the TM helices bundle and seems to be important for the receptor's stability [Venkatakrishnan Nature 2013]. In the structure of β 2AR obtained in complex with cognate G_s protein, ICL2 was observed to directly interact with the N-terminus of the $G\alpha$ subunit [Rasmussen Nature 2011]. In contrast, the ICL3 and C-terminal tail are long, with variable regions and are probably intrinsically disordered in many GPCRs. During crystallographic studies these regions are deleted in order to stabilize the receptors, and to facilitate the crystallogenesis.

- The molecular changes during the receptor's activation.

Biochemical, biophysical, and structural studies suggest that some GPCRs exist in dynamic equilibrium between the inactive (R , R') and active states (R'' , R^*), which can subsequently be converted to the signalling state in the presence of G proteins (Figure 20) [Katritch Annu Rev Pharmacol Toxicol 2013]. The distribution of this state, in ligand-free receptors, varies and reflects different levels of their basal activity. The binding of neutral agonists does not affect the receptors' basal activity, whereas inverse antagonists shift the equilibrium towards inactive states (thus decreasing the basal activity of the receptor) [Nygaard Trends Pharmacol Scie 2009]. The most drastic changes are triggered by the binding of agonists which shift the receptor towards active states. Crystallographic studies revealed large-scale conformational changes at the receptors intracellular region. The existence of these different conformational states has until now been characterized crystallographically with three receptors: rhodopsin, β 2-AR and A_{2A} AR [Katritch Annu Rev Pharmacol Toxicol 2013].

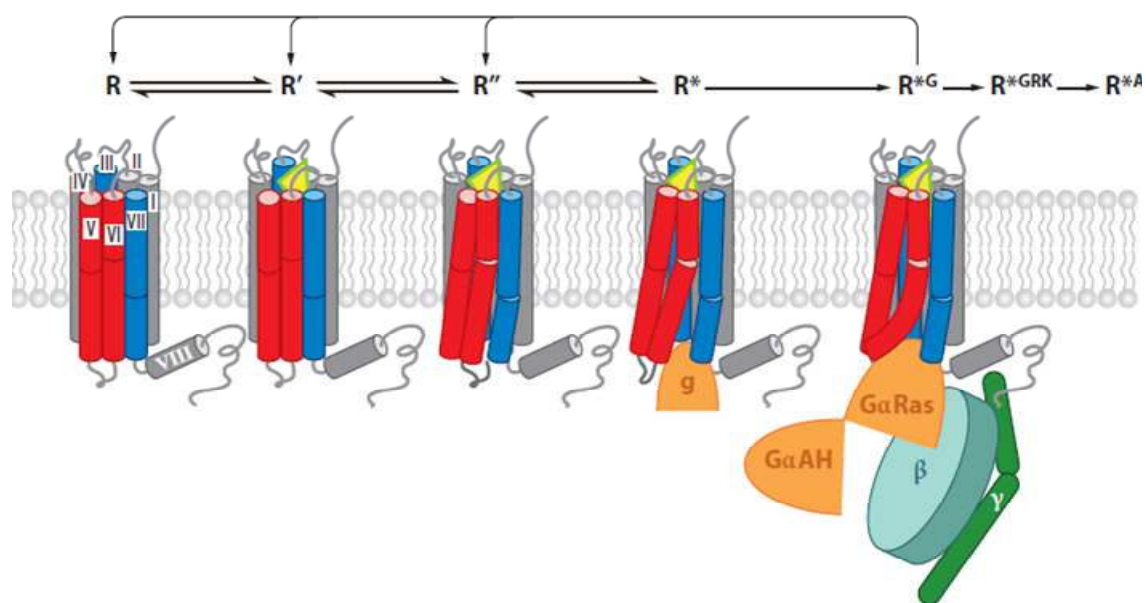


Figure 20. Key intermediate states in the GPCR activation mechanism, characterized by crystallographic studies.

R represents inactive states, which can be stabilized by the inverse agonists or neutral antagonists binding. R' represents inactive low-affinity agonist-bound states (characterized with small local changes in the ligand binding pocket). R'' represents activated state(s) with large-scale rearrangements of TM helices and side chain microswitches in the IC regions. R* represents activated substates which characterizes with initial insertion of the G protein C-terminus. Finally, R*G is a distinct signalling conformation of GPCR bound to G protein, which can exclusively be achieved upon the full interaction and activation of the GPCR-Gαβγ complex. Other conformationally distinct states are: R*GRK and R*A with the receptor bound to GRKs and β-arrestins, respectively ^[Katritch Annu Rev Pharmacol Toxicol 2013].

The comparison of active- and inactive-state structures of different GPCRs disclosed common activation-related changes on the extracellular site of the receptor. The strongest rearrangements have been shown for helices VI and V. The magnitude of these motions on the intracellular part varies among different receptors and different activated states, and can range from 3.5 Å (in A_{2A}AR) to 14 Å (in β₂-AR in complex with Gαβγ). Substantial rearrangements during the receptor's activation have been observed for helices III and VII as well. However, in contrast to helices V and VI, which movements are absolutely indispensable for G protein binding and activation and seem to be conserved in class A; the motions of helices III and VII are more specific to the particular receptor and applied ligand, and their role in G protein activation is still not so clear ^[Katritch Annu Rev Pharmacol Toxicol 2013].

During GPCR activation, global movements of the transmembrane core helices take place, but also local 'microswitches' in the intracellular part of the receptor. Their role has been proposed to stabilize the 'global' conformational changes and help in G protein binding.

The comparison between multiple active and inactive structures of GPCRs demonstrated also modest, but well-defined changes in the binding pocket residues of each crystallized receptor. The ligand-receptor interactions play a leading role in the controlling of the equilibrium between the receptor's functional states. Strikingly, these ligand-dependent changes vary dramatically among different receptors, but in the end induce common large-scale rearrangements on the intracellular part of different GPCRs [Venkatakrishnan Nature 2013].

Unquestionably, invaluable crystallographic informations were provided by the structure of $\beta 2$ -AR in the complex with $G\alpha\beta\gamma$ [Rasmussen Nature 2011]. This structure shows a series of additional conformational changes in the receptor which are controlled by the binding and activation of the full $G\alpha\beta\gamma$. Moreover, structure of $\beta 2$ AR- $G\alpha\beta\gamma$ revealed the large protein's interface between the receptor and $G\alpha_s$ subunit. This interface is formed by ICL2 and helices V and VI of the receptor as well as by two helices and one β -strand of $G\alpha_s$. Strikingly, the crystal structure of the complex did not demonstrate any interactions with the $G\beta\gamma$ subunits and no contacts of the G proteins with $\beta 2$ -AR helices VII and VIII, suggesting that the activation-induced changes in these helices might not be important for G protein activation but might possibly mediate other GPCRs' interactions e.g. with β -arrestins [Katritch Annu Rev Pharmacol Toxicol 2013].

III.3.3 Project #1: Functional assessment of GPCR(T4L) with the ICCR technology

Due to the reason that optimized-GPCR for crystallization, by T4L insertion in the i3 loop, are unable to activate the G proteins we examined if ICCR could stand as the tool for the characterization of global conformational changes of these engineered-GPCRs (with inserted T4L domain). Invaluable advantage of ICCRs is detection of global conformational changes of GPCRs, independently of downstream signalling pathway. Moreover, using unlabelled ligand, they generate real-time results including estimation of apparent affinities and efficacies. In our investigations we examined three engineered ICCRs based on $\beta 2$ -adrenergic, M2 muscarinic and oxytocin receptor.

III.3.3.1 The $\beta 2$ -adrenergic receptor

The $\beta 2$ -adrenergic ($\beta 2$ AR) receptor is a member of the Rhodopsin-like family (class A) of GPCRs [Fredriksson Mol Pharmacol 2003]. The human $\beta 2$ AR contains 413 amino acids. This

receptor is activated by natural agonists such as adrenaline and noradrenaline. Moreover, it is targeted by numerous synthetic molecules with therapeutic properties (e.g. timolol). The β_2 -adrenergic receptor binds predominantly the G_s class of G proteins. The activation and subsequent dissociation of G_s proteins stimulates adenylyl cyclase, catalyzing the formation of cAMP. Increase of intracellular cAMP triggers the activation of PKA which in turn phosphorylates numerous effector proteins assuring the spread of the intracellular signalling. The β_2 -adrenergic receptor has also been found to couple with G_i proteins. It has been shown that β_2AR - G_i coupling plays an important role in heart protection. Released $G\beta\gamma$ subunits activate phosphoinositol 3-kinase (PI3K)-Akt cell survival pathway (cardiomyocytes) [Zhu Proc Natl Acad Sci 2001]. In heart canonical β_2AR - G_s signalling is involved in the regulation of cardiac contractility and metabolism [Pérez-Schindler J Eur Pharmacol 2013]. Moreover, the β_2 -AR receptors are expressed in many other organs including lung, kidneys, liver and eyes, where they are implicated in a variety of functions.

In terms of crystallographic studies, the β_2 -adrenergic receptor is one of the most extensively studied GPCR. It has been crystallized in agonist- and antagonist- bound states, in the presence of a stabilizing nanobody (mimicking G protein) and in the complex with its cognate G_s protein [Cherezov Science 2007] [Rosenbaum Nature 2007] [Rasmussen Nature 2007] [Rasmussen Nature 2011] [Chung Nature 2011].

III.3.3.2 The M2 muscarinic acetylcholine receptor.

The M2 muscarinic receptor is a 466 amino acids protein that belongs to the Rhodopsin-like family [Fredriksson Mol Pharmacol 2003]. It is activated by the natural agonist acetylcholine, but as well by other molecules like muscarine or carbachol, which action is antagonized by atropine. The M2 receptor is one of the representatives of muscarinic acetylcholine receptors (mAChRs). There are four other subtypes named M1, M3, M4 and M5. The M2 receptor regulates the inhibition of adenylyl cyclase via G proteins of the G_i and G_o classes which leads to a decrease in the cellular cAMP levels [Ockenga Genes 2013]. This action of the receptor can be inhibited by the pertussis toxin, PTX, which catalyzes the ADP-ribosylation of the $G\alpha_i$ subunit that prevents G proteins from interaction with receptors. The $G\alpha_i$ subunits remain locked in the GDP-bound inactive state, thus are unable to interact with adenylyl cyclases and inhibit their activity. Moreover, M2 receptors play important role in the regulation of potassium conductivity via released $G\beta\gamma$ subunits of $G_{i/o}$ proteins. $G\beta\gamma$ bind to the cardiac potassium channels I_{KAch} , which are heterotetrameric proteins composed of

Kir3.1/Kir3.4 (GIRK1/GIRK4) subunits, and activate them ^[Karpivinsky Nature 1995]. In humans, the M2 muscarinic receptors are predominantly expressed in the heart where they slow the heart rate down to normal sinus rhythm by slowing the speed of depolarization after the stimulatory action of the parasympathetic nervous system at sinoatrial and atrioventricular nodes ^[Brass GPCR Pathways 2010]. The M2 receptors can also be found in some neurons of central and peripheral nervous system. In neuronal cells, mostly at the synaptic termini, liberated G $\beta\gamma$ subunits directly interact with high-voltage activated Ca²⁺ channels, and inhibit the calcium influx into the nerve cells and terminals. This mechanism is responsible for presynaptic muscarinic autoinhibition of Ach release in both central and peripheral cholinergic neurons ^[Krejčí Physiol Res 2004]. They are also localized in the bladder and the gastro-intestinal tract where they are involved in muscle contraction.

The structure of the human M2 receptor in the presence of the antagonist provides insights into allosteric and orthosteric regulation of muscarinic receptors ^[Haga Nature 2012]. Crystallographic data are invaluable especially due to the essential role of the M2 receptor in physiological control of cardio-vascular functions. Such structural information may facilitate the development of highly subtype-selective pharmacological agents that will act, either exclusively as allosteric ligands or as ligands that occupy both orthosteric and allosteric sites, and will limit side-effects. Works are currently in progress in this way ^[Kruse Mol Pharmacol 2013].

III.3.3.3 The Oxytocin Receptor.

The oxytocin receptor (OXTR) is a typical member of the rhodopsin-like (class A) GPCR family. Its natural agonist is the nonapeptide hormone, oxytocin. Oxytocin is produced in magnocellular neurons of the hypothalamus, and is secreted from the pituitary into the systemic circulation in response to a variety of stimuli ^{[Gimpl Physiol Rev 2001] [Gimpl Prog Brain Res 2008]}. The effect of oxytocin is antagonized by OVTA. OXTRs are functionally coupled to G_{q/11}, G_i and to a minor extent, to G_s classes of G proteins ^[Reversi J Biol Chem 2005]. Nevertheless signalling events induced by G_{q/11} activation have been extensively investigated. Agonist-induced activation of these proteins results in the stimulation of phospholipase C activity which is followed by the production of inositol trisphosphate (IP₃) and 1,2-diacylglycerol (DAG). IP₃ triggers Ca²⁺ release from the intracellular stores, whereas DAG stimulates the protein kinase C, which phosphorylates unidentified target proteins. Finally, in response to an increase of intracellular [Ca²⁺] a variety of cellular events are initiated ^[Gimpl Physiol Rev 2001]. The

physiological role played by OXTRs coupled to G_i proteins is far less clear. However, it has been shown that OXTR- G_i coupling may play an essential role in mediating an anti-proliferative effect [Rimoldi *Oncogene* 2003]. Moreover, G_i pathway has been proved to be exclusively activated by the biased agonist of OXTR, atosiban.

OXTRs were originally described in the uterus, mammary gland, and central nervous system. In the myometrium and endometrium of the uterus, the level of OXTR expression increases during pregnancy and peaks immediately before the onset of labor. Then, the receptor activation promotes uterine contractility (increase in intracellular Ca^{2+} concentration). The OXTRs in the mammary gland are located on myoepithelial cells, where they stimulate contractility and promote milk ejection. In the central nervous system oxytocin receptors are implicated in numerous processes. They modulate hippocampal synaptic plasticity during pregnancy. Moreover, they are as well modulators of social behaviors including sexual behaviors, bonding (affiliation) and maternal behavior, as well as stress, anxiety, social memory and recognition [Gimpl *Physiol Rev* 2001]. OXTRs have been detected in a vast number of tumor cells of various origin and have been proposed to be involved in the differentiation of cardiomyocytes and myoblasts [Riversi *J Biol Chem* 2005].

There is no atomic resolution structure of the oxytocin receptor, however, to our knowledge there are intensified trials to obtain it.

IV ATP-sensitive potassium channel (K_{ATP})

IV.1 The molecular composition and stoichiometry of K_{ATP} channels.

K_{ATP} channels are formed by the unique association of two dissimilar proteins. They are composed of the sulfonylurea receptor (SUR, ~140-170 kDa) belonging to ATP binding cassette (ABC) transporter family, and the inwardly rectifying potassium channel (Kir6.x, ~40 kDa) (Figure 21). The SUR and Kir6.x channel are obligatory partners and until now, there is no evidence that they are able to function alone outside of the K_{ATP} complex.

In mammals two genes encode the SUR subunit, ABCC8 (SUR1) and ABCC9 (SUR2) located on two different chromosomes (11 and 12 respectively). Moreover, gene encoding the SUR2 protein undergoes alternative splicing resulting in two isoforms of the protein: SUR2A and SUR2B. Three isoforms of the SUR receptor characterizes with different tissues distribution, with SUR1 predominantly expressed in pancreas, SUR2A in heart and skeletal muscles, and SUR2B in smooth muscles of blood vessels.

In the case of Kir6.x channels there are two isoforms: Kir6.1 and Kir6.2, encoded by the KCNJ8 (in human chromosome 12) and KCNJ11 (chromosome 11) genes respectively. Kir6.1 channel is expressed mostly in smooth muscles, whereas Kir6.2 characterizes with more widespread distribution including pancreas, brain, heart and skeletal muscles.

The K_{ATP} channel adopts an octameric conformation. Four Kir6.x subunits assemble to form the K⁺-selective pore which is surrounded by four SUR subunits, playing a regulatory role. The simultaneous presence of both subunits has been proved to be essential for the presence of functional K_{ATP} channels in the plasma membrane [Inagaki Genomisc 1995]. Co-immunoprecipitations studies confirmed the physical association between the SUR and Kir6.2 subunits of cardiac K_{ATP} channels [Lorenz J Mol Cell Cardiol 1999]. Moreover it has been demonstrated with the fusion constructs of Kir6.2 and SUR1, that the 1:1 stoichiometry is necessary and sufficient for the assembly of the active K_{ATP} channels [Clement Neuron 1997][Inagaki FEBS Lett 1997]. The octameric structure of the K_{ATP} channel was confirmed with biochemical studies demonstrating that the molecular weight of the purified complex is around 950 kDa. Additionally, this stoichiometry and architecture was supported by a low resolution (18 Å), three-dimensional structure of K_{ATP} channel obtained with single-particle electron microscopy which confirmed the existence of the octameric complex with four Kir6.2 subunits constituting the central pore surrounded by the four SUR subunits [Mikhailov EMBO J 2005].

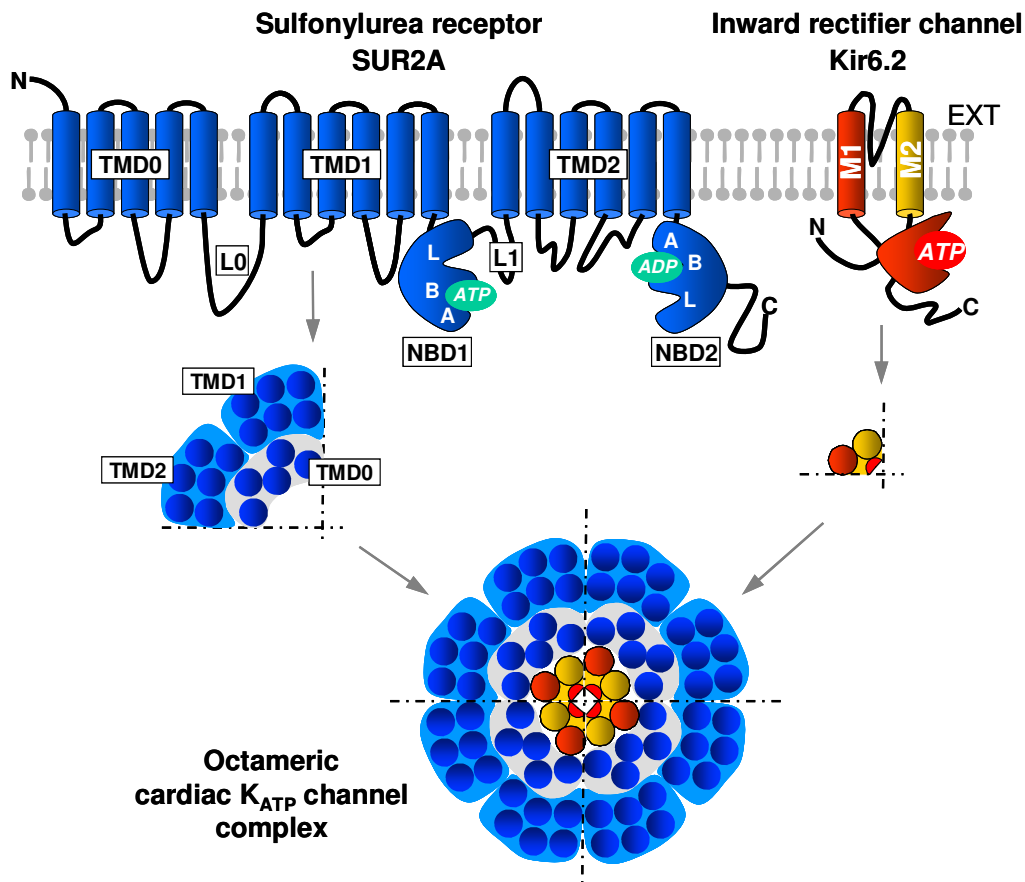


Figure 21. Topology and stoichiometry of the K_{ATP} channel.

Four subunits of the inwardly rectifying Kir6.2 channel associate with four subunits of the sulfonylurea receptor forming a functional K_{ATP} channel. Kir6.2 has two transmembrane helices (TM1 and TM2) and a large cytoplasmic domain containing an ATP-binding site. The SUR subunit is composed of three transmembrane domains (TMD0, TMD1 and TMD2) and two nucleotide-binding domains (NBD1 and NBD2) enclosing the Walker A, Walker B and Linker L consensus sequences ^[Moreau J Mol Cell Cardiol 2005].

IV.2 Physiological relevance.

The K_{ATP} channels link cellular metabolism to electrical activity of the plasma membrane and are found in a variety of tissues, including endocrine cells (pancreas), neurons, cardiac, skeletal and smooth muscles. Both subunits of K_{ATP} , SUR and Kir6.x, contribute to the metabolic regulation of the channel activity through the nucleotides. Binding of ATP to Kir6.x results in channel inhibition, whereas binding of Mg-nucleotides (MgADP and MgATP) to SUR subunits stimulates channel opening ^[Tucker Nature 1997]. These metabolism sensing proteins are involved in the control of major physiological processes, including glucose-dependent secretion of insulin from pancreatic β -cells, glucose uptake in skeletal muscles and cardio-protection during ischemia. Metabolic inhibition leads to K_{ATP} channel

opening, hyperpolarization and suppression of cellular responses, such as insulin secretion or muscle contractions. On the other hand, enhanced cellular metabolism triggers closure of the K_{ATP} channel, depolarization and stimulates insulin secretion/muscles contraction [Miki J Mol Cell Cardiol 2005].

IV.3 Inwardly rectifying potassium channels: Kir

IV.3.1 General characteristic of Kir channels.

Inwardly rectifying potassium channels (Kir, inward rectifiers) constitute a large family of voltage-independent K^+ channels, structurally and functionally distinct from K_V channels. Kir channels have two main physiological roles: they stabilize the resting membrane potential close to K^+ equilibrium potential and mediate transport of K^+ across the membranes [Nichols Annu Rev Physiol 1997] [Doupnik Curr Opin Neurobiol 1995].

To date, 15 Kir channels have been identified and classified into seven subfamilies (Kir1.x to Kir7.x), identified by distinct properties such as the degree of rectification, unitary conductance, and their sensitivity to different mediators. These subfamilies can be categorized into four functional groups: (1) classical Kir channels (Kir2.x), (2) G protein-gated Kir channels (Kir3.x), (3) ATP-sensitive K^+ channels (Kir6.x), and (4) K^+ -transport channels (Kir1.x, Kir4.x, and Kir7.x) (Figure 22) [Hibino Physiol Rev 2010]. Kir1.x plays a crucial role in the kidney, where it is involved in transepithelial transport. Kir2.x is implicated in the excitability control of the heart and the brain [Reimann Curr Opin Cell Biol 1999]. Kir3.x channels (G protein-activated), mediate the effects of some GPCRs on electrical activity in cardiac, neuronal and neurosecretory cells [Yamada Pharmacol Rev 1998]. In turn, Kir4.x can exist as homo- or heteromers with Kir5.x. It is expressed in glia cells of the brain, cochlea and kidney. In the kidney, Kir4.x/Kir5.x heteromers are involved in the reabsorption of the Na^+ by supplying K^+ to the extracellular site of the Na^+-K^+ ATPase, thus maintaining its activity [Hibino Physiol Rev 2010]. The Kir7.x channels are expressed in epithelial cells; however their physiological functions are largely unknown. Nevertheless, their localization suggests a role in cellular ion transport mechanisms [Hibino Physiol Rev 2010].

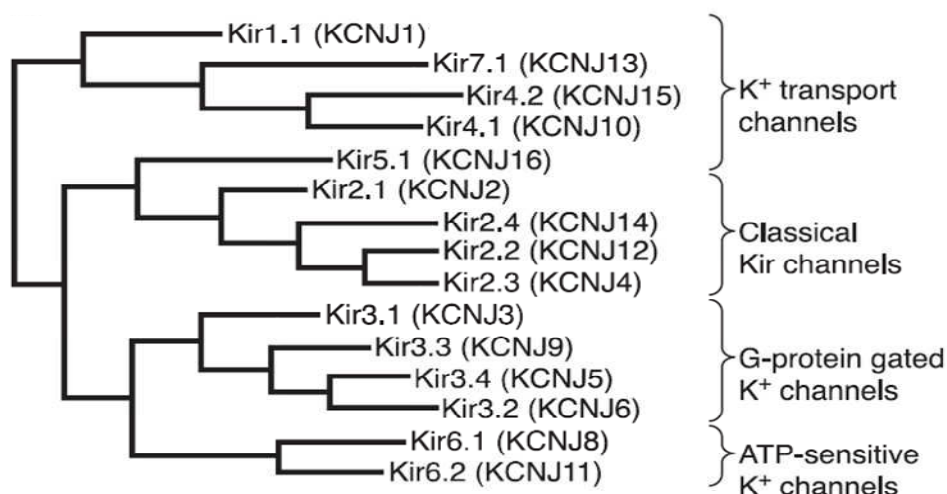


Figure 22. Kir channel phylogenetic tree.

Alignment of amino acid sequences and phylogenetic analysis allows the grouping of 15 known Kir channels into seven subfamilies (Kir1.x to Kir7.x) that can be categorized into four functional groups [Hibino *Physiol Rev* 2010].

IV.3.2 Rectification.

Inward rectification is a term that describes the fact that inward current evoked by a given hyperpolarisation is greater than that elucidated by a depolarization of the same amplitude (Figure 23). Thus, under physiological conditions, when the membrane potential (V_m) is depolarized, outward conduction of K^+ ions is low, whereas in hyperpolarizing conditions the inward flux of K^+ is much stronger [Lu *Annu Rev Physiol* 2004]. The inward rectification results from the rapid and highly voltage-dependent block of outward currents by cytoplasmic cations (mainly Mg^{2+} and polyamines) that move into the inner mouth of the pore under the influence of the voltage field and block the outward flux of K^+ ions [Nichols *Annu Rev Physiol* 1997] [Doupnik *Curr Opin Neurobiol* 1995]. Two residues appear to be important for Mg^{2+} and polyamines blocking: one is located in the TM2 (transmembrane segment 2) and the second one in the C-terminus of the channel [Lu *Nature* 1994] [Yang *Neuron* 1995]. All Kir channels show inward rectification however, some of them, for example Kir2.1 and 3.1, are strong rectifiers while others, such as Kir1.1 and Kir6.2 are weak inward rectifiers. It has been demonstrated that the strength of rectification depends on the amino acid residue present in the TM2. This amino acid (position 171 in Kir1.1) is neutral (asparagine) in weak rectifiers, but negatively charged (aspartate in position 172 in Kir2.2) in strong rectifiers [Stanfield *J Physiol* 1994] [Lu *Nature* 1994]. It has

been shown that the substitution of aspartate for asparagine allows to convert weak inwardly rectifying Kir1.1 channel into a strong rectifier [Lu Nature 1994].

Thus the interaction of the intracellular cations with two residues in TM2/C-terminus induces an inward rectification. A proposed mechanism of this phenomenon suggests that polyamines bind to the C-terminal site, which in turn become more positively charged and acts as the inactivation domain that plugs the pore from its cytoplasmic side. The TM2 side is proposed to be part of the inactivation domain [Reimann Curr Opin Cell Biol 1999].

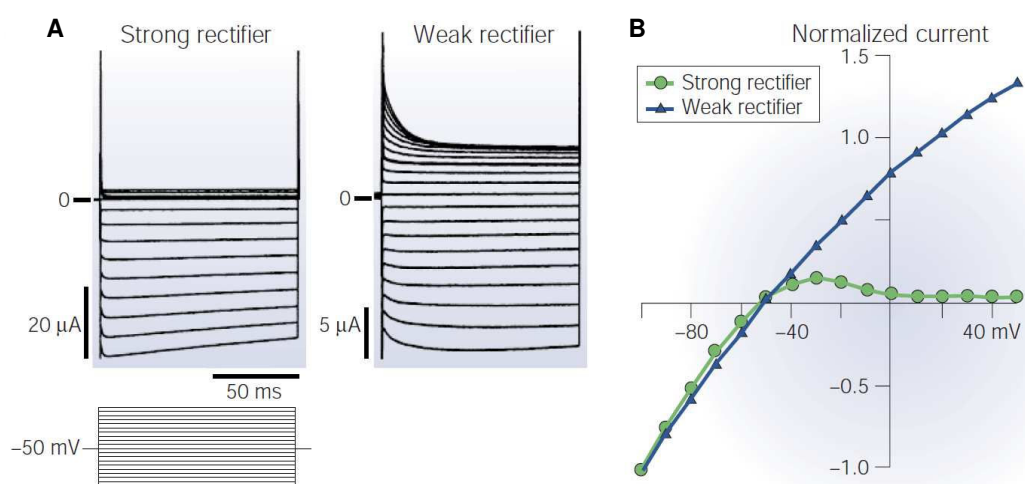


Figure 23. Inward rectification.

Inwardly rectifying channels enable more current to pass in response to a hyperpolarizing voltage (negative potentials) step than to a depolarizing (positive potentials) step of the same amplitude. a) Currents elicited by a series of voltage steps (from -140mV +50 mV) recorded from *Xenopus* oocytes expressing wt strong rectifier Kir2.1 channel and its weakly rectifying mutant. b) Associated current-voltage relation (I-V curve) [Bichet Nat Rev Neurosci 2003].

IV.3.3 Structure of Kir channels.

Kir channels are tetramers of four identical (homomeric) or related (heteromeric) subunits. Kir1.x, Kir2.x and Kir6.x probably exist *in vivo* as homotetramers. In contrast, Kir3.x channels have been shown to create heterotetramers, composed of Kir3.2 and Kir3.4 subunits in brain, and Kir3.1 and Kir3.4 subunits in heart [Kofuji Proc Natl Acad Sci 1995] [Karpivinsky Nature 1995]. However, it has been shown that homomeric Kir3.2 and Kir3.4 complexes might occur *in vivo* [Corey J Biol Chem 1998]. In general, heteromerization takes place mostly between two members of the same subfamily rather than between members of different families. An interesting exception are the heterotetrameric channels composed of Kir4.1 and Kir5.1 subunits that have been shown to create functional complexes in glia and kidneys [Hibino Physiol Rev 2010]. Nevertheless, the most unique subfamily constitutes Kir6.x channels that co-assemble

with SUR subunits in order to form fully functional K_{ATP} channels [Sakura FEBS Lett 1995] [Clement Neuron 1997].

A breakthrough in ion channel studies was the atomic-resolution structure of the bacterial K^+ channel (KcsA) from *Streptomyces lividans*. The crystal structure, though a bit fuzzy, however revealed the molecular design of the pore (Figure 24). It showed that KcsA channel is a homotetramer with four identical subunits creating an inverted teepee. Each subunit is composed of two transmembrane helices (TM1 and TM2) which are linked together by a short stretch, ~30 amino acids, that form a turret (extracellular loop), a pore helix and a selectivity filter (containing conserved signature of potassium channels, TV-GYG) [Doyle Science 1998]. The narrow selectivity filter has a tunnel-like shape and is lined with carbonyl oxygen atoms from main chains which provide multiple closely spaced sites allowing proper coordination of dehydrated K^+ ions [Doyle Science 1998] [Zhou Nature 2001].

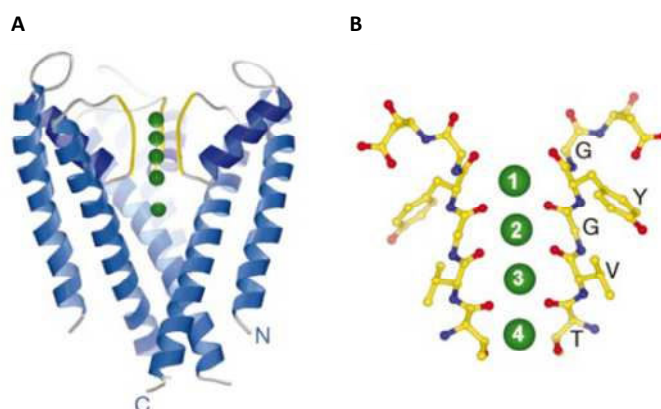


Figure 24. Binding sites for K^+ ions in the KcsA K^+ channel.

a) Ribbon representation of the KcsA channel with K^+ ions bound at four locations in the selectivity filter and in the water-filled cavity. b) Close-up view of the selectivity filter. The four ions are numbered to indicate the location of the binding site in the filter; position 1 is the closest to the extracellular solution and position 4 is the closest to the cavity [Zhou Nature 2001] [Morais-Cabral Nature 2001].

An important step in studies of inward rectifiers was the crystal structures of the bacterial homolog, KirBac1.1 (Figure 25). This atomic-resolution picture revealed that KirBac1.1 can be structurally divided into five regions. The extracellular part is the selectivity filter, followed by the water-filled cavity, the gate, flexible linkers and the cytoplasmic vestibule. It has been found that the ion conduction pathway at the intracellular face of the membrane (the bundle crossing) contains physical constriction created by the hydrophobic side chains of four phenylalanines (Phe146). These phenylalanines have been proposed to

create a so-called ‘activation’ gate (bundle-crossing gate). The sequence alignment of other Kir channels indicates that residues with large hydrophobic, aromatic or aliphatic, side chains are favoured in this position [Kuo Science 2003].

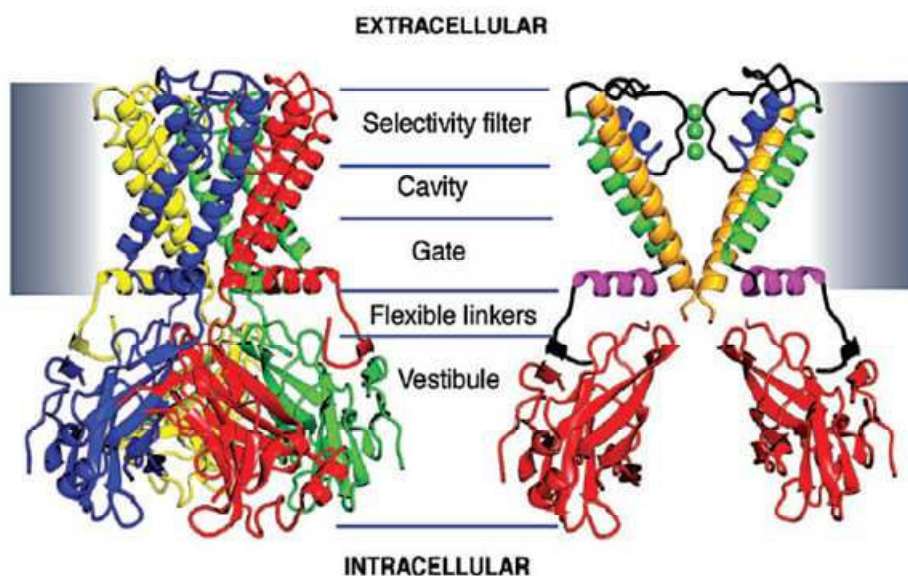


Figure 25. Overview of the KirBac1.1 structure.

Five structural elements can be distinguished: the pore helix (blue), the inner helix (yellow), the outer helix (green), the slide helix (pink) and the C-terminal intracellular domain [Kuo Science 2003].

Another milestone achieved in the studies of the inwardly rectifying K^+ channels was the crystal structure of eukaryotic Kir2.2, a typical strong rectifier channel [Tao Science 2009] [Hansen Nature 2011] (Figure 26). The main-chain structure of the filter in Kir2.2 is the same as in other K^+ channels [Zhou Nature 2001] [Kuo Science 2003]. However, at a detailed structural level, Kir2.2 is quite different from the prokaryotic Kir channels. The first substantial difference is presence of the extracellular cysteine pair that is absolutely conserved among all eukaryotic Kir channels. These cysteines create a circularized pore region through covalent linkage of the segment preceding the pore helix (C123) and the segment following the selectivity filter (C155). It has been proposed that the disulfide bridge is important not only for the proper folding of the channel, but as well for its function (Figure 26b). Besides disulfide bond, the second new protein interaction, is created by the ionized hydrogen bond between the arginine (R149) in the filter sequence T-X-G-Y-G-F-R and the glutamate (E139) [Tao Science 2009]. It has been shown previously that mutations disturbing this interaction result in altered channel function [Dibb J Biol Chem 2003] [Yang Proc Natl Acad Sci 1997]. The structure obtained for another

representative of eukaryotic Kir channels, G protein-activated Kir3.2 (GIRK2), is very similar to the structure of the classical inward rectifier Kir2.2 channel. However, two differences have been found. First one considers the extracellular entry to the pore. In GIRK2 channel, turrets surrounding the entry form a wider, more open vestibule (Figure 27). This structural difference thus provides an explanation of observed different pharmacological properties between Kir3.x and Kir2.x channels. Whereas Kir3.x channels are inhibited by some toxins (e.g. tertiapin), the classical inward rectifiers are not. More open turrets of Kir3.x channels probably allow the toxin to fit into the vestibule. The second significant difference concerns the interface between the TMD (transmembrane domain) and CTD (cytoplasmic domain). In Kir2.2 structure (obtained in absence of PIP₂) the channel characterizes with an elongated shape in which TMD and CTD are distant. In contrast, in GIRK2, the two compartments are tightly juxtaposed ^{[Tao Science 2009] [Whorton Cell 2011]}.

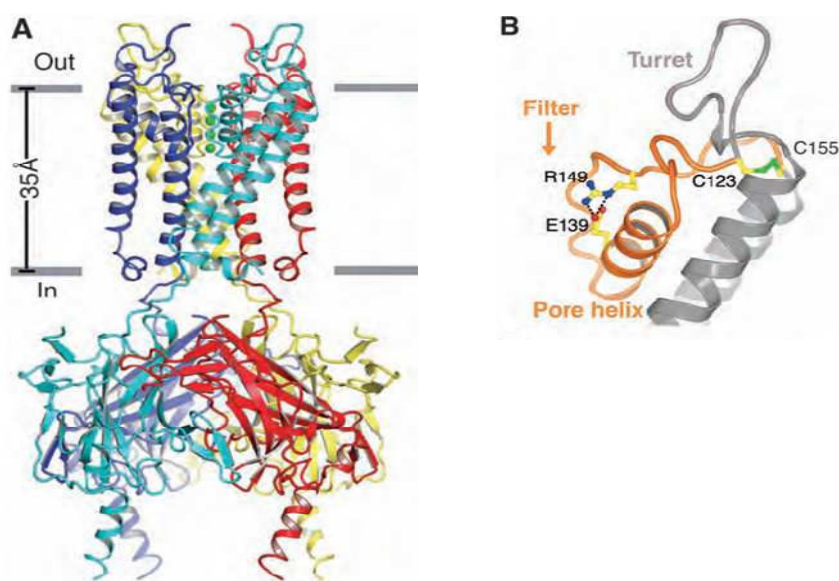


Figure 26. The structure of Kir2.2 channel.

a) The representation of Kir2.2 tetramer. The transmembrane (TMs) and cytosolic (CT) domains are spatially separated by a flexible, extended linker b) Close-up view of the pore region with present turret, pore helix and selectivity filter. The two interactions: disulfide bond (C123 and C155) and ionized hydrogen bond (R 149 and E139) are essential for proper folding and function of the channel. The region flanked by the two disulfide bond cysteines is shown in orange ^[Tao Science 2009].

Nevertheless, there are essential similarities between the structure of Kir2.2 and Kir3.2 channels. The structures of both eukaryotic channels reveal the presence of two constrictions along the ion conduction pathway which have been proposed to act as functional gates. One

gate, called the inner helix gate, is formed by the inner helices of the TM domains, just inside the membrane, above the level of the interfacial helix [Tao Science 2009] [Whorton Cell 2011]. In the case of the Kir2.2 channel, it has been shown that two residues present in the TM2, I177 and M181, form two hydrophobic seals which are able to close off the pore at the cytoplasmic interface [Jiang Nature 2002] [Tao Science 2009]. Interestingly, in other eukaryotic Kir channels, amino acids corresponding to the positions 177 and 181 are as well large and hydrophobic. In contrast, many other K⁺ channels including KcsA, K_v channels and prokaryotic Kir channels are characterized with a small and sometimes polar amino acid in the position corresponding to 177 (Val/Thr). The second gate – the G loop gate - is formed by the G-loop at the apex of the CTD, just outside the membrane, below the level of the interfacial helix [Tao Science 2009] [Whorton Cell 2011]. In the PIP₂-free structure of Kir2.2 channel, where CTD is extended away from the TMD, both gates are as well positioned far away from each other [Tao Science 2009]. Nevertheless, in the structure of Kir2.2 obtained in the presence of a short-chain derivative of PIP₂, as well as in the structure of Kir3.2 channel (GIRK2), both gates are closely associated suggesting their possible cooperation [Hansen Nature 2001] [Whorton Cell 2011].

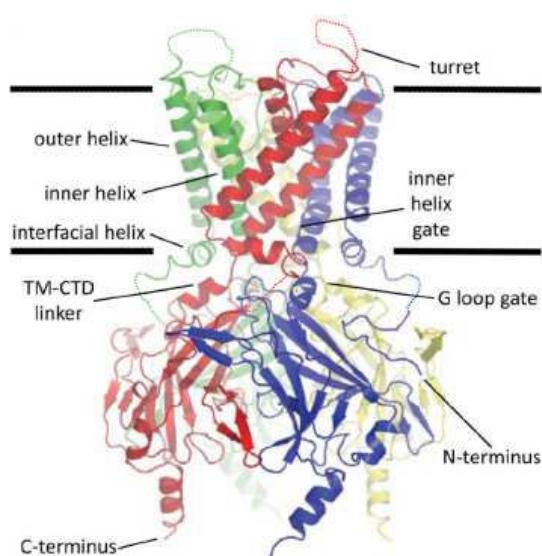


Figure 27. Cartoon diagram of GIRK2's structure.

TM and CT domains are closely juxtaposed thus bringing closer two potential gating regions of the channel (inner helix gate and G-loop gate) [Whorton Cell 2011].

IV.3.4 Regulation of the Kir channels.

Many different stimuli can open or close K⁺ channels, a process known as gating. These signals are received by the domains attached to the pore-forming subunit. It is known

that inwardly rectifying Kir channels are modulated by a wide spectrum of factors including proteins (G $\beta\gamma$ subunits of G proteins, kinases), membrane lipids (phosphatidylinositol-4,5-bisphosphate, cholesterol), nucleotides (ATP) and ions (H⁺, Na⁺). There are intensified studies dedicated to elucidate the most pervaded questions about the precise localization of the ligand-binding sites, the number of ligands that bind, and the molecular mechanisms by which the ligand binding induces channels gating. Importantly, very significant progress has been made in many of these areas thanks to the combination of functional and structural studies.

IV.3.4.1 Regulation by phosphatidyl-4,5-bisphosphate (PIP₂)

PIP₂ is a minor, but functionally critical component of the cell membrane (present in the inner leaflet). It is known as a regulator of many different ion channels including members of the Kir family [Hansen Nature 2011]. It has been demonstrated for many representatives of this family, including Kir1.x, Kir2.x, Kir3.x and Kir6.x that PIP₂ directly activates these channels by increasing their open probability [Ruppertsberg Eur J Physiol 2000]. In excised membrane patches the Kir channel's activity gradually declines. This 'run-down' of activity can be reversed by an application of ATP to the intracellular side which allows the replenishment of PIP₂, via the action of specific lipid kinases [Hilgeman Science 1996].

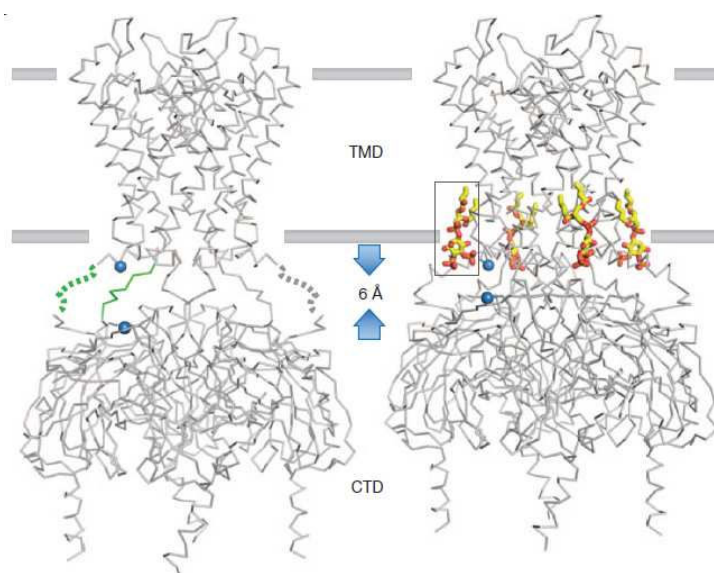


Figure 28. Effect of the short-chain PIP₂ on Kir2.2 channel.

X-ray crystal structures of apo- and PIP₂-bound Kir2.2 channel. In apo structure, the CTD is extended away from the TMD, and the gate in the TMD is tightly closed. Four PIP₂ molecules bind to the channel at the interface between TMD and CTD. Upon their binding, the flexible linker between CTD and TMD form helical structures inducing the translocation of the CTD towards the TMD resulting in opening of the inner helix gate [Hansen Nature 2011].

In the case of Kir2.x channels, PIP₂ is the primary agonist. The crystal structure of Kir2.2 channel in the complex with a short-chain (dioctanoyl) derivative of PIP₂ revealed its binding site and enabled understanding the mechanism of channel's activation by this lipid [Hansen Nature 2011]. PIP₂ binds at the interface between TMD and CTD, and produces a large conformational change in Kir2.2. In the presence of PIP₂, the flexible and extended linker contracts to a compact helical structure (Figure 28&29). Then the entire CTD moves 6 Å towards the TMD in association with the formation of two new helices. This motion induces the opening of the inner helix gate. This high resolution structure revealed that PIP₂ binding site consists of a conserved non-specific phospholipid-binding region present in the TMD, and a specific phosphatidylinositol-binding region in the CTD.

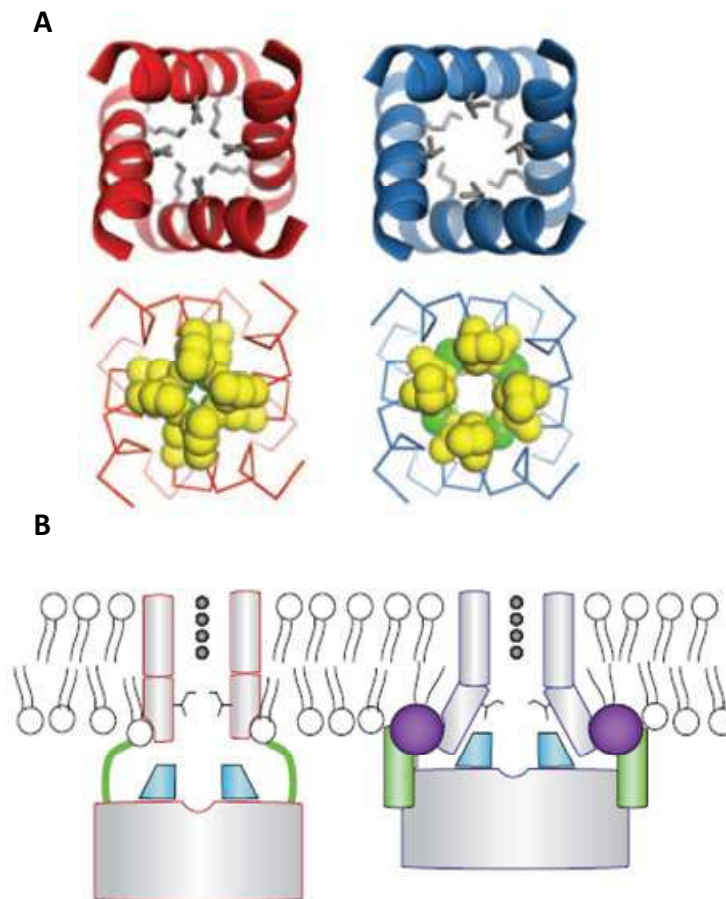


Figure 29. Proposed mechanism of Kir2.2 channel activation by PIP₂.

a) Comparison of the inner helix gate in PPA-bound (phyrophosphatidic acid, unable to induce conformational changes) Kir2.2 (red) and PIP₂-bound (inducing conformational changes of protein) Kir2.2 (blue). B) PIP₂ (purple spheres) binds at the interface between CTD and TMD inducing large conformational changes that result in the movement of the CTD and its attachment to the TMD. Located at the apex of CTD G-loop gate (cyan wedge) inserts into TMD and the inner helix gate opens [Hansen Nature 2011].

A sequence alignment shows that the residues implicated in the binding of PIP₂ are highly conserved among the whole Kir channels' family. Thus, the PIP₂ binding site found in Kir2.2 channel will be probably observed in many other inward rectifiers which activity have been proved to be modulated by this lipid (in some cases in concert with other ligands like ATP or G proteins). This assumption was confirmed by the crystal structure of Kir3.2 (GIRK2) channel. This structure revealed one ordered PIP₂ molecule per subunit, like in the case of Kir2.2, near the TMD-CTD interface. However, unlike in Kir2.2 channel, the interaction of PIP₂ with Kir3.2 produces only a modest displacement of the protein's main chain near the PIP₂ binding site. Both gates, the inner helix gate and the G-loop gate remain closed in the presence of PIP₂. This structure stays in agreement with results obtained from electrophysiological experiment demonstrating that PIP₂ alone is not sufficient to open GIRK channels [Whorton Cell 2011].

IV.3.4.2 Regulation by cholesterol

Multiple studies have shown that an increase of the cholesterol's level in the plasma membrane regulates the function of a variety of channels, including different types of K⁺, Ca²⁺, Na⁺ and Cl⁻ channels. It is also known that the effect of cholesterol on different types of channels is highly heterogeneous [Rosenhouse-Dantsker Channels 2010]. It has been demonstrated that eukaryotic Kir channels are as well modulated by cholesterol content in the plasma membrane [Rosenhouse-Dantsker Channels 2010] [Leviatan IUBMB Life 2009]. The first representative of this family this has been proved to be modulated by cholesterol was the Kir2.1 channel. The Kir2.1 channel activity is suppressed by the elevation of the membrane's cholesterol and enhanced by cholesterol depletion [Epshtein Proc Natl Acad Sci 2009] [Romanenko Biophys J 2004]. Cholesterol sensitivity of Kir2.x channels has been demonstrated to critically depend on a specific loop, called CD-loop, present in the C-terminus domain of these channels (Figure 30). Mutations of the residues present in this loop (especially L222I) significantly decrease the sensitivity of Kir2.1 to cholesterol [Epshtein Proc Natl Acad Sci 2009].

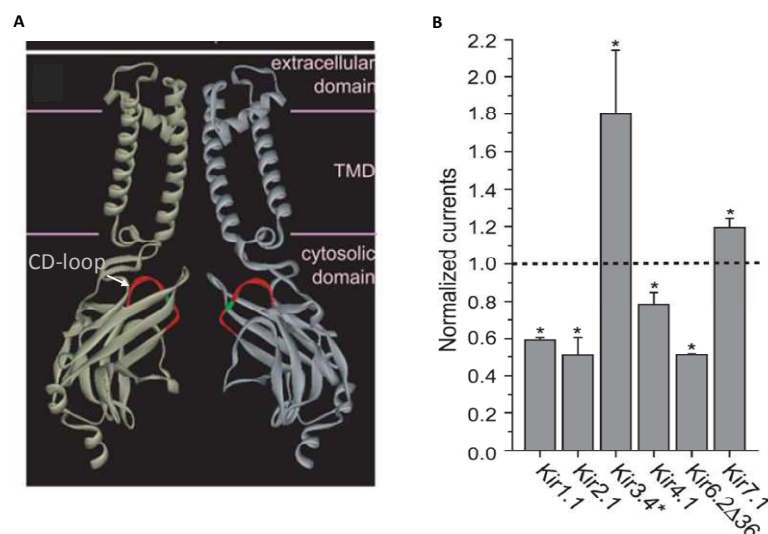


Figure 30. Cholesterol effect on Kir channels.

a) Homology model showing two opposite facing subunits of Kir2.1. The CD-loop is coloured in red and the position of L222 is highlighted in green. b) The effect of cholesterol enrichment on Kir channels activity [Rosenhouse-Dantsker Channels 2010].

Similarly to Kir2.1, Kir1.1 and Kir6.2 have been shown to be inhibited by the increase of the cholesterol content in the plasma membrane. In contrast, the G protein-activated Kir3.4 channel seems to be regulated in an opposite way. The increase of cholesterol enhances the channel's activity thus resulting in the generation of strong K^+ currents. Strikingly, it has been shown that the same CD-loop is responsible for cholesterol sensitivity of Kir3.4 channels. Moreover, Kir4.1 and Kir7.1 channels are affected as well by cholesterol but to a lesser extent. Nevertheless, the opposite effects of cholesterol on different Kir channels may be mediated by a common mechanism [Rosenhouse-Dantsker Channels 2010].

IV.3.4.3 Regulation by other proteins

Protein-protein interactions are involved in the control of some Kir channels' function. They include a protein-based regulation of Kir3.x channels with heterotrimeric G proteins and the association of SUR with Kir6.x channels [Hibino Physiol Rev 2010] [Ruppertsberg Eur J Physiol 2000].

GIRK (Kir3.x) channels are activated by the $G\beta\gamma$ subunit released from $G_{i/o}$ proteins upon the ligand binding to specific GPCRs [Huang Neuron 1998] [Wickman Physiol Rev 1995]. The inhibition of Kir3.x channels by the $G\alpha$ subunit has been as well reported, however, it seems to be less potent than the activation by $G\beta\gamma$ heterodimers [Slesinger Neuron 1995].

Introduction

The crystal structure of GIRK2 (Kir3.2) alone and in complex with $G\beta\gamma$ provided important insights in the regulation of channel gating by $G\beta\gamma$ subunits (Figure 31). The $G\beta\gamma$ subunits interact directly with the cytoplasmic domains (CTDs) of the channel through $G\beta$ and with the membrane through the covalent lipid attached to $G\gamma$. The binding site for $G\beta\gamma$ occurs at the interface between the two adjacent channel subunits which produces detectable relative motions. Binding of $G\beta\gamma$ induces 4° rotation of the CTD around the channel axis, which is associated with a partial unwrapping and splaying of the bundle of the four inner helices. These helices, in the absence of $G\beta\gamma$, form the ‘closed’ inner helix gate, in which four side chains of the phenylalanine (Phe 192) come together and form the narrowest contraction in ion conduction pathway. Intriguingly, according to obtained crystallographic data, even in the presence of $G\beta\gamma$ this gate is closed [Whorton Nature 2013].

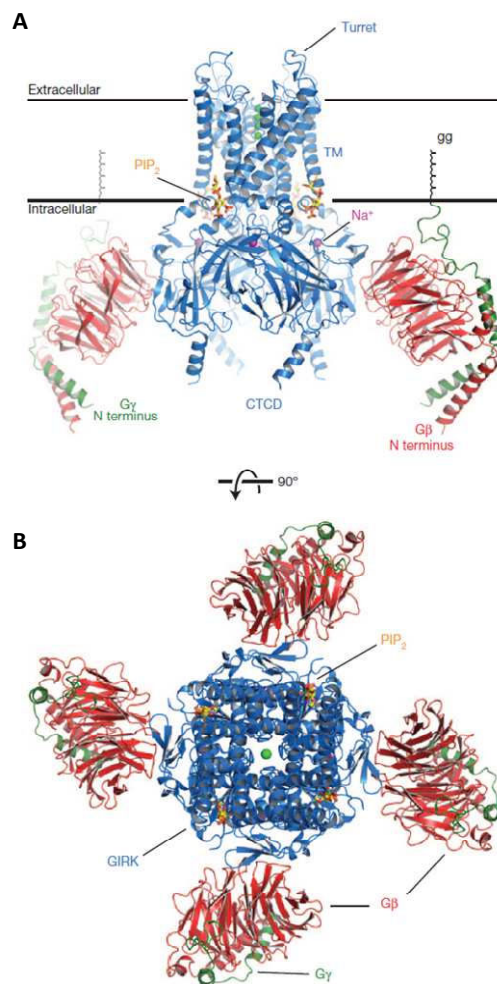


Figure 31. The overall structure of the GIRK-G $\beta\gamma$ complex.

a) A side view of GIRK (blue), G β (red) and G γ (green) complex. b) Top-down view of the complex from the extracellular side of the cell [Whorton Nature 2013].

Thus, there has been proposed a model of GIRK channels' regulation with a structural description of the conformational states that might underlie their sequential activation (Figure 32). In that model, the presence of PIP_2 in the inner leaflet of the membrane presumably results in the strengthening of the interface between the CTD and TMD thus enabling the binding of the $\text{G}\beta\gamma$. Binding of these subunits produces a pre-open conformation from which the channel readily flickers between the open (conductive) and pre-open (non-conductive) conformations. This model stays in agreement with the electrophysiological recordings revealing that GIRKs display 'burst kinetics' during which the $\text{G}\beta\gamma$ -bound channel undergoes rapid transitions between the conductive -and non-conductive state [Whorton Nature 2013].

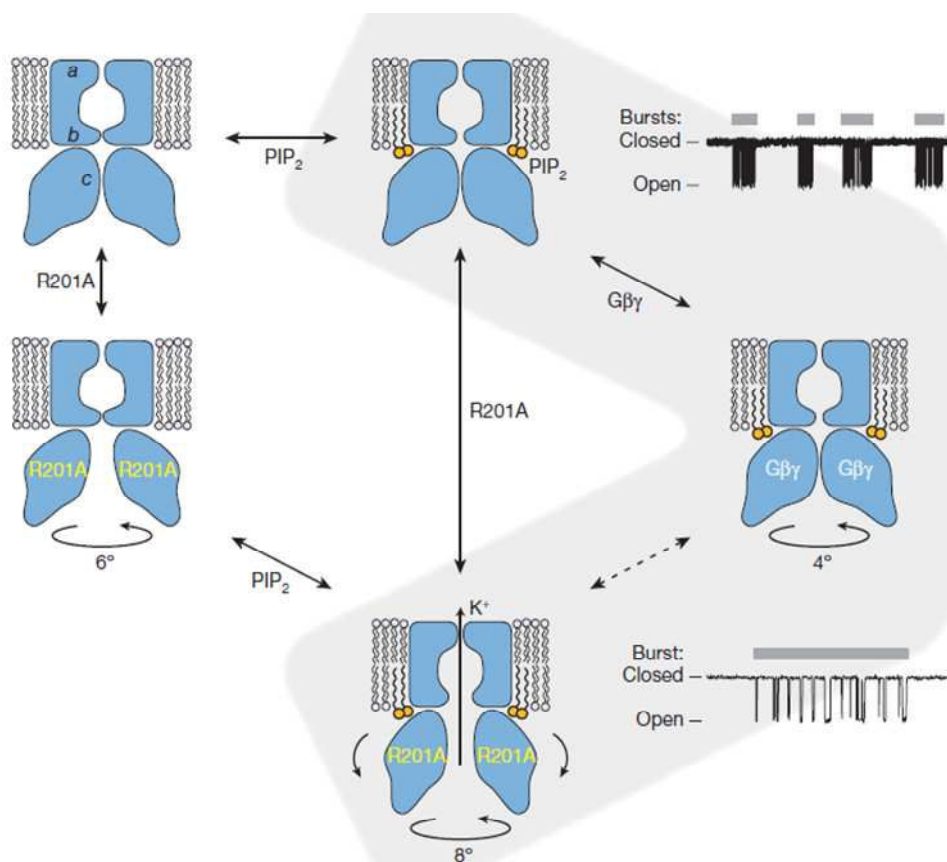


Figure 32. Model of gating regulation of GIRK channels.

The blue shapes depict a GIRK channel with a selectivity filter (a) and two gates: inner helix gate (b) and G-loop gate (c). In the presence of PIP_2 channel remains closed, however this lipid probably facilitates the binding of the $\text{G}\beta\gamma$ which in turn results in GIRK activation. The conformation of the GIRK in complex with $\text{G}\beta\gamma$ is an intermediate between the closed ($\text{G}\beta\gamma$ -free) and opened (GIRK R201A) channel structures. In the constitutively open mutant channel (considered as the conductive conformation) the CT domain is rotated additional 4° beyond the rotation induced by $\text{G}\beta\gamma$ and undergoes internal conformational changes associated with a widening of the membrane-facing apex. This widening is proposed to further open the inner helix gate allowing for the conduction of K^+ ions through the pore. Inter-burst periods correspond to the channel with only PIP_2 bound (top), whereas burst (grey bars) correspond to the channel with PIP_2 and $\text{G}\beta\gamma$, which fluctuates rapidly between non-conductive (right) and conductive (bottom) conformations [Whorton Nature 2013] [Whorton Cell 2011].

The gating of GIRK channels is controlled by several regulators (PIP₂, G protein subunits, Na⁺ ions), which activate the channel only partially when bound individually. However, their combination is required to result in a channel opening.

IV.3.4.4 Regulation by protein kinases.

Phosphorylation of the Kir channels' subunits by protein kinases such as kinases A and C (PKA and PKC, respectively) can modulate their activity. It has been demonstrated for the Kir1.1 channel that its activity requires a PKA-dependent phosphorylation of the Ser residues [McNicholas Proc Natl Acad Sci 1994]. PKA-mediated phosphorylation increases the channels' open probability (P_O), possibly by enhancing the interaction between Kir1.1 and PIP₂ [Liu Proc Natl Acad Sci 1999]. On the other hand, it has been shown that the phosphorylation of Ser residues by PKC results in the suppression of the channel's activity [Liu J Biol Chem 2002]. Moreover, it has been shown as well for smooth muscle K_{ATP} channels, that PKA-mediated phosphorylation of both subunits (Kir6.1 and SUR2B) enhances its activity [Quinn Circ Res 2004].

IV.3.4.5 Regulation by other ligand

Another important factor for the regulation of the channel activity is the pH. It has been shown for several Kir channels that they are inhibited by a decrease in intracellular pH. In contrast to indirect regulation by protein kinases, the effect of intracellular pH on Kir1.1 and Kir4.1 channels is mediated by the channel protein itself. Intracellular acidification closes Kir1.1 and Kir4.1 channels very rapidly with steep pH dependence [Schulte J Physiol 2001] [Schulte Proc Natl Acad Sci 1996]. Moreover, the external concentration of H⁺ may also influence the channel's activity, as it is the case for Kir2.3 where proton sensitivity is conferred by the presence of histidine residue (His177) in the extracellular loop linking TM1 to the pore [Coulter Neuron 1995].

It has also been demonstrated for GIRK channels (GIRK2 and GIRK4) that elevated levels of intracellular Na⁺ can induce the activation of these channels [Ho J Biol Chem 1999] [Petit-Jacques J Gen Physiol 1999]. The Na⁺-specific binding site has been as well confirmed in the crystal structure of the GIRK2 channel [Whorton Cell 2011] [Whorton Nature 2013]. Sodium activation of GIRK channels is thought to play an important physiological function by producing a negative feedback on excessive electrical excitability. During the excitation, Na⁺ entry elevates the

concentration of the intracellular Na^+ above the normal range, which is enough to activate the GIRK channels and restore the negative membrane potential [Whorton Cell 2011].

The intracellular ATP is another example of crucial regulator of the activity of Kir6.x subfamily.

IV.4 Focus on Kir6.1 and Kir6.2 channels.

IV.4.1 Structural features and localization.

Both members of the Kir6.x subfamily share the same topology as the other inwardly rectifying K^+ channels. They are composed of two transmembrane helices (TM1 and TM2) linked by the H5 loop carrying the canonical signature of K^+ channels (GYG or GFG); a short N-terminus, and a large C-terminal cytoplasmic domain.

Kir6.x channels are very ubiquitous. Kir6.1 channels, were first identified in pancreas, but they are mainly expressed in smooth muscle cells [Inagaki Genomics 1995] [Suzuki Circ Res 2001]. In turn Kir6.2 channels are highly expressed in α (glucagon-secreting), β (insulin-secreting), and γ (somatostatin-secreting) cells of pancreatic islets [Karschin FEBS Lett 1997]. Moreover, at a lower level, they are expressed in heart, skeletal muscles and brain [Suzuki Diabetes 1997].

IV.4.2 Physiological regulation of Kir6.x channels.

Several ligands have been identified to control the gating of Kir6.1 and Kir6.2 including ATP, PIP_2 and intracellular pH. Moreover, their activity is as well strongly modulated by kinases-mediated phosphorylation.

➤ Nucleotides

Fully assembled K_{ATP} channels are inhibited by the intracellular ATP. Due to the reason that SUR receptor possesses two nucleotide-binding sites, it has been proposed as the main site for ATP inhibition [Aguilar-Bryan Science 1995]. However, the discovery that the truncation of the 26 C-terminal residues (ER retention signal) of the Kir6.2 resulted in functional channels present in the plasma membrane revealed that Kir channels are directly inhibited by ATP [Tucker Nature 1997]. Many functional data suggest that the ATP-binding site involves residues from both C- and N-terminal domains of Kir6.x [Tucker EMBO J 1998] [Drain Proc Natl Acad Sci

1998] [Proks J Physiol 1999] [Trapp EMBO J 2003]. Homology modeling and ligand docking application allowed identifying of the putative ATP-binding site [Antcliff EMBO J 2005].

According to this data and functional studies, there are four ATP-binding pockets [Markworth Diabetes 2000]. Each one is located in the upper part of the intracellular domain (IC) on the outer face of the protein what may facilitate access of the cytosolic ATP molecule to its binding site. The main binding pocket lies at the interface between the N- and C-terminal domains of the same subunit, and including contribution from the N-domain of the adjacent subunit (Figure 33). The phosphate tail of ATP interacts with R201 and K185 in the C-terminus of one subunit, and with R50 in the N-terminus of another; the N6 atom of the adenine ring interacts with E179 and R301 in the same subunit. It has been demonstrated that naturally occurring mutations at some of the positions that frame the ATP-binding site (R201) or lie close to it (F333, Y330) results in neonatal diabetes [Haider J Mol Cell Cardiol 2005].

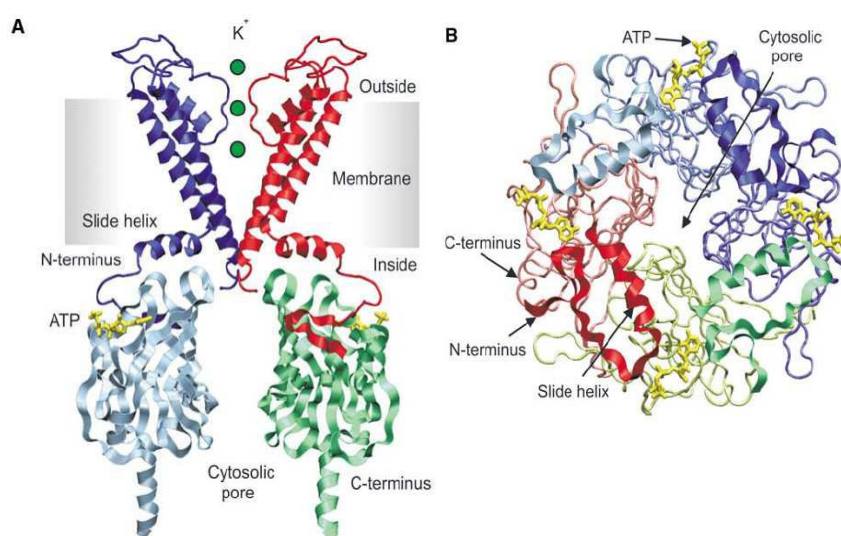


Figure 33. Localization of a putative ATP-binding site in the Kir6.2 channel [Antcliff EMBO J 2005].

Binding of the ATP to a single Kir6.2 subunit appears to be sufficient to induce the tetrameric channel closure [Markworth Diabetes 2000]. It has been hypothesized that gating of the Kir6.2 by ATP probably involves changes in inter-subunit interactions. Possibly the flexible loops present in the C-terminal domain may help to translate these ATP-induced conformational changes into movement of the slide helix and thereby closing of the channel [Antcliff EMBO J 2005]. Importantly, the ligand binding site is very selective for nucleotides with adenine ring [Tucker EMBO J 1998] [Dabrowski J Physiol 2004]. It as well requires the presence of at least

three phosphates. Removal of these phosphates decreases nucleotide binding affinity by several-fold of magnitude [Tucker EMBO J 1998].

When expressed alone, activity of Kir6.2 channels is half maximally inhibited by ~100 μ M ATP. Co-assembly with SUR subunit decreases this value to ~10 μ M, possibly by reshaping the ATP binding pocket [Dabrowski J Physiol 2004].

➤ Lipids

Likewise in the case of most representatives of Kir channels, Kir6.x have also been shown to be dependent on PIP₂. It plays a dual role in Kir6.x regulation: 1) it increases the open probability of run-down channels in the absence of ATP; and 2) it antagonizes the ATP inhibition by significantly decreasing the apparent affinity of the channel for ATP [Hilgemann Science 1996] [Fan J Biol Chem 1997] [Shyng Science 1998] [Fan J Gen Physiol 1999]. The negatively charged molecule of PIP₂ modulates the activity of Kir6.x channels by electrostatic interactions with the positively charged residues of the channel. It has also been demonstrated that positively charged amino acids present in the N-terminal (K39, R54), transmembrane (K67) and C-terminal regions (R176, R177, R301) of Kir6.2 interact with the polar head of PIP₂, whereas the aliphatic chain is anchored in the plasma membrane [Haider EMBO J 2007]. Interestingly, the PIP₂ binding site has been proposed to partially overlap with the one of the ATP [Haider J Mol Cell Cardiol 2005].

Another family of anionic lipids proven to regulate the activity of Kir6.x channels are the long chain co-enzyme A esters (LC-CoA). They have been demonstrated to activate Kir6.x channel in the similar manner as PIP₂. Moreover, they also reduce the 'run-down' of channels and decrease the sensitivity to ATP [Gribble J Biol Chem 1998] [Shumilina J Physiol 2006].

It has been proposed that the activity of Kir6.x channels depends as well on the level of cholesterol present in the plasma membrane. However, the role of the cholesterol in the regulation of Kir6.x channels is quite controversial. Some studies have suggested that Kir6.x channels' activity is enhanced by the elevation of cholesterol content in the plasma membrane; others suggest the opposite, inhibiting effect of this sterol [Mathew Arterosc 2001] [Genda J Am Coll Cardiol 2002]. The most recent studies performed with Kir6.2 Δ C36 heterologously expressed in *Xenopus* oocytes shows that this channel is significantly suppressed by the elevation of membrane cholesterol [Rosenhouse-Dantsker Channels 2010].

➤ Impact of pH

Activity of Kir6.x channels has been demonstrated to be pH-dependent. Protons modulate the activity of Kir6.x channels in three ways:

- activation by moderate acidification,
- inactivation by a significant acidification
- increase of the inward rectification by the alkalization,

When the intracellular pH decreases, at first there is observed a reversible activation (pKa ~7.2) of the channel which is subsequently followed by an irreversible inactivation (pKa ~6.8) [Xu J Biol Chem 2001a]. The observed transient activation phase results from the shortening of the closure periods [Wu J Physiol 2002]. The activation event involves the His 175 residues which is located in TM2, while the inactivation engages three other histidines (His 186, 193 and 216) located in the C-terminal domain of the channel [Xu J Biol Chem 2001b]. Moreover, the deprotonation of the His 216 has been demonstrated to be essential for observed increase in channel rectification [Baukrowitz EMBO J 1999].

➤ Phosphorylation

Kir6.1 and Kir6.2 channels are as well regulated by PKA- and PKC-mediated phosphorylation of some particular Ser/Thre residues. It has been demonstrated that Kir6.2 (co-expressed with SUR1) is activated as a result of the phosphorylation by both kinases, while Kir6.1 (co-expressed with SUR2B) is activated by PKA-mediated phosphorylation but inhibited by PKC [Beguin EMBO J 1999] [Quinn Circ Res 2003]. The T224 and S372 from Kir6.2 and S385 from Kir6.1 have been proved to be implicated in the activation via PKA, while T180 in the Kir6.2 in activation via PKC [Beguin EMBO J 1999] [Lin EMBO J 2000] [Quinn Circ Res 2004] [Light Proc Natl Acad Sci 2000].

IV.5 SUR receptor, a particular member of the ABC transporters family.

Sulfonylurea receptors belong to the huge family of ABC (ATP-binding cassette) transporters. Together with three other transporters, MRP (multi-drug resistance protein), CFTR (cystic fibrosis transmembrane conductance regulator) and YCF1 (yeast cadmium factor), they constitute the ABCC subfamily. Most members of this ABC family of proteins are implicated in the active transport across membranes, against the concentration gradient of

substrates, using energy from ATP hydrolysis. They are involved in the transport of many biological (hormones, peptides, sugars, ions) as well as toxic substances (heavy metals, drugs). Strikingly, the SUR receptor, despite its affiliation, is a very particular representative of this family, because it does not transport known substrates across the plasma membrane. It is implicated in the regulation of Kir6.x channel activity.

IV.5.1 Isoforms and localization

In humans three SUR isoforms are present: SUR1, SUR2A and SUR2B; and they are encoded by two distinct genes (ABCC8 for SUR1 and ABCC9 for SUR2). The SUR1 receptor contains 1581 amino acids and is predominantly expressed in the pancreas where it associates with Kir6.2 [Aguilar-Bryan Science 1995] [Inagaki J Biol Chem 1995]. Moreover, it has as well been found in the brain coupled to both isoforms of Kir6.x [Karschin FEBS Lett 1997]. SUR2A and SUR2B isoforms of SUR2 have respectively 1549 and 1591 residues and are obtained by alternative splicing of the ABCC9 gene. SUR2A is mainly expressed in heart and skeletal muscles and at a lower level in the brain, ovaries, and pancreatic Langerhans islets [Inagaki J Biol Chem 1995] [Isomoto J Biol Chem 1996]. In turn, SUR2B receptor is expressed mostly in smooth muscles and certain neurons [Isomoto J Biol Chem 1996].

IV.5.2 Structural organization

There is no atomic resolution structure of the SUR receptor. However, based on sequence alignment with other members of the ABC transporter family, characteristic structural elements can be distinguished. The SUR receptor possesses an N-terminus exposed to the extracellular medium and a C-terminus orientated into cytoplasm. It is characterized with the presence of three transmembrane domains: TMD0, TMD1 and TMD2 which respectively contains five, six and six hydrophobic transmembrane α -helices. TMD0 is connected to TMD1 by a cytoplasmic loop (L0). This domain constitutes a distinctive feature of some receptors which belongs to the ABCC subfamily (SUR1-2, MRP1-3, 6, 7 and YCF1). TMD0s from different proteins show different amino acid sequence, and their functions are largely unknown [Chan EMBO J 2003]. However, it has been proposed that this N-terminal region containing TMD0 and L0 (TMD0-L0) region of SUR is implicated in the interaction with Kir6.x channels and controls the gating [Babenko J Biol Chem 1999b].

Moreover, SUR receptors possess two nucleotide-binding domains: NBD1 and NBD2. NBDs contain characteristic motifs: Walker A and B, and the ABC transporters signature (Figure 34).

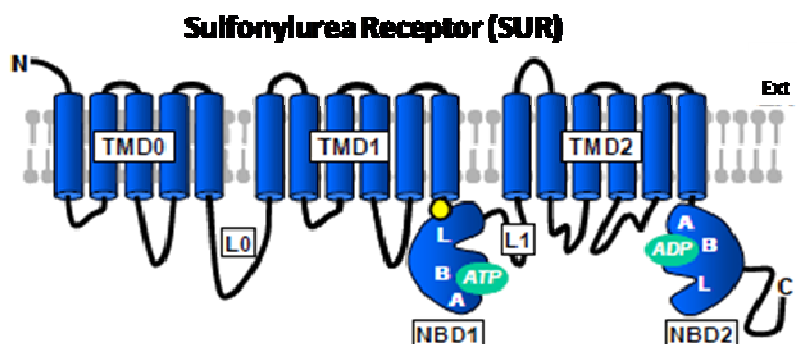


Figure 34. Structural organization of sulfonylurea receptors (SUR).

SUR (~16000 a.a.) possesses three transmembrane domains (TMD0, 1 and 2) and two nucleotide-binding domains (NBD1 and NBD2) containing Walker A, Walker B and linker L (with the signature of ABC transporters) ^[Channels Group].

IV.5.3 Regulation of K_{ATP} channel mediated by SUR receptor.

IV.5.3.1 Physiological agents

Sulfonylurea receptors are regulated by the intracellular nucleotides associated with Mg^{2+} ions (Mg-nucleotide) which interact with the nucleotide-binding domains (NBDs). Occupancy of the NBDs of the SUR receptor by MgADP stimulates K_{ATP} channel's activity (by antagonizing Mg^{2+} -independent ATP inhibition), a circumstance that can be achieved either by direct binding of MgADP or by binding and subsequent hydrolysis of MgATP ^[Nichols Science 1996]. Each nucleotide-binding domain contains a highly conserved Walker A and Walker B which characterize with an inherent catalytic activity hydrolysing the ATP γ -phosphate ^[Gribble EMBO J 1997]. It has been demonstrated that SUR receptors preferentially bind MgATP at the NBD1 and MgADP at NBD2, and both domains work cooperatively ^[Ueda J Biol Chem 1997] ^[Gribble EMBO J 1997]. Thus, MgADP through direct interaction or as a product of hydrolysis at NBD2, stabilizes nucleotide binding to NBD1 of SUR, suggesting that the cooperation between NBDs is an essential conformational rearrangement for ADP-induced K_{ATP} channel activation ^[Karger J Gen Physiol 2008]. Additionally, some other nucleotides associated with Mg^{2+} ions are as well capable to stimulate K_{ATP} channels via the SUR receptors including MgGDP, MgGTP, MgUDP and MgUTP ^[Trapp Proc Natl Acad Sci 1997] ^[Satoh J Physiol 1998].

IV.5.3.2 Pharmacological agents

K_{ATP} channels' activity can as well be regulated by pharmacological agents that bind directly to the SUR subunit. Depending on their way of action they can be divided into two major classes: inhibitors of the channel's activity (mainly sulfonylureas) and potassium channel openers/activators (KCO).

K_{ATP} channel inhibitors can be divided into two groups: these which interact with Kir6.x (imidazolines and antimalarias), and these which interact with the SUR regulatory subunit (sulfonylureas and benzoamido derivatives). Binding of inhibitors triggers the closure of Kir6.x pore which leads in turn to the depolarization of the plasma membrane. All these molecules stimulate insulin secretion from the pancreatic β -cells, and some are used in the treatment of type II diabetes, such as the sulfonylureas (with their hypoglycemic properties). The sulfonylurea receptors SUR1 (β -cell type) and SUR2 (cardiac and smooth muscle types) are characterized with different affinities for hypoglycemic sulfonylureas. This phenomenon provides basis for the selectivity of these compounds for different K_{ATP} channel isoforms [Vila-Carriles FASEB J 2007]. Whereas tolbutamide and gliclazide block channels containing SUR1, in turn glibenclamide, repaglinide and meglitinide block both types of channels [Proks Diabetes 2002]. Sulfonylureas also interact with Kir6.2, but with much lower affinity [Gribble J Physiol 1997b] [Gribble Diabetes 1998b]. TMD2 of SUR has been proved to be a critical component for the high-affinity inhibition of the channel's activity. It has been demonstrated that the transmembrane helices 14-16 of SUR1 are functionally important for inhibition by tolbutamide, and serine 1237, located in the cytoplasmic loop between transmembrane helices 15 and 16, is crucial for the blocking. On the other hand, binding of glibenclamide requires the interaction with the intracellular loops between transmembrane helices 5- 6 and 15-16 [Mikhailov FEBS Lett 2001]. The loop between transmembrane helices 15 and 16 has been identified as the binding site for sulfonylurea moiety, whereas benzamido derivatives have been shown to interact with the intracellular loop between helices 5 and 6 (L0) and the N-terminus of Kir6.2 [Ashfield Diabetes 1999] [Babaneko FEBS Lett 1999] [Mikhailov FEBS Lett 2001].

K_{ATP} channel openers, as their name suggests, induce opening of the channel which leads to membrane's hyperpolarization and reduction of electrical excitability. K_{ATP} channel openers are a very diverse group of pharmacological agents with a wide range of therapeutic applications. The most commonly studied K_{ATP} channel openers are:

- diazoxide - vasodilator in the treatment of malignant and pulmonary hypertension, and inhibitor of insulin secretion in insulinoma or congenital hyperinsulinism,
- pinacidil, cromakalim, minoxidil sulfate – vasodilators used in the treatment of hypertension,
- nicorandil – vasodilator used in the case of cardiac arrhythmias and angina,

Interestingly, native K_{ATP} channels from different tissues display varying sensitivities to $K_{ATP}CO$ [Ashcroft Trends Pharmacol Sci 2000]. This tissue-specificity to $K_{ATP}CO$ can be attributed to the different types of SUR that make up K_{ATP} channels in β -cells (SUR1), cardiac (SUR2A) and smooth muscle (SUR2B). Most of the openers preferentially targets SUR2A and SUR2B isoforms. As an exception stands the diazoxide, opener that has effect mainly on K_{ATP} channels comprising of SUR1 found in the pancreas and brain [Moreau Mol Pharmacol 2005]. This phenotypic difference between SUR1 and SUR2 receptors is connected with some essential distinctions in primary sequences. It has been shown that two residues within TM helix 17 of SUR2 (L1249 and T1253 in SUR2A) are essential for the high affinity binding of non-diazoxide openers (Figure 35). When these residues were mutated in SUR2A, subsequent loss-of opener action was observed [Moreau J Mol Cell Cardiol 2005] [Moreau Mol Pharmacol 2005]. Moreover, the cytoplasmic loop connecting the transmembrane helices 13 and 14 also appears to participate in this process of openers binding [Uhde J Biol Cell 1999].

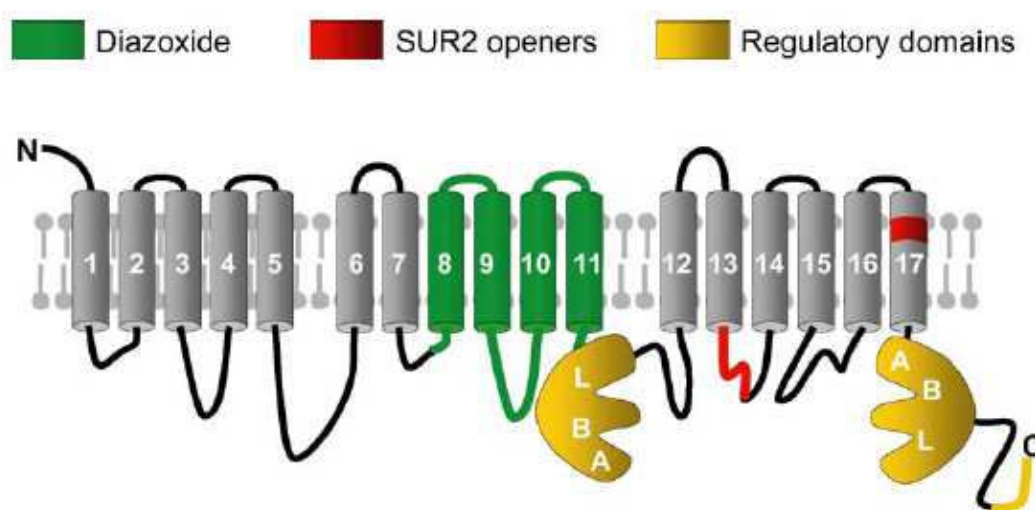


Figure 35. Segments of SUR engaged in the binding of diazoxide and non-diazoxide openers.
 Activation of K_{ATP} channels by openers is regulated by nucleotides through their binding to both NBDs [Moreau Mol Cell Cardiol 2005].

In turn diazoxide, a selective opener acting on SUR1, but also on SUR2A and SUR2B in presence of MgADP, activates K_{ATP} channels through its binding to the region located in the TMD1 (including helices 8-11) [Babenko J Biol Chem 2000] [Moreau Mol Cell Cardiol 2005].

It has been demonstrated that binding of the openers is positively modulated by MgATP nucleotides through both NDBs [Swanstecher 1998]. However, there are studies which show that MgATP is not absolutely indispensable for the openers to bind to SUR2. In the absence of MgATP, openers can still bind to SUR2B, but the binding affinity is then reduced by at least two orders of magnitude [Russ Br J Pharmacol 2003].

IV.5.4 The assembly with Kir6.2 and addressing of K_{ATP} channels.

In vivo assembling of K_{ATP} channels is still a poorly understood process. Nevertheless, there are some data which suggest that the interaction between monomeric forms of SUR1 and Kir6.2 channel takes place early in the endoplasmic reticulum. Subsequently SUR1-Kir6.2 heterodimers interact with each other leading to the formation of the heterooctameric K_{ATP} complexes [Crane J Biol Chem 2004]. The assembly of Kir6.x pore-forming subunits with SUR receptors increases surface expression, $P_{o(max)}$ and sensitivity to inhibitory ATP, and induces responsiveness to stimulatory MgADP, K_{ATP} openers, inhibitory sulfonylureas and other insulin secretagogues. Due to the lack of the three-dimensional structures of K_{ATP} channels, the precise map of interaction between its two components is not known [Babenko J Biol Chem 2003]. Several regions of interaction have been proposed between both components of the K_{ATP} channel which seem to play an important role in the assembling process. TMD0 (N-terminal bundle of five transmembrane helices) has been proved to strongly interact with the Kir6.2 channel and control its trafficking and gating (Figure 36). The relevance of this domain in the K_{ATP} channel's function underscores the fact that two mutations in TMD0 (A116P and V187D) domain are associated with persistent hyperinsulinemic hypoglycemia of infancy (PHHI). PHHI mutations have been shown to completely abolish the association between SUR1 and Kir6.2 [Aguilar-Bryan Endocr Rev 1999]. It has been shown that TMD0 greatly enhances surface expression of Kir6.2 Δ 26, however, it is unable to mask the retention signal of the full length Kir6.2 thus indicating that there are other regions of the SUR receptor also involved in the direct interaction with the channel [Chan EMBO J 2003] [Fang J Physiol 2006]. A second piece of evidence for the contribution of different region of SUR in its interaction with Kir6.x channel, is the modified ATP-sensitivity. SUR receptor increases the ATP-sensitivity of Kir6.x, but

TMD0 does not. On the opposite, it has been proved that TMD0/Kir6.2 Δ C26 complexes are characterized with lower ATP-sensitivity ($IC_{50} \sim 300\mu\text{M}$) than for Kir6.2 Δ C26 channels ($IC_{50} \sim 100\mu\text{M}$) [Chan EMBO J 2003]. This can be explained by their higher P_o compared with the Kir6.2 Δ C26 channels [Enkvetehakul Biophys J 2000]. It has been demonstrated that the cytoplasmic segment linking TMD2 and NBD2 of SUR is as well very crucial for the physical interaction with Kir6.x. It has been shown that this fragment (between residues 1295-1358), when expressed, co-precipitates with Kir6.2. Moreover, its co-expression with the full length receptor and Kir6.2 results in an altered surface expression of the native channel [Rainbow Biochem J 2004] [Rainbow J Physiol 2004].

In the case of the Kir6.x channel there are numerous reports that indicate the N-terminal and proximal C-terminal parts as well as the transmembrane helix 1 (TM1) as major regions involved in the physical interaction with SUR subunits [Schwappach Neuron 2000] [Babenko J Biol Chem 2002] [Bryan Diabetes 2004] [Tammara J Physiol 2007a] [Tammara J Physiol 2007b].

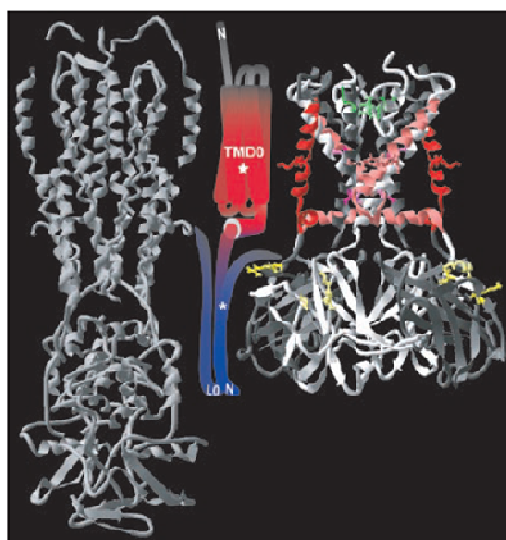


Figure 36. Proposed interactions between Kir6.x subunit and TMD0-L0 region critical for the assembly and gating of K_{ATP} channels [Bryan Diabetes 2004].

Each Kir6.2 subunit possesses a unique stretch of three residues, RKR, present in its C-terminus that act as the endoplasmic reticulum (ER) retention signal. Exposition of this signal precludes Kir6.2 from exiting the ER/Golgi network [Zerangue Neuron 1999]. Only when it co-assembles with the SUR subunit, is the ER retention signal masked (Figure 37). Moreover, it has been shown that the SUR1 receptor contains the same RKR motif located in the N-terminal region of the NBD2 domain that, likewise in the case of Kir6.2, prevents individual SUR1 proteins to reach the plasma membrane, unless it is masked by neighboring SUR1. Due

to the reason that the exposition of only one retention signal can be detected by COPI (coat protein complex I) watchdog and is sufficient to halt forward trafficking, only fully assembled octameric complexes of K_{ATP} can reach the membrane [Zerangue Neuron 1999] [Yuan Curr Biol 2003]. It has been proposed that in the masking process, the members of the 14-3-3 protein family are implicated. Thus, Kir6.2 signals are sterically masked by SUR1, while SUR1 signals are recognized and masked by 14-3-3 proteins [Heusser J Cell Sci 2006].

Interestingly, truncation of the last 36 residues from the Kir6.2 C-terminus allows the channel to reach the plasma membrane even in the absence of the SUR subunit [Tucker Nature 1997].

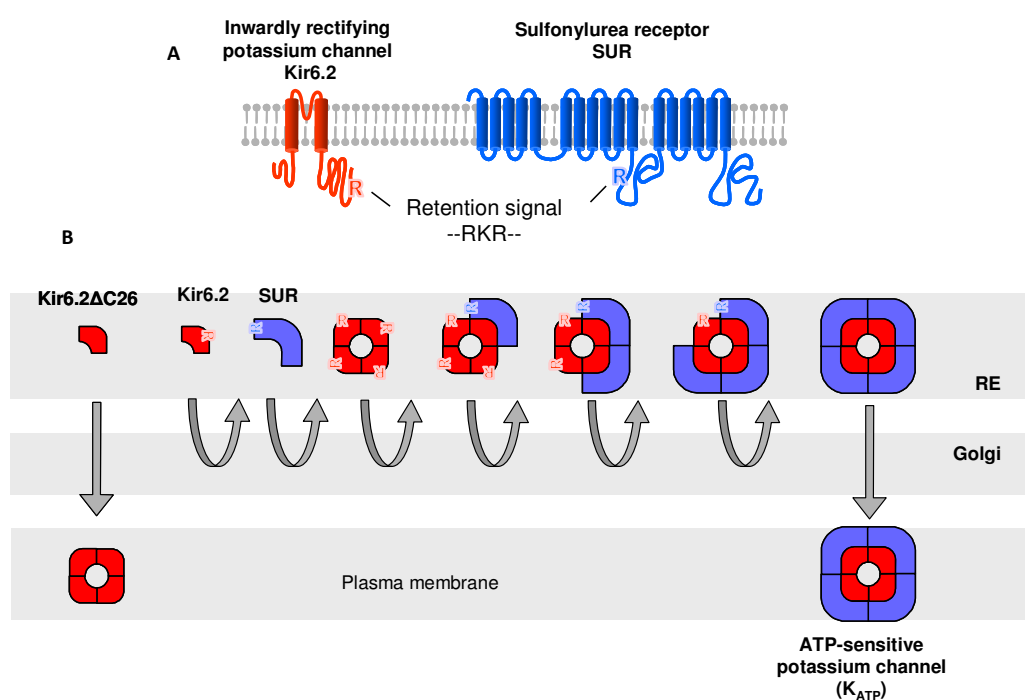


Figure 37. Model representing the mechanism of the masking of retention signals.

a) The RKR endoplasmic reticulum retention signal is present in Kir6.2 as well as in the SUR receptor. b) Kir6.2 channels with deleted C-terminus, carrying the ER retention signal, traffic to the plasma membrane in the absence of SUR subunits. Detention of that signal allows only fully assembled octameric complexes SUR-Kir6.2 to reach the cell surface [Channels Group].

IV.5.5 Functional coupling with Kir6.x

K_{ATP} channels are composed of two distinct proteins and the fundamental question is how does the sulfonylurea receptor ‘communicate’ in molecular terms with the ion conducting Kir6.x subunit. Multiple studies suggest that the TMD0 domain of SUR seems to be highly involved in the functional coupling between the receptor and pore-forming Kir6.x channel. It has been shown that, besides affecting the trafficking of Kir6.2ΔC26, TMD0 also

modulates its gating. Kir6.2 pores, normally retained in the ER display a low maximal open probability in ligand-free solutions (P_{omax}), when forced to the cell surface by deleting the C-terminal RKR retention signal (the pores are nearly silent) [Babenko J Biol Chem 2003]. Full-length SUR receptor affects the gating of the channel by increasing its burst duration and open probability (P_o) [Babenko FEBS Lett 1999a]. Interestingly, TMD0/Kir6.2 Δ C26 and SUR1/Kir6.2 Δ C26 characterize with very similar single channel kinetics, thus indicating that the TMD0 domain by itself is sufficient to confer most, but not all, of the wild-type K_{ATP} intrinsic gating pattern to Kir6.2 Δ C26 [Chan EMBO J 2003]. Moreover, it has been demonstrated that the presence of the cytosolic linker L0 that connects TMD0 to the core of the SUR receptor also contributes to the regulation of the intrinsic gating of Kir6.2 Δ C26. These findings suggest that under nucleotide-free conditions other SUR1 domains do not functionally couple to Kir6.2 [Fang J Physiol 2006]. However, these 'other' domains are crucial for the effects of MgADP, sulfonylureas and potassium channel openers to the Kir6.2 pore-forming subunit because TMD0/Kir6.2 Δ channels seem to be insensitive to these agents. However, this was contested by the finding that mutation present in TMD0 (F132L) of SUR1, precisely within the short cytosolic loop that links transmembrane helices 3 and 4 (CL2), dramatically decreases the sensitivity of K_{ATP} channel to inhibitory effect of ATP and tolbutamide (sulfonylurea) [Proks Hum Mol Gen 2006].

One of the candidates proposed to be involved in the ligand-induced functional coupling between SUR and Kir6.x is the aspartate/glutamate rich domain (ED). The ED has been proposed to act as an allosteric transducer securing functional communication between the Kir6.2 subunit and SUR2A receptor in cardiac K_{ATP} channels. SUR2A contains a stretch of 15 negatively charged Asp and Glu residues, designed as ED localized downstream of NBD1, in the cytoplasmic loop six (CL6) between TMD1 and TMD2. The ED domain does not overlap with the known binding sites for the K_{ATP} channel ligands. It is known that cooperative interaction between the NBDs domain is critical for conformational rearrangement of ADP-induced K_{ATP} channel activation. It has been proved that the ED domain essentially contributes in that cooperative interaction and plays an essential role in mediating of MgADP-dependent channel activation. While inhibition of the wild-type K_{ATP} channels with ATP is readily reversed by equivalent concentration of MgADP, the ED mutants SUR2A/Kir6.2 are unable to reverse that inhibition. Moreover, it has been shown that the disruption of the ED domain antagonized K_{ATP} channel opening induced by the potassium channel opener (KCO, e.g. pinacidil) and highly reduced sensitivity toward the inhibitory

effect of the sulfonylurea drug, glyburide. Thus, it seems that ED domain is an integral element of the allosteric machinery that controls the readout of NBDs states and transduces this signal to Kir6.2 pore (however it has not yet been proved if the communication between SUR2A and Kir6.2 involves direct electrostatic interaction via the ED region) [Karger J Gen Physiol 2008].

Another region demonstrated to be involved in the cross-talk between the K_{ATP} channel subunits is a proximal C-terminus of the SUR receptor. A 65-residue fragment, located between the last transmembrane helix 17 and NBD2, has been proved to stand at the interface between the SUR2A receptor and Kir6.2 [Rainbow Biochem J 2004a] [Rainbow J Physiol 2004b]. Importantly, it has been shown that besides the physical interaction, this fragment is also crucial for the functional coupling. Particularly, three residues present in the N-terminus of NBD2 (E1350, I1310 and L1313) are strongly involved in the K_{ATP} channels regulation with MgADP and K^+ channel openers mediated by SUR2A. Mutation of these residues resulted in a drastic reduction of the channel's activation by MgADP and KCO compared to the wild-type K_{ATP} . Thus, these three residues have been proposed to serve as part of the transducing machinery linking the effector binding to SUR and Kir6.2 activation [Dupuis J Physiol 2008]. Interestingly, in contrast to ED-mediated transduction of inhibiting and activating signals, it has been shown that mutations of three N-terminal residues of NBD2 do not affect channel inhibition induced with sulfonylureas (glibenclamide) and ATP [Karger J Gen Physiol 2008] [Dupuis J Physiol 2008]. This finding supports the idea that there may exist multiple transduction pathways functionally linking SUR2A to Kir6.2; one for activation processes engaging the TMD2-NBD2 linker, and one for the inhibition, possibly involving the TMD0 domain.

IV.5.5.1 Project #3: Mapping of Kir6.2 gates regulated by N-terminus actuation

Sulfonylurea receptors regulate the gating of Kir6.x pore-forming subunits in two ways: (1) directly by opening and closing the pore through conformational changes induced in the receptor upon binding of MgADP, KCO and sulfonylureas and (2) indirectly, through modulation of Kir6.x's sensitivity to ATP (as a result of the co-assembly with SUR, Figure 38). This dual modulation renders very laborious the identification of the gates in Kir6.x directly regulated by SUR receptor. Mutagenesis of potential 'gate' residues can either result in actual opening/altering of gate or it can alter the protein-protein interaction between both SUR and Kir6.x. Therefore, straightforward interpretation of mutation phenotypes is limited

by possible effects on the SUR/Kir6.2 association. Thus, we have decided to use ICCR tool in order to investigate potential gate(s) present in the Kir6.2 channel. This approach allows to exclude the indirect effects as the GPCR does not alter this parameter.

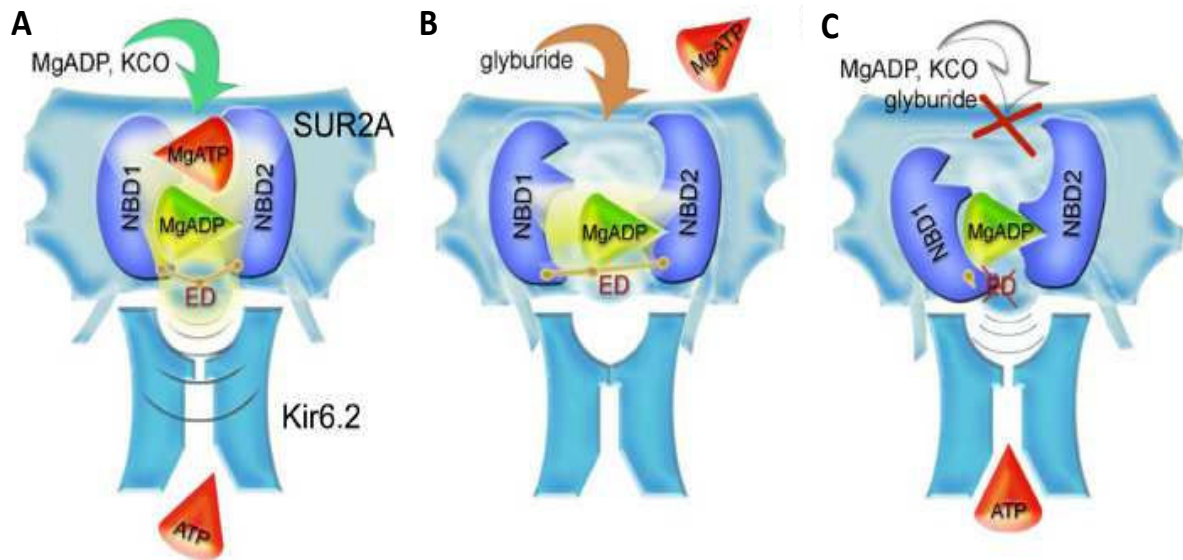


Figure 38. Suggested role of the ED domain in K_{ATP} channel gating.

a) In the presence of Mg-nucleotides intact SUR2A ED domain assists in the optimal coordination of NBDs, which is substantial for antagonism of ATP-induced Kir6.2 inhibition. This cooperative NBDs formation supported by the ED domain is as well important for K_{ATP} channel activation in response to KCO. b) Binding of the sulfonylurea (glyburide) destabilizes ED-mediated cooperative interaction between NBDs and results in the dissociation of MgATP from NBD1. This conformational rearrangement of NBDs/ED is essential for the transduction of an inhibitory signal originating in SUR2A to Kir6.2 pore closure. c) Disruption of ED domain stabilizes NBDs in suboptimal conformation in which Kir6.2 and SUR subunit can communicate partially. Hypothetic occlusion of MgADP within this suboptimal conformation of NBDs allows only for residual channel activation with KCO, impairs MgADP-induced channel opening and precludes glyburide induced channel inhibition [Karger J Gen Physiol 2008]

MATERIALS AND METHODS

Le mode opératoire mis en oeuvre au cours de ces travaux pour la caractérisation fonctionnelle des Récepteurs Couplés à un Canal Ionique (ICCRs) peut être résumé selon un schéma en trois étapes:

- première étape: génération des constructions d'ADN par biologie moléculaire, puis transcription *in vitro* en ARN messagers codant pour les protéines d'intérêt.
- deuxième étape: injection des ARN messagers dans l'ovocyte de Xénope permettant l'expression hétérologue des protéines modifiées.
- troisième étape: caractérisation fonctionnelle des ICCRs par microélectrode sur ovocytes.

The strategy that we applied to investigate functional properties of the Ion Channel-Coupled Receptors with modified G Protein-Coupled Receptor or Kir6.2 channel can be divided into five main phases (Figure 39):

- Phase one: construction of cDNA encoding fusion proteins.
- Phase two: mutagenesis to obtain ICCR with modified GPCR/Kir6.2.
- Phase three: synthesis of mRNA during *in vitro* transcription.
- Phase four: heterologous expression of mRNA in *Xenopus laevis* oocytes.
- Phase five: functional characterization of the protein of interest with two-electrode voltage clamp technique (TEVC)

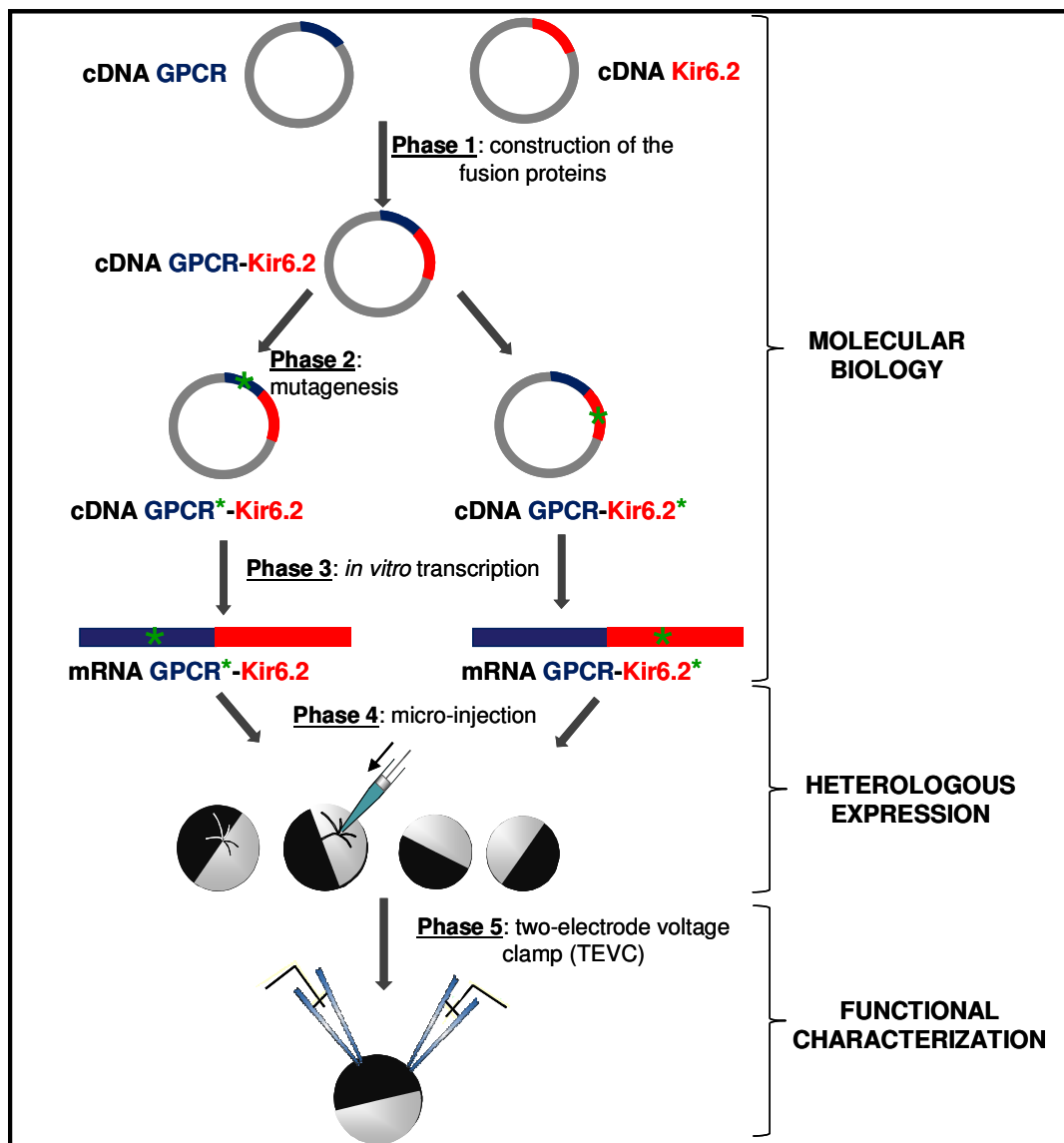


Figure 39. Schematic representation of all steps implemented in this study [Channels Group].

V Molecular Biology

V.1 Clones and expression vectors.

In our experiments, we used the clones of the mouse Kir6.2 channel (KCNJ11, Genbank accession number: D50581) given to us by Dr. S. Seino (Chiba University School of Medicine, Japan). We also used following clones: TMD0-F195 of hamster SUR1 (ABCC8, Genbank accession number: L40623) and the mouse Kir6.2 Δ C36 [Chan EMBO J 2003] [Hosy J Physiol 2007] [Tucker Nature 1997]. The human clone of the M2 muscarinic receptor was provided by Dr. D. Logothetis (Virginia Commonwealth University). The clone of the human β 2 adrenergic receptor derived from the company Origene (Genbank accession number: NM_000024.3), whereas the clone of T4L-modified receptor was given to us by Dr. D. Rosenbaum and Dr. B. Kobilka. The wild-type and T4L-modified clones of the human oxytocin receptor were provided by our collaborator Dr. V. Cherezov.

The expression vector in which we subcloned our constructions was pGEMHE plasmid, provided to us by Dr. D. Logothetis (Virginia Commonwealth University). The plasmid currently used, called pGH2, was created in the laboratory by Dr. F. Pages. It has a bacterial origin of replication (ORI) and a β -lactamase coding region (Amp^R, ampicillin resistance), which allows for the selection of the recombinant bacteria with the incorporated plasmid. In addition, the vector contains both the T7 and SP6 polymerase promoters. The multiple cloning site (MCS) is flanked by 5'- and 3'-untranslated (UTRs) regions from *Xenopus* β -globin gene to stabilize injected mRNA and thereby boost by several-folds the expression of the protein of interest. Moreover, plasmid contains a poly-A tail at the 3' end of the cDNA that stabilizes both the mRNA transcripts and linearization site (Figure 40).

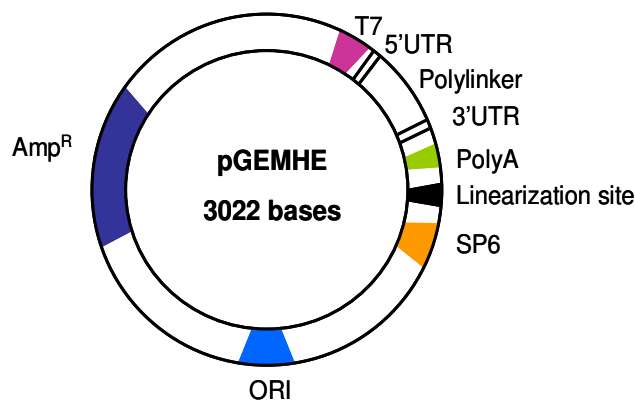


Figure 40. Schematic representation of pGEMHE, expression vector optimized for *Xenopus* oocytes [Channels Group].

V.2 Mutagenesis.

V.2.1 Site-directed mutagenesis.

The technique of *in vitro* site-directed mutagenesis allows to make point mutations, replacing amino acids, and deleting or inserting single or multiple adjacent amino acids. In our investigations, we used QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies), which principle is presented in Figure 41. This method is based on PCR reaction in which *Pfu*Ultra high-fidelity DNA polymerase conducts mutagenic primers-directed replication of the plasmid strands.

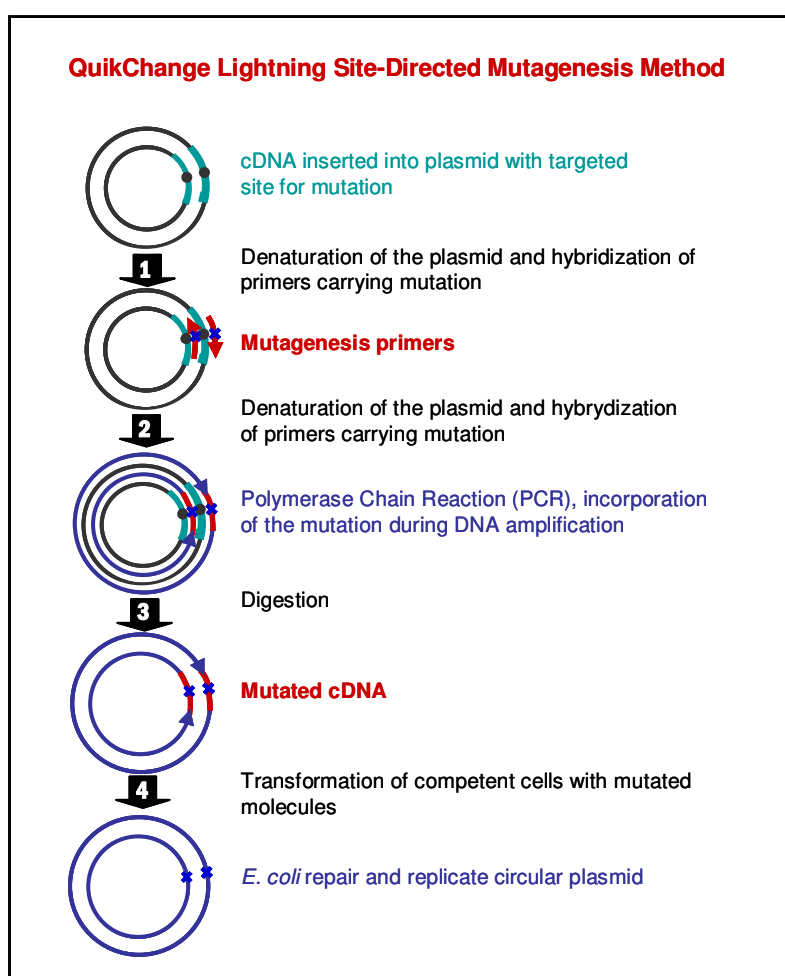


Figure 41. Principle of the site-directed mutagenesis carried out with QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies) ^[Channels Group].

Both primers complementary hybridized with DNA strands and are subsequently prolonged by *Pfu* polymerase giving the rise to a « new » mutated plasmid. After PCR, product of this

reaction is subjected digestion with *DpnI* endonuclease in order to eliminate parental methylated and hemimethylated template DNA and to select for mutation-containing synthesized DNA. At final stage mutated DNA molecules are incorporated into *E.coli* bacteria during transformation process.

V.2.2 Insertion/deletion of big fragments.

In order to create modified M2-based ICCR with an inserted T4 phage lysozyme domain, it was necessary to replace several dozens of residues present in the i3 loop with encoding sequences of the T4L domain.

In our manipulations we performed a two-step PCR. During the first PCR we used the β 2(T4L)-Kir6.2 (subcloned into the pGEMHE2 vector) as the template to obtain the cDNA encoding only the T4L domain flanked by 3' and 5' termini complementary to the targeted M2 receptor. In order to do this we used oligonucleotides of about 50 bp, complementary to the region of the T4L domain at the 3', thus allowing for its elongation and containing a floating terminus at the 5' complementary to the M2 receptor region targeted for insertion (Fig. 42a). Product of this first reaction was subsequently loaded into 1% agarose gel and purified using GeneClean turbo kit (MP Biomedicals). After, in-gel DNA-purification, the obtained product served as a "megaprimer" (~500bp) for a second PCR in which we used as the template M2-Kir6.2 ICCR subcloned in the pGEMHE2 vector.

In order to create the fusion protein lacking the i3 loop, we used primers that are complementary only to flanking regions of the area to be deleted (Fig. 42b). This way during the elongation step the DNA polymerase synthesizes a new strand excluding the region of i3 loop.

The obtained PCR products were digested with *DpnI* enzyme and used for the transformation with XL-10GOLD bacteria (Stratagene). Subsequently positive clones were selected according to their restriction pattern and sent to sequencing (Genomics).

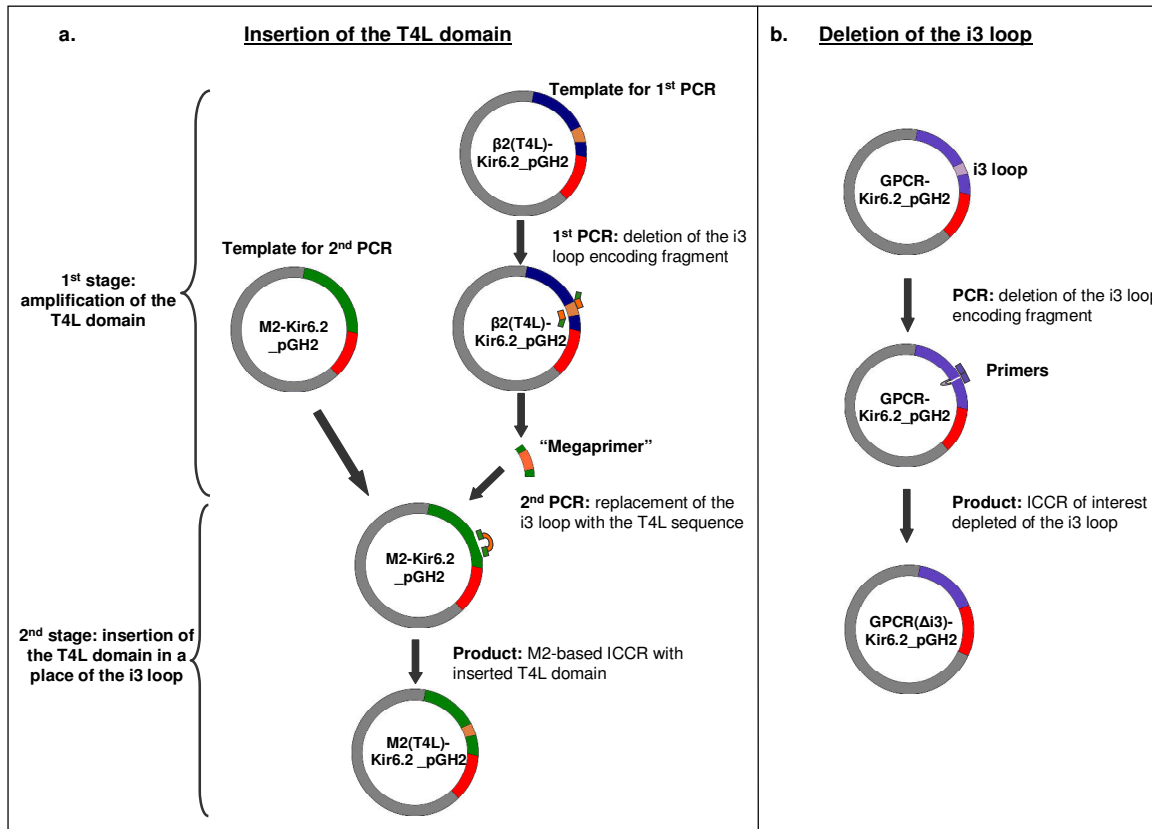


Figure 42. Schematic representation of strategy used for insertion/deletion of big fragments during PCR.

a) Insertion of the T4L domain in a place of the receptor's third intracellular loop (i3 loop). b) Deletion of the i3 loop.

V.3 Amplification of genetic material.

V.3.1 Transformation of competent bacteria.

Subsequent step after genetic engineering manipulations was the transfer of the obtained DNA encoding fusion protein of interest into bacteria in order to amplify the genetic material. In our investigations, for products of the PCR, we use «ultracompetent» bacteria *Escherichia coli* XL-10 Gold (provided by QuickChange Lightning Site-Directed Mutagenesis kit, Agilent Technologies). We mixed 50 μ l of competent bacteria with cDNA and incubate the mixture on ice for 30 minutes. The «entry» of DNA is induced during a thermic shock: 45 seconds at 42°C and afterwards 2 minutes on ice. Subsequently, recombinant bacteria are selected based on their resistance to ampiciline: recombinant plasmids possess ampiciline resistance gene, thus only bacteria carrying these plasmids are capable to grow on LB (Luria-Bertani) agar culture medium containing this antibiotic (100 μ g.mL⁻¹)

V.3.2 Amplification and purification of the plasmid DNA.

Extraction of the plasmid DNA from the bacteria cells is based on the alkaline lysis procedure done by solutions containing a detergent, sodium dodecylsulfate (SDS), and an alkali: sodium hydroxide (NaOH). The SDS detergent is used to lyse the cells and denature the proteins. The alkaline conditions denature the genomic DNA, plasmid DNA and proteins. The principle of this method is based on the different physical properties of linear genomic DNA and supercoiled plasmid DNA. Thus, when the neutralizing solution of potassium acetate (pH 5,2) is added to the cellular lysate, only the plasmid DNA renatures while the genomic DNA undergoes irreversible denaturation. The precipitate (plasma membrane, proteins, RNA, genomic DNA) is separated from the plasmid DNA by a high speed centrifugation (14000 rpm, at 4°C, for 15 minutes) or with columns (MidiPrep Quiagen kit). The plasmid from the supernatant is recovered by precipitation using pure isopropanol (14000 rpm, 4°C, ≥ 30 minutes), and then purified with 70 % ethanol (to remove contaminant salts). At the end, the DNA pellet is dissolved in distilled water once dry.

In our investigations we used two methods of amplification and purification of plasmid DNA: miniprep for screening a high number of clones, and midiprep after selecting a positive clone from the results of the miniprep.

V.3.3 Miniprep and Midiprep.

The Miniprep method is applied in order to select positive clones of recombinant bacteria. Bacteria clones are transferred to 5 mL of LB medium + Ampiciline (100 $\mu\text{L}\cdot\text{mL}^{-1}$). The resuspension, lysis and neutralization solutions are provided by the Quiagen kit. All steps of purification are described above. The selection of positive clones is done by the digestion with specific restriction enzymes and further analysis of the restriction profile. Selected, positive clones are then sent to sequencing.

The Midiprep method allows for the obtention of high quality (purity) plasmid DNA subsequently used for *in vitro* transcription. The purity of the obtained plasmid DNA increases due to the filtration step enabling the elimination of all bacterial remainings, and the anion-exchange column which selectively binds the plasmid DNA. For the Midiprep method, we start with 50 mL of bacteria culture in LB medium, supplemented in ampiciline, which results in a larger quantity of genetic material when comparing to Miniprep. Likewise in the case of Miniprep, all the solutions are provided by the Quiagen Plasmid Purification kit. The

concentration of purified plasmid DNA is then estimated using the NanoDrop 2000 spectrophotometer by measuring the optic density at a wavelength of 260 nm.

V.3.4 Sequencing.

A volume of 20 μL at a concentration of 100 $\text{ng}/\mu\text{L}$ of the samples are sent to Beckman Coulter Genomics for sequencing. The sequencing technique used by this company is based on the Sanger et al. method (1977), which requires a single-stranded DNA, a DNA primer, normal dioxynucleosidetriphosphates (dNTPs) and modified nucleotides (ddNTPs). The DNA sample is divided into four separate sequencing reactions which run simultaneously. Each reaction contains all four standard deoxyribonucleotides triphosphate (dNTP) and DNA polymerase. To each reaction, only one of the four dideoxynucleotides (ddNTP) is added, labeled with a fluorescent probe. ddNTPs are characterized with an ability to terminate the synthesis of the new strand of DNA. Thus, the migration of the PCR products in the capillary gel electrophoresis, allows for the separation of the DNA fragments of different lengths and the presence of the fluorochromes facilitates the reading in an optical system. Depending on the size of the synthesized fragments is it possible to rebuilt the sequence of the DNA of interest in its totality.

V.4 In vitro transcription.

RNA manipulations require maintenance of «RNase-free» conditions. It is necessary to wear gloves and prepare transcription mixture under the fumehood, to avoid any contamination. Moreover, all used materials: pipettes, tips and Eppendorf tubes, as well as reagents, are exclusively designed for mRNA manipulations. Water used for transcription and mRNA dilutions is treated with diethyl pyrocarbonate (DEPC, Sigma Aldrich) which deactivates RNAses. Water with 0.05% DEPC is incubated overnight at room temperature and then autoclaved (20min, 121°C) in order to degrade the DEPC compound which is toxic for oocytes.

We linearize the plasmid DNA encoding sequence of the protein of interest with a specific enzyme (*NotI*) that acts at the linearization site localized downstream of the 3'UTR sequence. Twenty units of restriction enzyme are used to digest 10 μg of DNA, during overnight incubation at 37°C. Subsequently, the linearized DNA is extracted using the phenol/chloroform/isoamyl alcohol method. The quantity and quality of the purified DNA is

estimated with a 0.8% agarose gel. The intensity of the band allows to determine the quantity of DNA used for *in vitro* transcription.

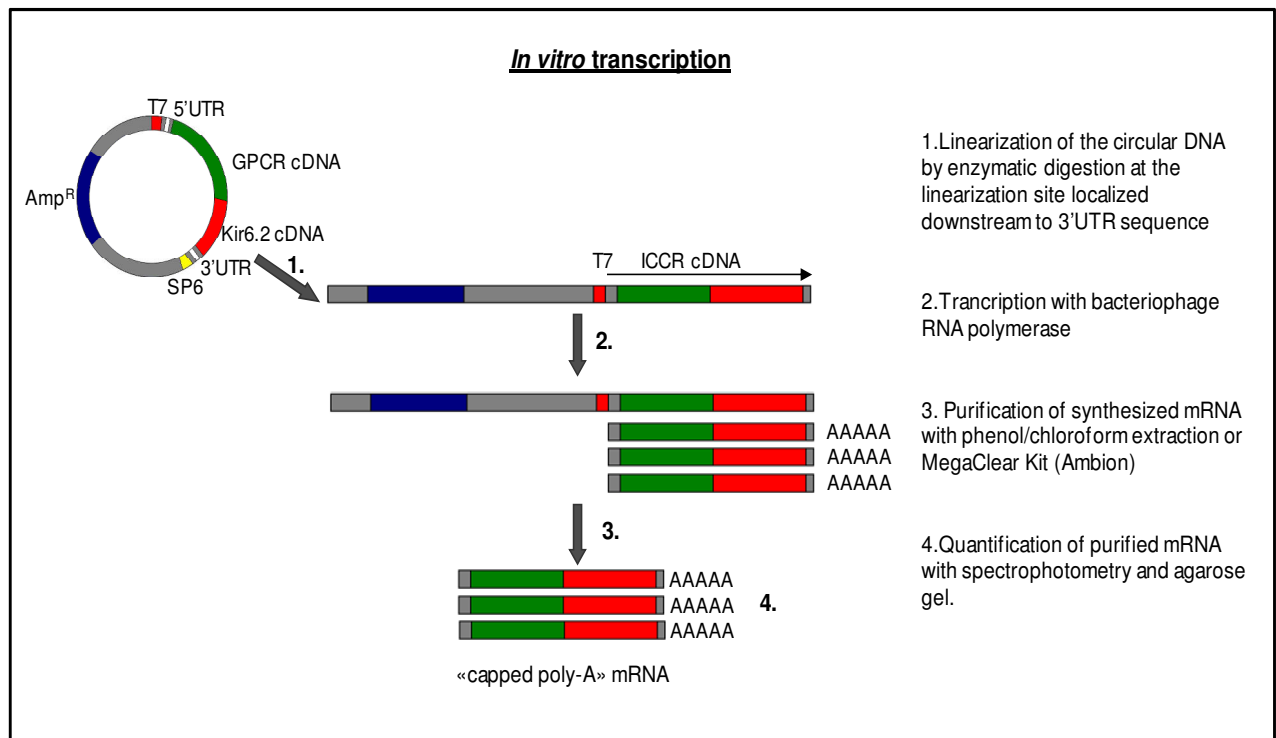


Figure 43. Principle and phases of *in vitro* transcription [Channels Group].

Transcription is performed by RNA T7 polymerase, which promoter is localized upstream of the sequence to be transcribed. All reagents used for the synthesis are provided by mMessage mMachine T7 kit (Ambion). This kit allows for the obtention large amount of RNA capped at the 5' with methylated guanosine, crucial for the initiation of transcription and protection from degradation by RNAses 5' -> 3'. After 4 hours of transcription, synthesized mRNA was purified using one of two different methods: extraction with phenol/chlorophorm mixture or with MegaClear kit (Ambion). Subsequently, the quantity and quality of purified mRNA was estimated with U.V. spectrophotometry method (Nanodrop2000c, Thermo Scientific) and by agorose gel (Figure 43). At the final stage of manipulations, mRNA was diluted to obtain concentrations used for injections (Table 3) and then frozen at -80°C in order to limit its degradation.

mRNA	Concentration ($\mu\text{g}/\mu\text{l}$)
ICCR	0.08
GPCR	0.04
TMD0	0.02
Kir3.4 S143T	0.04
Kir6.2 Δ C36	0.04

Table 3. Concentrations of mRNAs used for injections.

VI Heterologous Expression

VI.1 *Xenopus* oocytes, tool for functional expression.

After the observation that foreign RNA injected into *Xenopus laevis* oocytes can be translated into proteins and the first use of oocytes for the expression of receptors and channels, oocytes from *Xenopus laevis* have become a popular expression system for ion channel, receptors and transporters ^{[Gundersen Proc R Soc Lond 1983] [Miledi Proc R Soc Lond 1983]}.

The fully developed *Xenopus laevis* oocyte is a big, spherical cell of about 1-1,3 millimetre in diameter. Size of the cell makes it handling, manipulations and microinjections easy. During oogenesis, oocytes accumulate large amounts of storage proteins that provide the developing embryo with building blocks and energy metabolites. Thus, the mature cell is fully equipped to initiate protein synthesis (enzymes, ribosomes, tRNA, translation and maturation proteins). The size of the oocyte, the large reserve of storage proteins and its ability to synthesize on demand makes *Xenopus laevis*' oocyte an almost ideal single-cell expression system. This is the reason why *Xenopus laevis* oocytes are a versatile expression system particularly suited for membrane transporters and channels. Oocytes have little background activity and therefore offer a very high signal-to-noise ratio for transporter and channel characterization.

Nevertheless, it should be kept in mind that in case of other expression system (as well as mammalian, bacterial etc.) recorded responses can be influenced by differences in the lipidic composition of the plasma membrane or by intracellular signalling pathways specific to *Xenopus* oocytes.



Figure 44. *Xenopus laevis* [<http://www.flickr.com/photo>].

The *Xenopus* oocyte is divided into two hemispheres: a well-defined brown animal pole (containing the majority of the nucleus) and a weakly pigmented vegetal pole (containing the majority of the yolk platelets). The two hemispheres are separated by a thin, unpigmented ring called the equatorial belt (Figure 45).

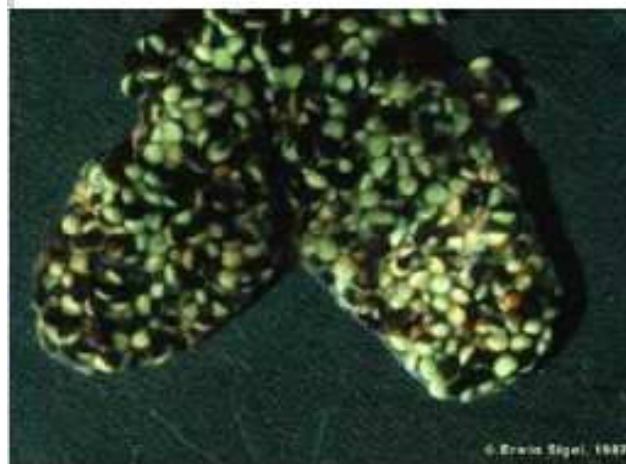
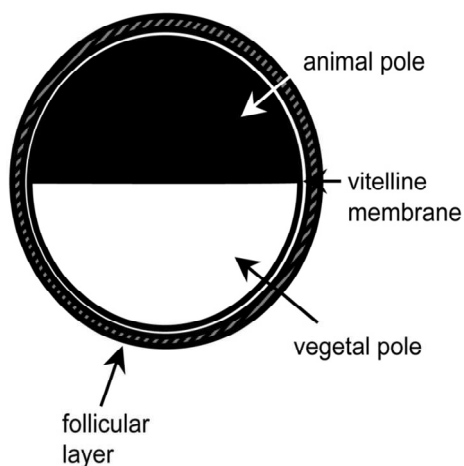


Figure 45. *Xenopus laevis* oocytes.

a) Representation of the oocyte in the V or VI stage of development. b) Two ovarian lobes containing hundreds of oocytes in different stages of development [<http://ibmmsrvlakit.u.unibe.ch/sigel/xenopicts.html>].

Oocytes are localized in two ovaries subdivided into multiple lobes. Each lobe contains hundreds of oocytes at all stages of development (I-VI), blood vessels and connective tissue. Moreover oocytes are surrounded by two distinctive layers:

- A vitelline membrane (an acellular layer of glycoprotein matrix which allows for the preservation of the oocytes' spherical shape). This layer is kept during the TEVC

recordings, but removed before Patch-clamp experiments in order to get access to plasma membrane.

- An external layer of follicle cells that extend small projections which cross the vitelline membrane to contact the oocyte surface and form gap junctions.

VI.2 Extraction and preparation of the oocytes.

The *Xenopus laevis* used for our experiments are either provided by the Centre d'élevage des Xénopes (CNRS, Montpellier), or by Xenopus Express (Vernassal). They are raised in tanks in a room where the temperature is maintained at 22°C, and a 12h/12h night cycle is imposed. Each week, surgery is performed in order to extract oocytes. The animal is first anesthetized with a solution of acid aminobenzoic ethyl ester at a concentration of 1 g.L⁻¹. After 30 minutes, the anesthetized *Xenopus* is placed on ice and remains on ice for the duration of the surgery in order to keep it asleep. The abdomen is then disinfected with iodine and an incision of approximately 1 cm of the animal's skin is performed. The muscle is then incised to enable access to the ovarian lobes (minilaparotomy). Extracted oocytes are transferred to Barth's isotonic solution (NaCl 88 mM, KCl 1mM, NaHCO₃ 2,4 mM, Hepes 16 mM, MgSO₄ 0,82 mM, Ca(NO₃)₂ 0,3 mM, CaCl₂ 0,41 mM, pH7,4). After getting the desired amount of oocytes, both incisions are stitched with absorbable thread (5-0, tip 16 mm 3/8c, 75 cm VICRYL Ethicon). After finishing the surgery, the *Xenopus* is rinsed and placed in a small container filled with water (with head out of the water to avoid the drowning risk) in order to wake it up. After a few hours, the awoken frog is transferred to the post-surgery tank.

Oocytes are isolated by enzymatic defolliculation in 2 mg.ml⁻¹ type 1A collagenase (Sigma-Aldrich, C9891). The digestion undergoes for duration of 2 hour of incubation at 19°C, under gentle agitation. Separated oocytes are then rinsed at least three times (5 minutes each rinse) with Barth's solution shorting of Ca(NO₃)₂, and once with full Barth's solution. The oocytes in stages V-VI of development are selected based on visual criteria (size, shape and homogeneity of pigmentation). Selected eggs are stored at 19°C in Barth's solution supplemented with 100 U.mL⁻¹ penicillin, streptomycin and gentamycin.

VI.3 RNA microinjections

Micro-injections are performed the day after surgical extraction, either manually (Nanoject, Drummond), or automatically (RoboInject, Multi Channel Systems MCS GmbH). For manual injection, the oocytes are immobilized on the net situated in the Petri dish filled with Barth's solution supplemented with antibiotics. The injection is performed with glass microcapillary (3.5" Drummond # 3-000-203-G / X) pulled horizontally (Micropipette Puller P-97, Sutter Instrument Co.) and then broken to a final size of ~ 7 microns. The prepared pipette is filled with incompressible mineral oil (Sigma-Aldrich) and then placed on the plunger of the injector. Injected oocytes are then transferred to the 96-well plates filled with Barth's solution with antibiotics. For the automatic system, oocytes are placed in cone-shaped bottom 96-well plate fully filled with antibiotic-enriched Barth's solution before injection (Figure 46b). The prepared plate is then set in the carrier which moves during the injection. The pre-pulled borosilicate glass micropipette (ID 0.49 mm, tip opening approx. 25 μ M, Multi Channel Systems MCS GmbH) is filled with mineral oil (Sigma-Aldrich), similar to the one for manual injection.

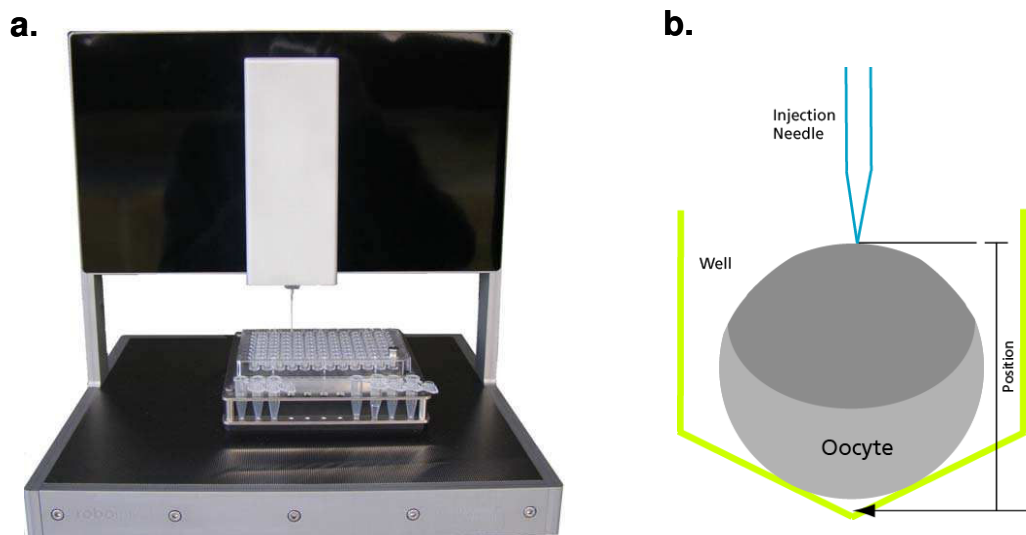


Figure 46. Automated injections of oocytes.

a) RoboInject - microinjection robot (allows to inject >500 oocytes/h). b) Oocyte plated in cone-shaped well with vertically orientated injection micro-pipette [<http://www.multichannelsystems.com>].

For both systems 3 μ L of mRNA is sucked into the micropipette. Each egg is pricked with the micropipette and 50 nL of mRNA mixture is injected. Between 40 and 50 eggs by construction are thereby obtained. The amounts of RNA injected for each construct are shown in Table 4.

mRNA	Quantity per oocyte (ng)
ICCR	4
GPCR	2
TMD0 (SUR1)	1
Kir3.4 S143T	2
Kir6.2 Δ C36	2

Table 4. Quantity of mRNA encoding indicated constructs injected per single oocyte.

Microinjected oocytes are then incubated individually in 96-well plates containing Barth's solution with antibiotics, for more than 2 days at 19°C.

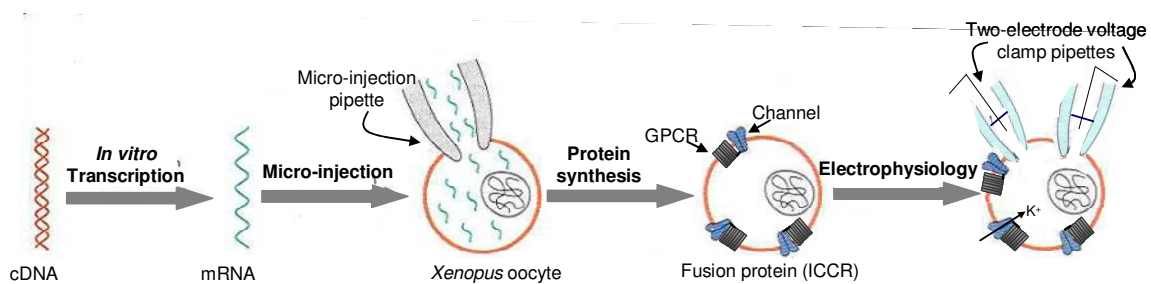


Figure 47. Following phases of functional expression of the Ion Channel-Coupled Receptors in *Xenopus oocyte* [Channels Group].

VII Functional characterization of the fusion proteins.

VII.1 Manipulation of the cholesterol content in the plasma membrane of oocytes.

In order to investigate properties of the expressed proteins in a cholesterol-deficient environment, we used methyl- β -cyclodextrin (M β CD, Sigma-Aldrich) dissolved in A+B solution to extract cholesterol from the plasma membrane of tested oocytes. Cells expressing the protein of interest were individually incubated in a 96-well plate; each in a well filled with 300 μ L of 20mM M β CD. Treatment with the cholesterol-complexing agent was performed for

3 h at 19°C, under gentle agitation. After a set time, oocytes were functionally characterized with the two-electrode voltage clamp technique.

To restore the cholesterol in the plasma membrane of the oocytes pre-treated (for 3h) with 20mM M β CD, we used inclusion complexes of M β CD and cholesterol (Sigma-Aldrich) in a molar ratio of 8:1 in order to saturate cyclodextrins with exogenous cholesterol molecules. For the preparation of the steroid cyclodextrin inclusion complexes, we dissolve M β CD in A+B solution and vortex it for 5 minutes in order to obtain a homogenous mixture. Subsequently, we add cholesterol to 5 mM M β CD and vortex the prepared mixture for another 5 minutes. Similarly we prepare inclusion complexes of M β CD and lanosterol. Oocytes were incubated in the mixture solution, of M β CD/cholesterol or M β CD/lanosterol for 1 hour at 19°C, under gentle agitation.

VII.2 Two-Electrode Voltage Clamp (TEVC) technique.

VII.2.1 Principle of the TEVC technique.

The voltage-clamp technique is the most powerful electrophysiological method for basic research as it allows ion flow across the plasma membrane to be measured as an electric current, as the membrane potential of the cell is held under constant experimental control ('clamped') with the feedback amplifier. The voltage-clamp technique was the basis for two milestones in electrophysiology: the Hodgkin-Huxley (1952) description of excitability and the demonstration of the single channel events by Neher and Sakman (1976).

For big cells, especially for *Xenopus* oocytes, ion channels present in the plasma membrane can be studied with two-electrode voltage clamp technique (TEVC). During TEVC experiments, the membrane of the cell is penetrated by two microelectrodes: one to monitor the membrane's potential (V_1) and the other to inject the current to adjust the membrane potential to the desired values (V_2). The membrane potential is measured by voltage-sensing electrode and a high input impedance amplifier (amp1) compares it with the command voltage. The obtained difference is brought to zero, by second, high gain amplifier (amp2). The injected current (to hold command potential) is monitored via current-to-voltage converter to provide a measure of the total membrane current ^[Baumgartner Biophys J 1999].

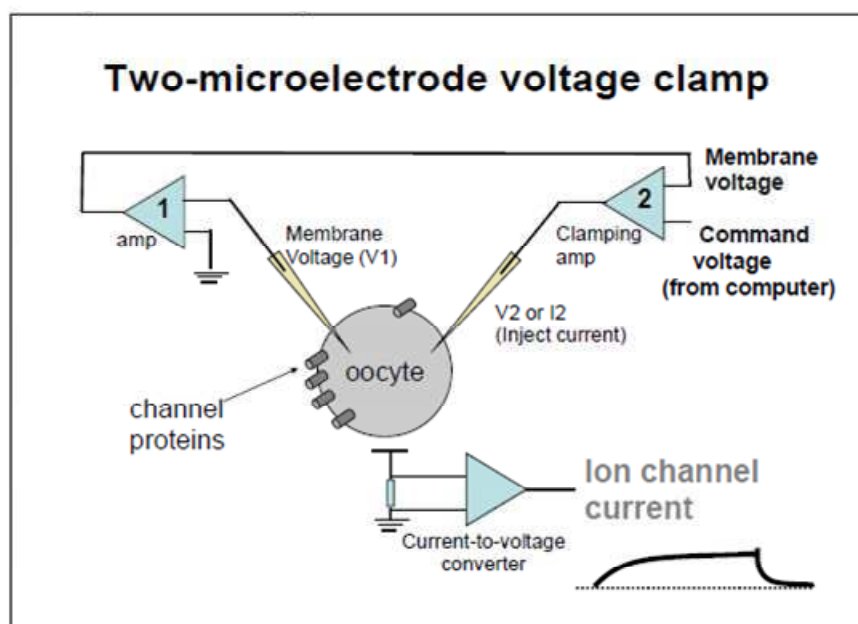


Figure 48. Principle of the two-electrode voltage clamp (TEVC) technique [Baumgartner Biophys J 1999].

VII.2.2 Experimental procedures.

The initial phase of the TEVC experiment does not differ significantly between the manual and automated technique. We prepare ligand solutions in TEVC bath of the following composition: 91 mM KCl, 5 mM HEPES, 1.8 mM CaCl₂, MgCl₂, 1 mM, 0.3 mM niflumic acid, pH 7.4. A prevailing advantage of the HiClamp robot is significant reduction of the ligand sample volume (~200 μ L) compared to classical setup (min. volume ~10 mL). Then we prepare micro-pipettes which are pulled from borosilicate capillaries (Kimble Product Inc.) to obtain a diameter of few micrometers and a resistance between 0.2-0.8 M Ω . Pulled pipettes of desirable parameters are subsequently filled with 3 M KCl solution and placed on the two Ag/AgCl electrodes. After this operation the way of following experiment differs between manual and automated techniques.

In the case of the manual setup, the oocyte is placed in a perfusion chamber (Automate Scientific) (Figure 49) and initially washed with TEVC bath solution. In order to test different ligands, we use ValveLink 8.2 perfusion system (Automate Scientific). We exploit the protocol in which the system applies 500 ms voltage pulses at three different command voltages: -50, 0 and +50 mV (in order to control rectification level) in 5 seconds intervals. For data acquisition we use Digidata 1440A system (Molecular Devices). The recorded variations in K⁺ current are visualized with Clampex 10.0 software (Molecular Devices).

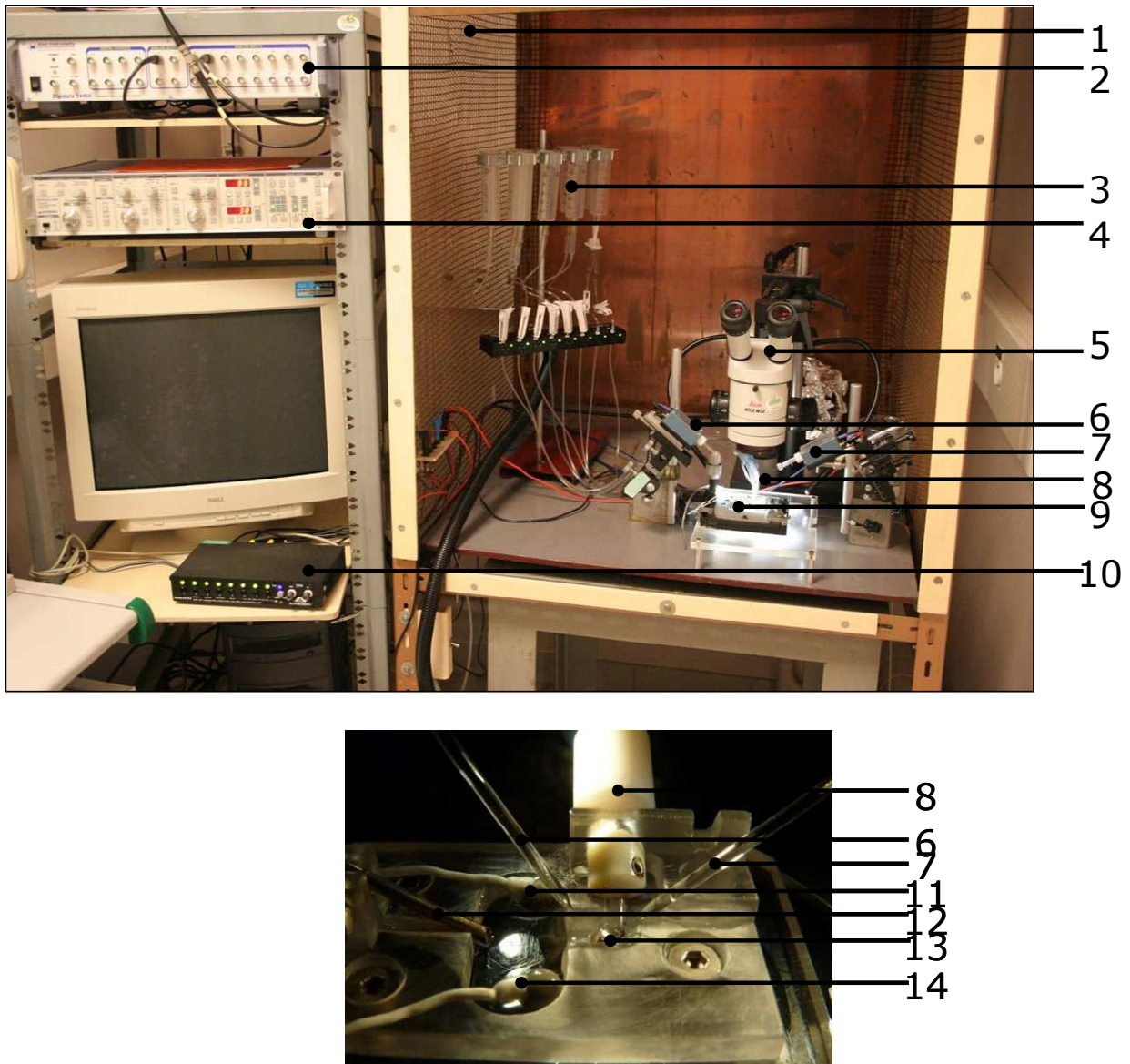


Figure 49. The classical (manual) setup for TEVC experiments [Channels Group].

During the experiment performed with the HiClamp TEVC robot, oocytes are stored in a 96-well plate, called the feeder plate, localized on the right site of the table. Subsequently, with the suction pump (manageable with simple Syringe System) the oocyte is transferred to the basket. In contrast to the manual technique, for the HiClamp robot, we apply a protocol in which we command voltage only at -50mV . Before testing the ligand (in Bath TEVC solution), the cell situated inside the basket is placed in the washing station (localized between the plates) and washed with ND96, low- K^+ solution (NaCl 91 mM, KCl 2mM, CaCl_2 1,8 mM, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 1mM, HEPES 5mM, pH 7.4). Application of the ND96 solution in the first stage of the experiment is very crucial. In these conditions, the robot tests the oocyte's

potential, thus is able to recognize the oocyte as a healthy cell (with no leak, in our setting current $<20\mu\text{A}$). After selection in ND96, the oocyte is washed with the TEVC bath solution. This step allows for the distinction between non-expressing (current $<0.1\mu\text{A}$), and dying cells (current $>20\mu\text{A}$, leak of ions from tested cell).

Manual set up of Two—Electrode Voltage Clamp
1. Faraday's cage
2. Digidata 1440a (Molecular Devices)
3. Perfusion system (reservoirs)
4. Geneclamp (Axon Instruments)
5. Microscope (Wild M3Z, Leica)
6. Stimulating electrode
7. Detection electrode
8. Head of the perfusion system
9. Perfusion chamber (Automate Scientific)
10. ValveLink 8.2 (Automate Scientific)
11&14. Reference Electrodes
12. Suction pump
13. Oocyte

Table 5. Composition of the manual TEVC setup.

After the examination with ND96 and TEVC bath solution, the tested oocyte (when fulfils established parameters: current $>0.1\mu\text{A}$, but $<20\mu\text{A}$) is exposed to a compound solution by moving it physically to the solution (whereas as it was mentioned in standard system the solution is applied on the cell). Placed in the basket, the oocyte is automatically transferred to the compound plate on the left side of the table in order to investigate the cell's response. Like in the case of the manual system we start our recording with TEVC bath solution and finish with 3 mM barium.

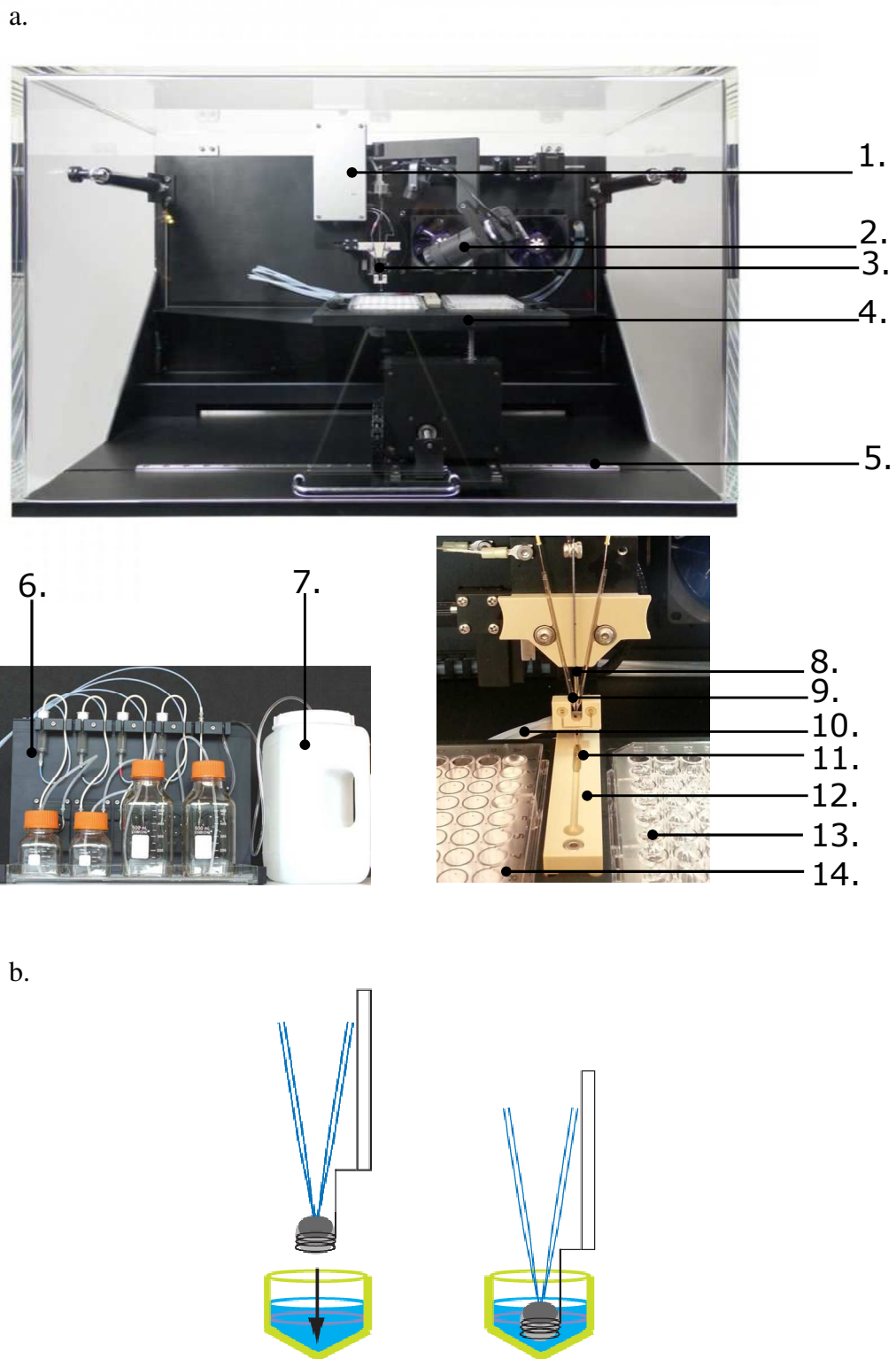


Figure 50. An automated setup for TEVC experiments.

a) HiClamp Robot ^[Channels Group], b) Motion of the basked with situated oocyte into tested solution ^[<http://www.multichannelsystems.com>]

HiClamp robot for Two—Electrode Voltage Clamp
1. Amplifier (ClampAmpC)
2. Camera
3. Suction pump (engaged by Syringe System)
4. Well plate table
5. Trackrail
6. Washing station
7. Waist container
8. Suction pump
9. Electrodes (stimulation and detection)
10. Perfusion system tubing
11. Perfusion chamber
12. Basket with Reference Electrodes
13. Feeder plate (with oocytes)
14. Compound plate (with tested ligands)

Table 6. Composition of the automated TEVC setup (HiClamp robot).

VII.2.3 Data processing.

Subsequently data is processed with the software Clampfit10 (Molecular Devices) and the obtained values are exported to an Excel file to calculate the currents generated by the studied channels. In order to standardize recorded current we determine the 0 current during the application of the 3 mM barium and 1 in the bath solution. The applied formula is as follows:

$$i_{\text{normalized}} = \frac{i - i_{\text{Ba}^{2+}}}{i_{\text{bath}} - i_{\text{Ba}^{2+}}}$$

where i is the current recorded upon the application of a specific ligand (agonist/antagonist), $i_{\text{Ba}^{2+}}$ current measured in the presence of barium ions, and i_{bath} in the TEVC bath solution. The percentage of the change in the current (activation or inhibition) induced by the applied compound is calculated from the following equation:

$$\%_{\text{change}} = (i_{\text{normalized}} - 1) \cdot 100$$

where $i_{\text{normalized}}$ is the value obtained through the application of a specific ligand. This value is calculated in respect to the 1 and 0 obtained in the bath solution and in presence of

the 3 mM barium respectively. Subsequently, data is transferred to the Origin 5.0 software (OriginLab) in order to fit ('fittings') according to a standard Hill equation:

$$f(x) = \frac{\text{Max}}{1 + \left(\frac{\text{EC}_{50}}{[x]}\right)^h}$$

where:

[x] is the concentration of the applied ligand

Max is the asymptotic maximal effect

EC₅₀ is the concentration at which half maximal effect of ligand is observed

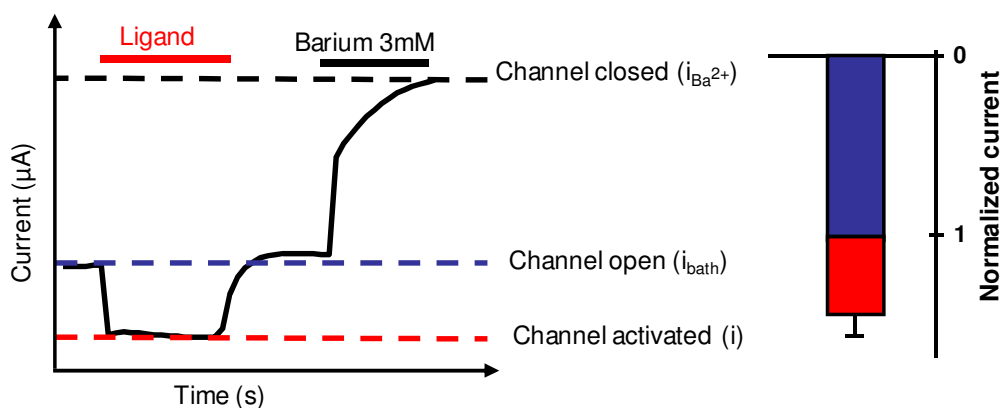


Figure 51. Typical recording obtained using manual TEVC setup with the bar representing normalized current.

RESULTS

Dans le cadre de cette étude, nous avons développé quatre applications des Récepteurs Couplés à un Canal Ionique (ICCRs) qui sont: 1) la caractérisation fonctionnelle des RCPG optimisés pour la cristallisation par insertion de domaine du lysozyme du phage T4 dans la boucle ICL3; 2) la détection de la dépendance des RCPGs au cholestérol; 3) la détection de ligands dits "biaisés" pour faciliter leur criblage; et 4) la cartographie fonctionnelle des portes du canal Kir6.2 régulées par des protéines membranaires interagissant par le domaine N-terminal.

VIII Project #1: Functional assessment of GPCR(T4L) with the ICCR technology

VIII.1 Problematic

As it was mentioned, G Protein-Coupled Receptors constitute the biggest class of membrane proteins involved in cell communications. Therefore, they play fundamental role in human physiology, making them a major pharmaceutical target. That is the reason of intensified trials to crystallize members of this family, in order to provide structural details essential to understand their molecular mechanisms and design rational drug-screening. However, the main hurdle that has suppressed progress in GPCRs crystallographic studies is the low expression level and the dynamic nature of these proteins, revealed by their inherent flexibility and proteolytic susceptibility. In consequence, after the publication in 2000 of the first crystallographic structure of a GPCR, the bovine rhodopsin, a long outage came before the crystallization of another GPCRs [Palczewski Science 2000].

Currently, to solve the structure of non-rhodopsin GPCRs, three successful methods are used, to overcome receptor intrinsic instability:

- co-crystallization with stabilizing antibodies [Rasmussen Nature 2007]
- insertion of the T4 phage lysozyme (T4L) [Cherezov Science 2007] [Rosenbaum Science 2007] or thermostabilized apocytochrome b562RIL (BRIL) domains [Liu Science 2012] [Chun Structure 2012]
- introduction of thermostabilizing mutations [Warne Nature 2008]

Quantitatively, the T4L insertion has been the most successful strategy with 14 distinct receptors crystallized in different states [Katritch Annu Rev Pharmacol Toxicol 2013]. However, to address the functionality of the crystallized GPCRs, with inserted T4L or BRIL domains in place of their 3rd intracellular loop, the classical G protein-based assays are excluded due to disruption of the interaction with the cognate G proteins. Nowadays, assessments of GPCR(T4L) function are partially performed by expensive and hazardous radiolabeled ligand assays or fluorescence spectroscopy with probes located only in close proximity to the i3 loop region. These results provide indications of the GPCR(T4L) activities, but spatially limited to the ligand binding site and the motion of the cytoplasmic side of helix VI.

We created the Ion Channel-Coupled Receptors (ICCRs) as a G protein-independent reporter of global conformational changes of GPCRs by genetic fusion of K⁺ inward rectifier channel (Kir6.2) to GPCR C-terminus. We investigated in this project if ICCRs could constitute an alternative tool to functionally characterize modified GPCRs that are unable to bind or activate G proteins, such as GPCR(T4L) (Figure 52). The ICCR concept has been already validated with four prototypical GPCRs: the M2 muscarinic, the D2L dopaminergic, the β 2 adrenergic and the opsin receptors [Moreau Nat Nanotechnol 2008] [Caro PloS ONE 2011] [Caro PloS ONE 2011]. Coupling between the receptors and the channel initially required extensive protein engineering, but the optimized constructs were able to detect both agonist- and antagonist-bound states independently of G protein involvement.

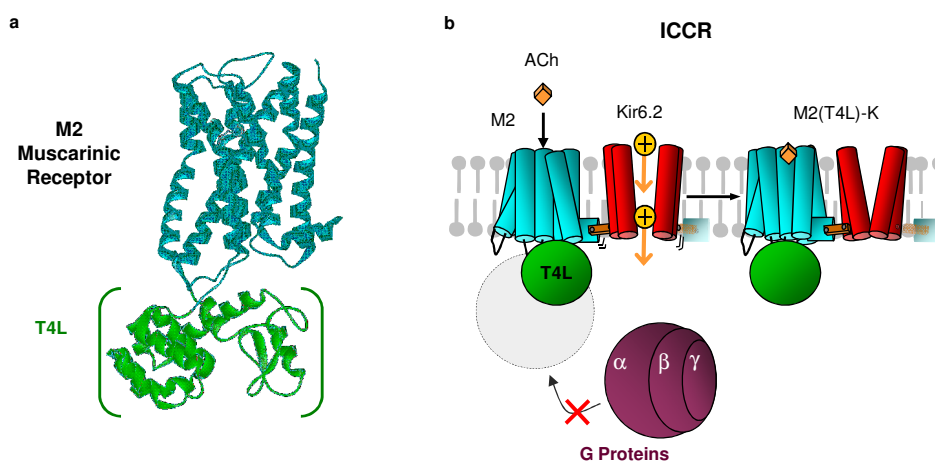


Figure 52. The T4L-modified Ion Channel-Coupled Receptor.

a) Crystallographic structure of the M2 muscarinic receptor (in blue, PDB code: 3UON) with the T4 phage lysozyme domain (T4L, in green) inserted in place of the 3rd intracellular loop of receptor. b) Partial representation of ICCR(T4L) showing, in the plane of the membrane, one of the 4 subunits of ICCR. Ligand-induced conformational changes of T4L-modified GPCRs are transduced into electrical signal generated by a fused channel.

To investigate the functional impact of the T4L domain insertion in GPCR we used ICCRs based on three different receptors: β 2 adrenergic, M2 muscarinic and Oxytocin. Using the two-electrode voltage-clamp technique on *Xenopus* oocytes, we functionally characterized the fusion proteins with the T4L domain.

VIII.2 Functional characterization of the first prototypical T4L-modified Ion Channel-Coupled Receptor: $\beta 2(\text{T4L})\text{-Kir6.2}$

In order to examine the impact of T4L domain on the GPCR-mediated channel regulation we started up our investigation with the ICCR based on the human $\beta 2$ -adrenergic receptor, the first G protein-coupled receptor crystallized in the presence of T4 lysozyme domain [Cherezov Science 2007]. As a template construct we used the well characterized $\beta 2\text{-K-62-25}$ ICCR created by Lydia Caro, throughout fusion of the C-terminal truncated form of $\beta 2\text{AR}$ ($\beta 2\Delta\text{C62}$) to a truncated Kir6.2 channel lacking the first 25 N-terminal residues (ΔN25). This construct requires co-expression of TMD0, a 195-residue N-terminal transmembrane domain of SUR1, for detectable surface expression of the ICCR and functional coupling between channel and receptor [Caro PloS ONE 2011].

The $\beta 2\text{AR}(\text{T4L})$ ICCR (Figure 53) was obtained by insertion of the T4L domain in place of the i3 loop (from residues 231 to 262), as it was done for the crystallized $\beta 2(\text{T4L})\text{-AR}$ [Rosenbaum Science 2007].

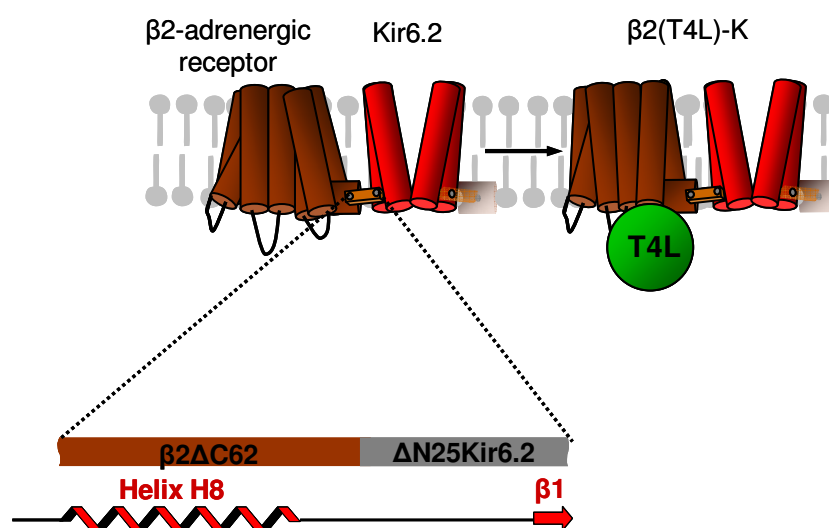


Figure 53. Design strategy of $\beta 2(\text{T4L})$ -based Ion Channel-Coupled Receptor.

T4L-modified $\beta 2$ -based ICCR was formed by a covalent linkage between truncated $\beta 2(\text{T4L})\Delta\text{C62}$ receptor and $\Delta\text{N25Kir6.2}$ channel [$\beta 2(\text{T4L})\text{-K-62-25}$]. Helix H8 and β -bridge $\beta 1$ were observed in the structures of $\beta 2\text{AR}$ (PDB code: 2RH1) and the chimeric Kir3.1 channel (PDB code: 2QKS), respectively.

VIII.2.1 Surface expression of the T4L-modified $\beta 2$ -ICCR

The first condition demanding fulfilment, before functional study, was the significant current amplitude generated by Kir6.2. Previous studies demonstrated that

fusion of GPCRs does not affect the channel activity. Consequently, the current amplitude is generally directly correlated to the surface expression of the ICCR. The simplest method to rapidly estimate the surface expression of the ICCRs is the measurement of the current amplitude generated by the channel in basal state, and called basal current. This basal current must be sufficient to allow the functional characterization with ligands. Due to this reason, we started the project by measurement of the basal current generated by $\beta 2(\text{T4L})$ ICCR. However, as it is shown in Figure 54, the basal currents produced by oocytes injected with $\beta 2(\text{T4L})\text{-K-62-25}$ fusion were as low as these generated by the negative control (non-injected oocytes). This result was anticipated as the wt $\beta 2\text{-K-62-25}$ ICCR requires TMD0 co-expression to reach the plasma membrane ^[Caro 2011 PloS ONE]. Unexpectedly, even in the presence of the chaperon-like TMD0 domain we were unable to detect any basal current from the $\beta 2(\text{T4L})$ ICCR. Due to this reason we looked for possible causes of abolished/undetectable surface expression of the fusion protein. We recognised three potential factors that might lie at the bottom of this problem:

- lack of the flexible, G protein interacting i3 loop
- incorporation of the stable T4L domain in the place of i3 loop
- disrupted interaction between receptor and protein partners. In this hypothesis, the partner(s) is/are not able to mask anymore the Kir6.2 endoplasmic reticulum (ER) retention signal, what results in trapping of the ICCR in the endoplasmic reticulum ^[Tucker 1997 Nature].

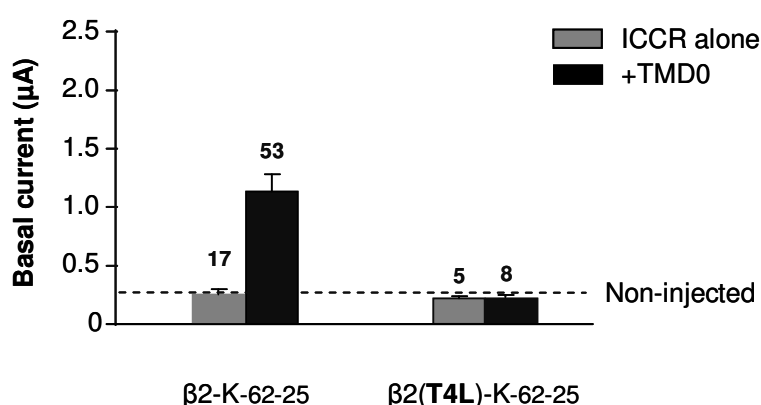


Figure 54. Surface expression of the T4L-modified $\beta 2$ -based ICCR.

Whole-cell basal current amplitude reflects the surface expression levels of indicated fusion proteins. To boost their surface expression constructs were co-expressed with TMD0 ^[Caro 2011 PloS ONE]. An introduced T4L domain abolished surface expression of the modified $\beta 2$ -based ICCR.

To address the question about possible cause of lack of expression of the T4L-modified ICCR we applied two strategies:

- creation of another mutant of β 2AR-based ICCR with depleted the third intracellular loop (β 2 Δ i3-K).
- removal of the Kir6.2 ER retention signal by truncation of the last 36 residues of the channel (K Δ) [Tucker 1997 Nature].

Data presented in Figure 55 show that the deletion in the Kir6.2 C-terminus allowed us to restore surface expression of both modified constructs: β 2(T4L)- and β 2(Δ i3)-based ICCR. Basal currents recorded from oocytes expressing β 2(T4L)-K Δ -62-25 were comparable with these generated by the control β 2 Δ i3-K Δ -62-25 ICCR. Interestingly, we could detect basal current even in the absence of TMD0. However, the co-expression with this domain significantly enhanced the surface expression level of both ICCRs. The resulting basal current with TMD0 was increased 5-fold for β 2(T4L)-K Δ -62-25, and 4-fold for β 2(Δ i3)-K Δ -62-25.

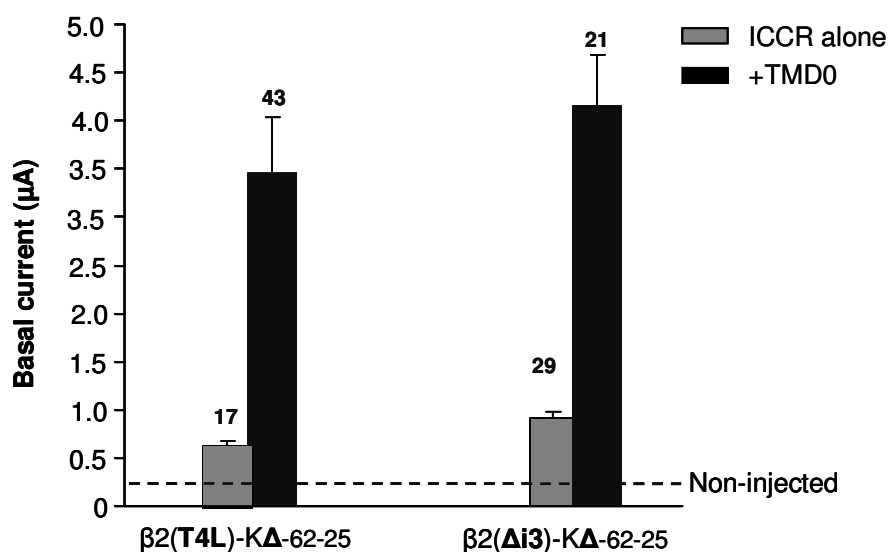


Figure 55. The whole-cell basal currents generated by oocytes expressing β 2(T4L)- and β 2(Δ i3)-based ICCRs with removed Kir6.2 endoplasmic reticulum (ER) retention signal (Δ C36 aa). Introduced into channel modification restored surface expression of the indicated constructs. Co-expression with TMD0 boosted resulting basal currents. Potential: -50mV.

Obtained results indicate that surface expression of β 2(T4L)-ICCR is restored when the C-terminus of Kir6.2 channel is truncated. Thus, abolished surface expression of β 2(T4L)-K-62-25 ICCR was not directly induced by the T4L domain insertion or the i3 loop removal, but by the exposition of the Kir6.2 ER retention signal in these

constructs. We suppose that the T4L domain disrupts the interaction with G proteins or other partners, and consequently does not form a complex big enough to mask the Kir6.2 ER retention signal. Thus, truncation of the Kir6.2 C-terminal region is a prerequisite for surface expression of the $\beta 2(\text{T4L})\text{-ICCR}$.

VIII.2.2 Does $\beta 2(\text{T4L})\text{-modified ICCR}$ remain functional?

The functional coupling between the $\beta 2(\text{T4L})$ receptor and Kir6.2 channel was tested with the adrenergic full agonist, isoproterenol (Iso, $0.5\mu\text{M}$). Despite sufficient surface expression of $\beta 2(\text{T4L})\text{-K}\Delta\text{-62-25 ICCR}$ in the absence of TMD0, we could not record any significant change in the current upon ligand application (Figure 56). In the case of our control construct, $\beta 2\Delta\text{i3-K}\Delta\text{-62-25}$, isoproterenol induced channel inhibition at the level of 25%. However, in the absence of control with the wt $\beta 2$ ICCR without TMD0, we cannot conclude on the functional effect of T4L insertion, or i3 loop deletion [Caro 2011 PloS ONE].

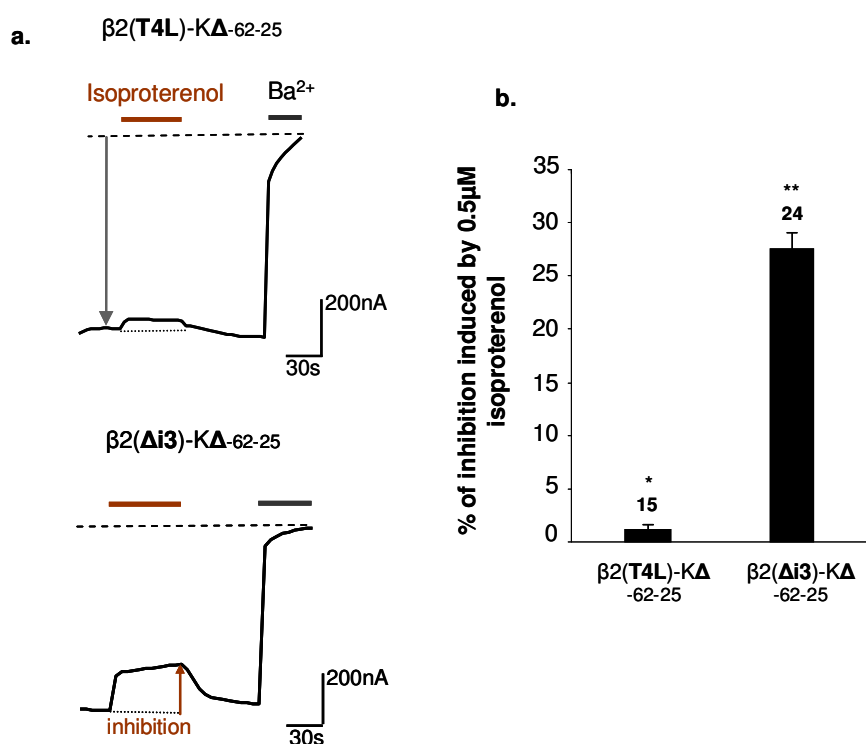


Figure 56. Functional properties of the modified $\beta 2$ -based ICCRs: $\beta 2(\text{T4L})\text{-K}\Delta\text{-62-25}$ and $\beta 2(\Delta\text{i3})\text{-K}\Delta\text{-62-25}$.

a) Representative TEVC recordings of $\beta 2(\text{T4L})\text{-}$ and $\beta 2(\Delta\text{i3})\text{-}$ based ICCRs showing responses to $0.5\mu\text{M}$ isoproterenol (Iso). Grey and brown arrows indicate basal current and channel inhibition by Iso, respectively. b) Average percent of channel inhibition in indicated constructs induced by agonist.

Importantly, co-expression of $\beta 2(\text{T4L})\text{-K}\Delta\text{-62-25}$ with TMD0 domain allowed us to measure clear agonist-induced response, as shown in Figure 57. Presence of the T4L domain did not affect properties of ICCR. Application of $0.5\mu\text{M}$ isoproterenol to $\beta 2(\text{T4L})\text{-ICCR}$ induced strong activation of Kir6.2 channel, similar to this observed for the wild-type $\beta 2\text{-ICCR}$, co-expressed with TMD0. The maximal channel activation was 140% of the basal current for $\beta 2\text{-K-62-25}$ and 100% for $\beta 2(\text{T4L})\text{-K}\Delta\text{-62-25}$ (Figure 57b).

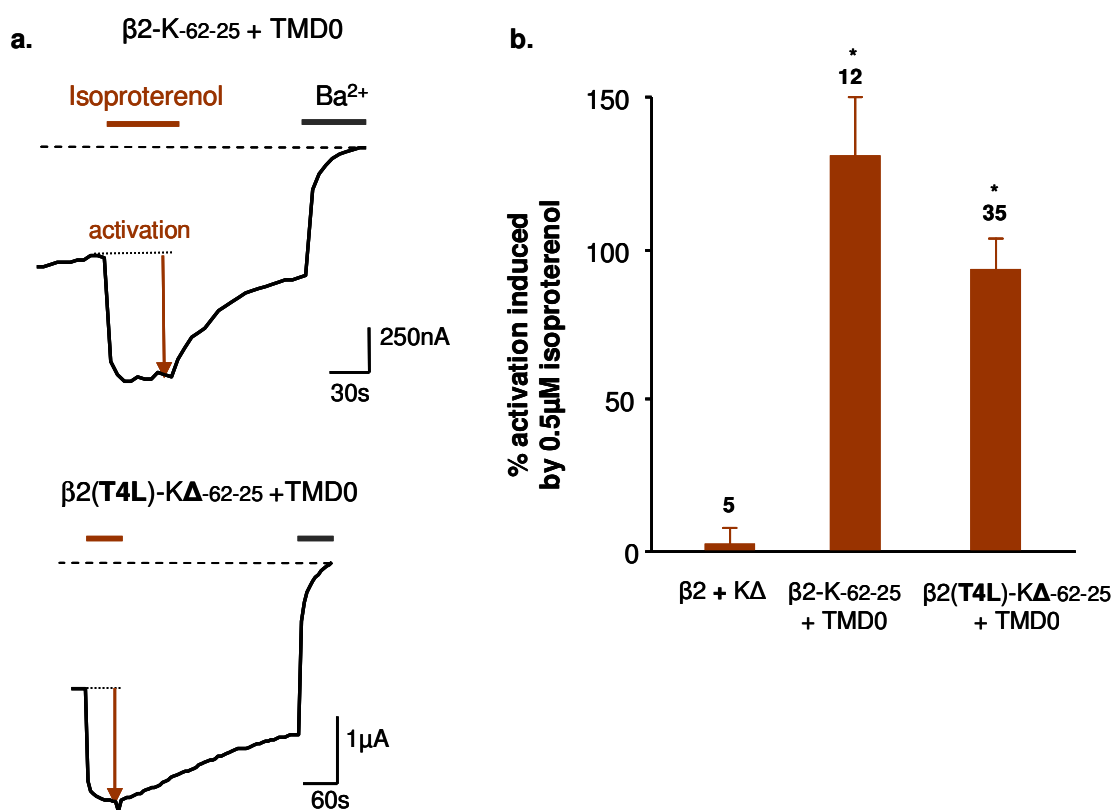


Figure 57. Functional characterization of $\beta 2(\text{T4L})\text{-K}\Delta\text{-62-25}$ and $\beta 2(\Delta\text{i}3)\text{-K}\Delta\text{-62-25}$ ICCRs co-expressed with TMD0 domain.

a) Representative TEVC recordings of indicated proteins. b) Average percent of channel activation induced by agonist, $0.5\mu\text{M}$ Iso.

Moreover, activation of $\beta 2(\text{T4L})\text{-ICCR}$ by isoproterenol is concentration-dependent with an $\text{EC}_{50} \sim 161\text{nM}$ (Figure 58). This value is consistent with EC_{50} obtained for wt $\beta 2\text{AR-ICCR}$ ($\sim 140\text{nM}$), and values obtained with other techniques that do not rely on G protein signalling, such as competitive radioligand binding or fluorescence spectroscopy [Yao Nat Chem Biol 2006] [Baker Br J Pharmacol Biol 2010]. These results demonstrate that T4L insertion reduces agonist efficacy as shown by the lower maximal amplitude. This observation is also reproduced with the $\beta 2(\Delta\text{i}3)\text{-K}\Delta\text{62-25}$. Two hypotheses could explain this lower efficacy: i) an enhanced receptor stability conferred by removal or replacement

of the i3 loop, which modifies the agonist-evoked conformational changes (what implies that the crystallographic structures depict a partial agonist-bound state); ii) the i3 loop modifications affect the channel regulation without changing the receptor activity.

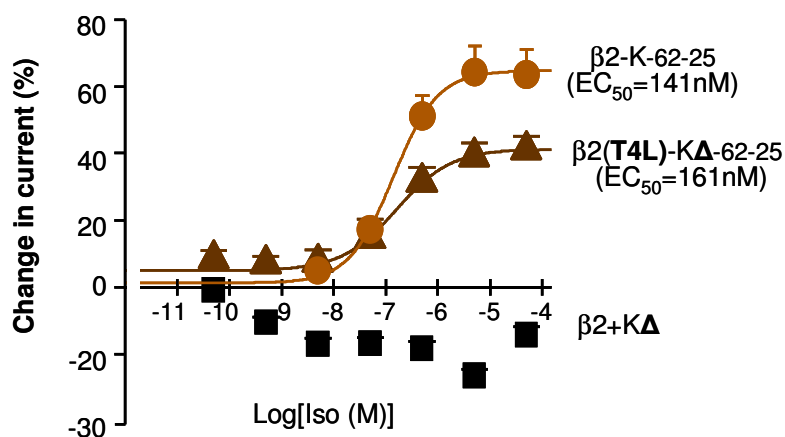


Figure 58. Concentration-dependent activation of $\beta 2$ -K-62-25 and $\beta 2$ (T4L)-KA-62-25 ICCRs, co-expressed with TMD0, by isoproterenol.

$\beta 2$ +KA, co-expression of non-fused receptor and channel, serves as a control for possible modulation of Kir6.2 directly by ligand or indirectly by receptor-mediated signalling cascades.

VIII.3 Extrapolation to another GPCR: characterization of the M2(T4L)-based ICCR.

In order to design M2(T4L)-based ICCR we used the M2-K-9-25 construct as a template. This ICCR was obtained by fusion of the C-terminally truncated M2-muscarinic receptor (M2 Δ C9) to the truncated Kir6.2 channel, lacking the first 25 residues (Δ N25). The M2(T4L)-ICCR was obtained by insertion of the T4L domain into the third intracellular (i3) loop replacing M2 residues 218-376 in similar positions to the $\beta 2$ (T4L). Our work on M2(T4L) was anterior to the crystallographic structure of this receptor obtained in 2012, however the T4L domain was inserted exactly in the same position as in our M2(T4L)-based ICCR ^[Haga Nature 2012].

VIII.3.1 Surface expression of M2(T4L)-ICCR.

In agreement with the results obtained with the $\beta 2$ (T4L)-ICCR, insertion of the T4L domain in place of the i3 loop resulted in abolished surface expression of the M2(T4L)-ICCR (Figure 59). To overcome this problem we also truncated the Kir6.2 C-terminus (Δ C36 residues) to remove the ER retention signal. Average basal current recorded from oocytes injected with the M2(T4L)-KA-9-25 was at the level of 5 μ A,

which was 25-fold higher compared to endogenous current generated by non-injected ones (basal current $\sim 0.2 \mu\text{A}$, dashed line). Simultaneously we tested as well the control mutant with truncated i3 loop [M2(Δ i3)-K-9-25]. Basal currents generated by oocytes expressing this construct were comparable with these recorded for M2(T4L)-K Δ -9-25. In the case of M2(Δ i3)-ICCR, in contrast to parallel β 2(Δ i3)-based fusion protein, truncation of the Kir6.2 N-terminus was not prerequisite. *Xenopus* oocytes endogenously express G_i proteins, which interact with the M2 receptor (but not G_s cognate for the β 2 receptor). We showed that the M2 receptor lacking its i3 loop is still able to interact with G_i proteins. Thus, we believe that this interaction allows to mask ER retention signal what results in surface expression of the M2(Δ i3) receptor fused to the full length Kir6.2 channel.

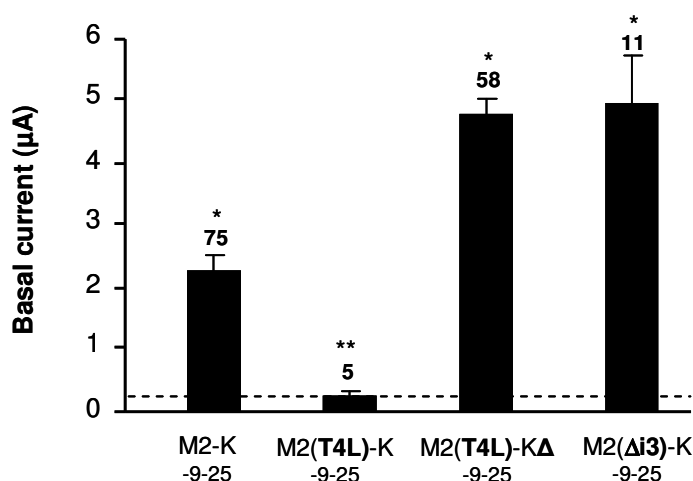


Figure 59. Surface expression of the M2-based ICCRs.

Basal currents in the first minute of TEVC recordings from oocytes expressing indicated proteins. Compared to this baseline, *: $P < 10^{-13}$ and **: $P = 0.011$.

VIII.3.2 Functionality of M2(T4L)-ICCR

Functionality of the coupling between M2(T4L) receptor and Kir6.2 channel was tested with the physiological agonist, $5 \mu\text{M}$ acetylcholine (ACh). As it is shown in Figure 60a, T4L modification did not disturb the function of the ICCR, which clearly detect the ACh-evoked conformational changes of M2(T4L). Binding of ACh to the fused M2(T4L) receptor induced strong inhibition of the Kir6.2 current, as in the wt M2-ICCR. The same response was observed for the control M2(Δ i3)-K-9-25.

We determined as well the agonist concentration-dependent response with carbachol (CCh), an ACh analogue. Inhibition of M2(T4L)-K-9-25 ICCR by CCh is dose-dependent, with half-maximal effective concentration $EC_{50} \sim 1 \mu\text{M}$, which is similar to the

EC_{50} of the wt M2-K-9-25 (~ 1.5 μ M). Consequently, the T4L domain did not alter the apparent affinity, indicating that the ligand binding site is preserved. The comparison with published values obtained for unfused M2 receptor is problematic because these are dispersed over several orders of magnitude.

Maximal inhibition for the reference M2-K-9-25 construct was 67% of the basal current, 28% for the modified M2(T4L)-K Δ -9-25, and 23% for the control M2(Δ i3)-K-9-25 ICCR. The same reduction of ligand efficacy was observed for β 2(T4L)-ICCR, which allows us to generalize the two hypothesis of i) partial agonist conformational changes, or ii) partial regulation of Kir6.2.

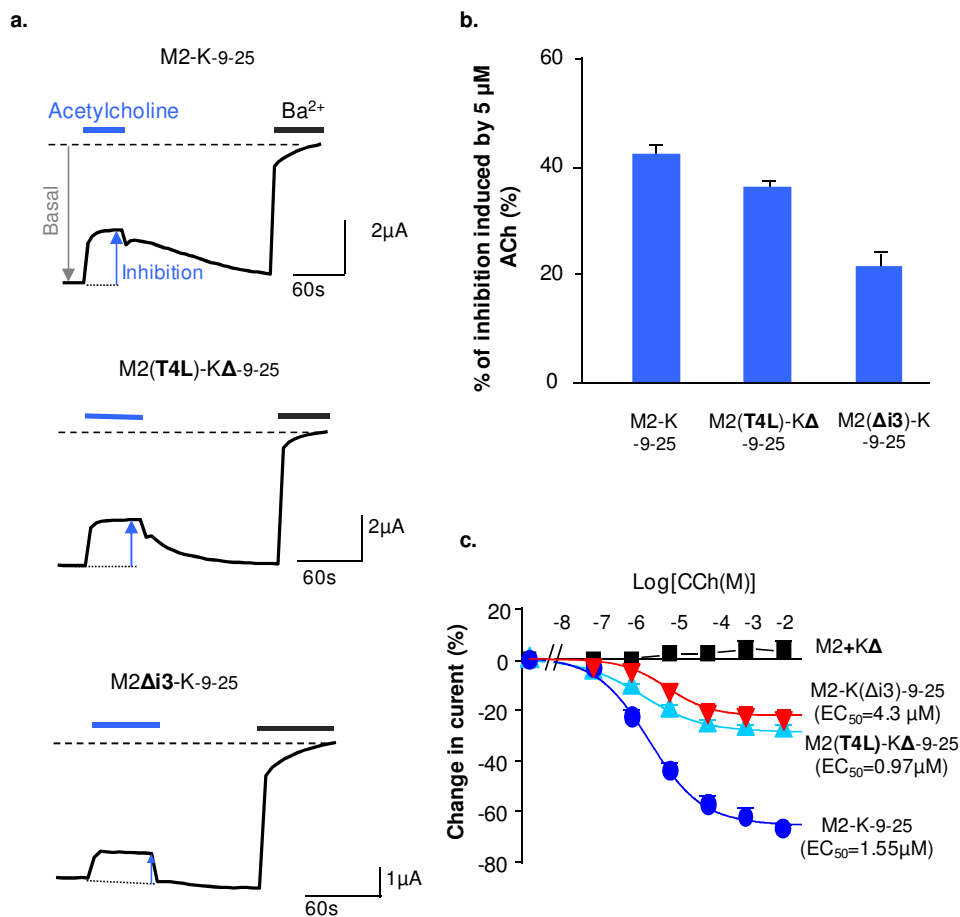


Figure 60. Receptor-channel communication in the modified (T4L, Δ i3) M2-based ICCRs.

a) Representative TEVC recordings obtained for indicated proteins. b) Changes in whole-cell current evoked by 5 μ M ACh. c) Concentration-dependent inhibition of M2-K-9-25, M2(Δ i3)-K-9-25 and M2(T4L)-K Δ -9-25 ICCR by carbachol (CCh, an ACh analogue). M2+K Δ , co-expression of unfused receptor and channel, serves as a control for possible modulation of Kir6.2 directly by ligand or indirectly by receptor-mediated signalling cascades. $n \geq 7$.

In order to fully characterize properties of M2(T4L)-ICCR, we investigated as

well the effect of atropine (1 μM), an orthosteric antagonist that prevents the M2 receptor activation. It has been shown for the wt M2-ICCR, that antagonist-bound state of M2 is detectable. Figure 61 shows that it is also the case for the M2(T4L)-ICCR, as atropine reversibly suppress ACh-induced channel inhibition. Obtained result confirmed that inserted T4L domain does not disturb or alter functional properties of the modified M2-based ICCR.

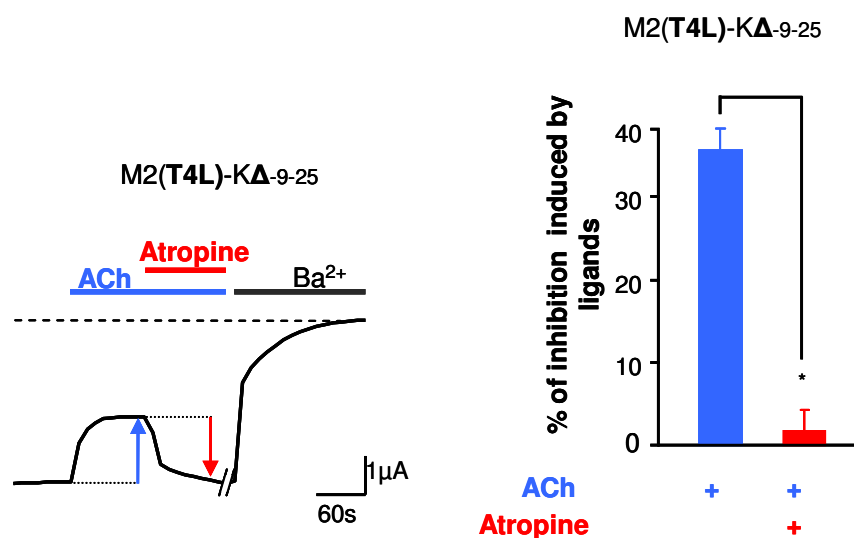


Figure 61. Antagonist effect on M2(T4L)-ICCR.

a) Atropine (1 μM , red arrow) blocks ACh-induced channel inhibition. b) Average percent inhibition by 5 μM ACh in control (blue bar) and in the presence of 1 μM Atropine (red bar). *: $P < 10^{-5}$

VIII.4 Extrapolation to another, not-yet crystallized GPCR: characterization of the Oxytocin Receptor ICCR, OXTR(T4L)-K.

Our collaborator, Vadim Cherezov from Scripps Research Institute, is trying to crystallize the oxytocin receptor using the T4L strategy. In order to functionally characterize this optimized receptor with T4L, he provided us the oxytocin(T4L) receptor gene, with 42 residues truncation at C-terminus [OXTR(T4L)DC42], which we fused with Kir6.2 channel lacking 25 N-terminal residues. Therefore we created OXTR(T4L)-K-42-25. We received also wild-type receptor gene encoding the full length protein, with Flag tag inserted in its N-terminus. In order to create functional ICCR, we further truncated its 42 C-terminal residues creating wt OXTR-K-42-25.

VIII.4.1 Surface expression of OXTR-based ICCR.

Like in the case of the two previously described ICCR(T4L), truncation of Kir6.2 C-terminus was required for surface expression of the OXTR(T4L)-K fusion protein. The average basal current at the level of 0.4 μ A, generated by OXTR(T4L)-K Δ -42-25, was sufficient to perform functional characterization of this ICCR. Despite intense efforts we were not able to detect any basal activity of Kir6.2 fused to the wt OXTR. This phenomenon could be evoked by the basal activity of the OXTR activating G_q proteins. Activated G α_q subunit triggers an activation of phospholipases C, which deplete the plasma membrane in phosphatidylinositol 4,5-bisphosphate (PIP₂), and subsequently induce closure of the Kir6.2 channel [Kobrinisky Nat Cell Biol 2000].

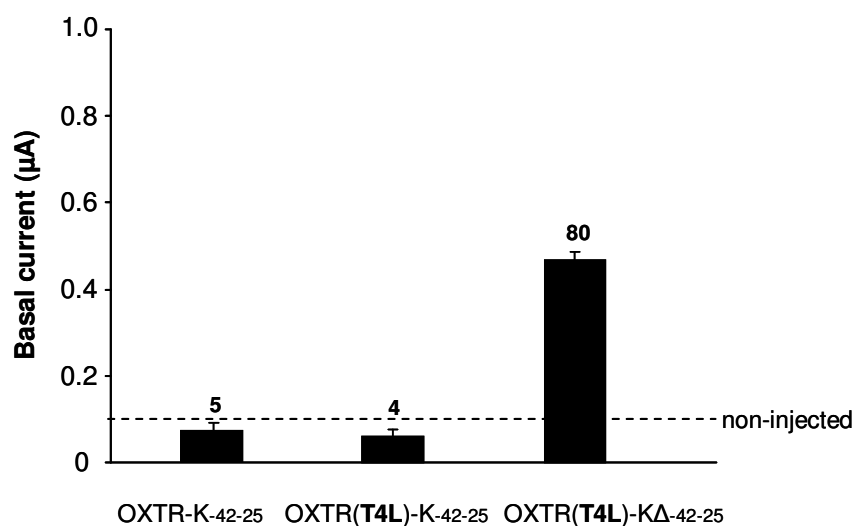


Figure 62. Basal currents in the first minute of TEVC recordings from oocytes expressing indicated proteins.

VIII.4.2 Functional characterization of OXTR(T4L)-K.

We studied the capacity of OXTR(T4L)-ICCR to regulate channel gating in the presence of an agonist, the oxytocin hormone. Application of oxytocin, at 1 μ M, resulted in great activation of the channel; with the maximal value reaching 150% of the basal current (Figure 63b).

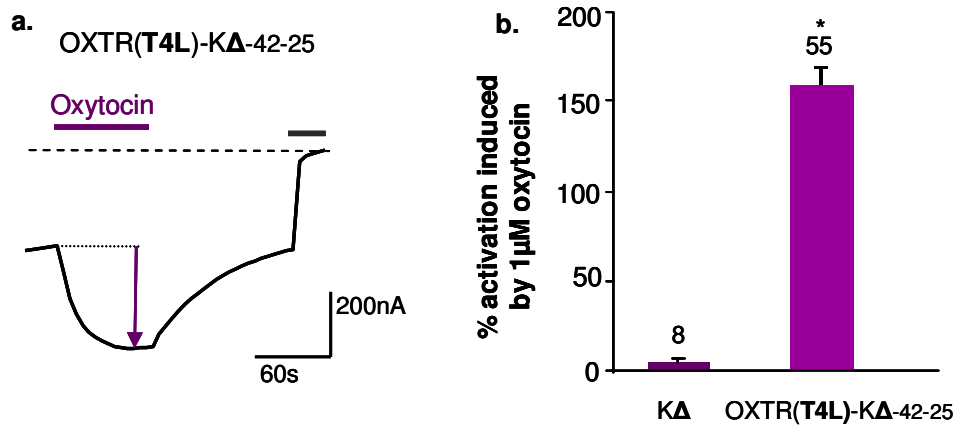


Figure 63. Functional characterization of the OXTR(T4L) ICCR

a) Representative TEVC recording showing OXTR(T4L)-ICCR response to an agonist, 1μM oxytocin. Violet arrow indicates channel activation. b) Average activation of OXTR(T4L)-KΔ-42-25 elicited by applied oxytocin.

We determined as well, the concentration-dependent response of the fusion protein, OXTR(T4L)-KΔ-42-25, to applied oxytocin. Obtained results proved that the OXTR(T4L) fused in ICCR is activated by an agonist, in the concentration-dependent manner, with $EC_{50} \sim 1.5\mu\text{M}$.

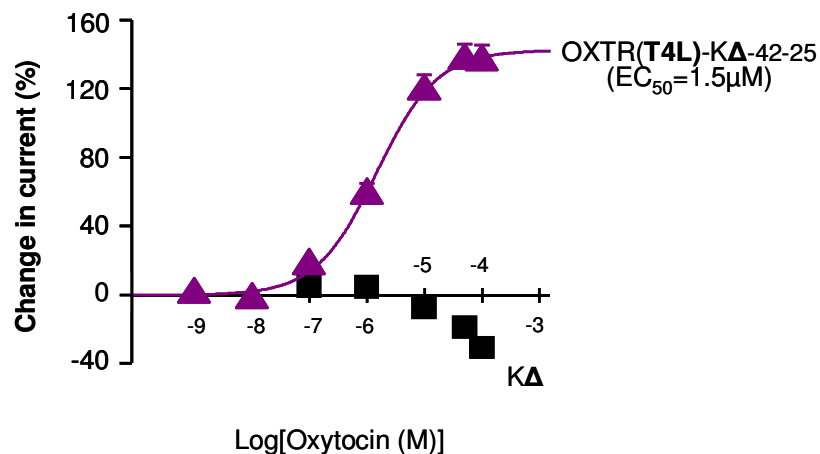


Figure 64. Concentration-dependent activation of OXTR(T4L)-KΔ-42-25 by oxytocin.

Expression of unfused channel, serves as a control for possible modulation of Kir6.2 directly by applied ligand. $n \geq 8$.

To complete functional characterization of OXTR(T4L)-based ICCR we tested Atosiban, a "biased" ligand inhibiting the G_q pathway, but activating the $G_{i/o}$ pathway.

Obtained results demonstrated that OXTR(T4L)-based ICCR is able to detect conformational changes induced by this “biased” ligand.

Application of Atosiban, at 1 μ M concentration, resulted in a great activation of the channel, with the maximal value reaching 120% of the basal current (Figure 65b). Obtained response is comparable with the effect triggered by natural agonist, oxytocin (maximal activation reaching 150% of the basal current, Fig. 63b). Moreover, we investigated as well the effect induced by Atosiban (1 μ M), in the presence of oxytocin, in order to test its antagonistic nature. Figure 61 c&d shows that Atosiban does not suppress oxytocin-induced channel activation. In this way ICCR tool allows to distinguish between a classical antagonist that suppresses an agonist-induced response, and a biased agonist which does not demonstrate this behaviour.

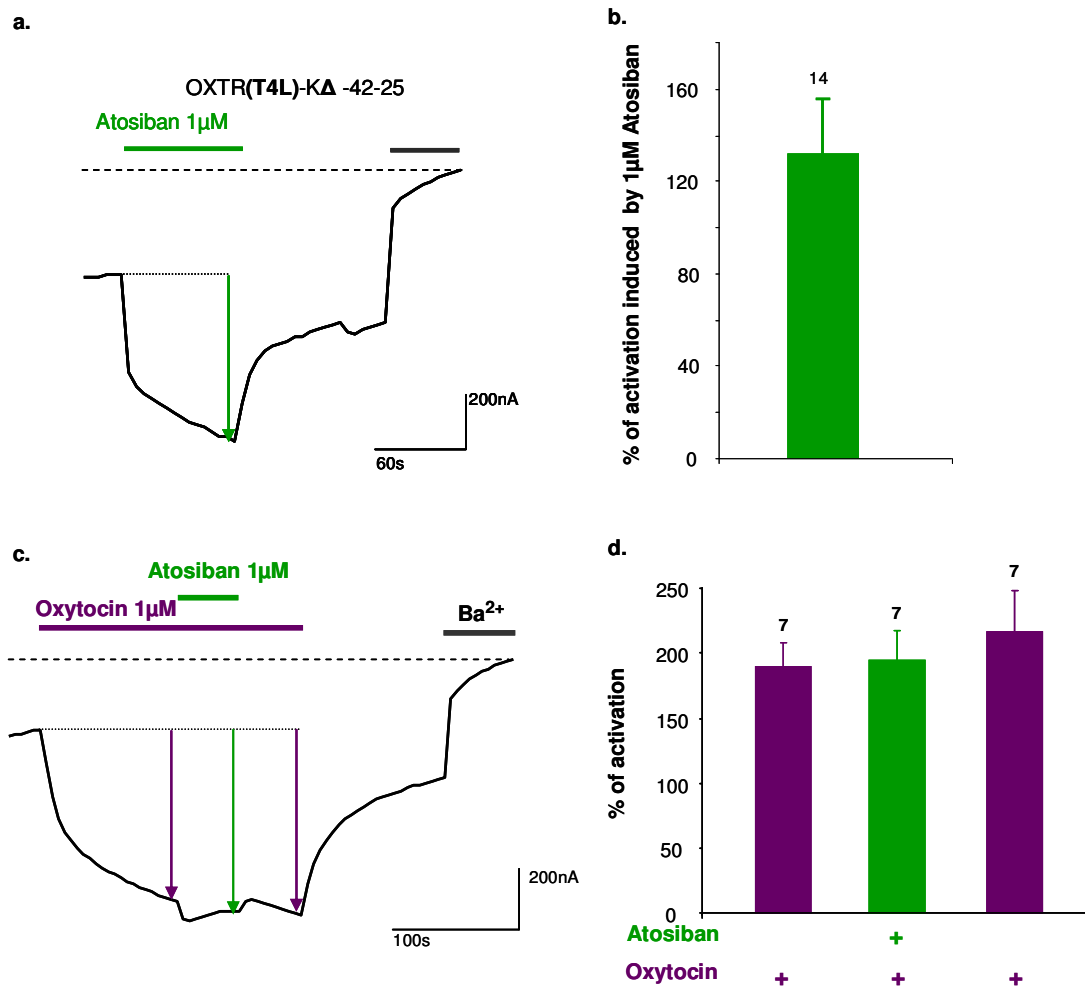


Figure 65. Biased agonist effect on OXTR(T4L)-ICCR.

a) Representative TEVC recording showing the OXTR(T4L)-ICCR response to a biased agonist, 1 μ M Atosiban. Green arrow indicates channel activation. b) Average activation of the OXTR(T4L)-K Δ -42-25 elicited by an applied Atosiban. c) Atosiban (1 μ M, red arrow) does not block oxytocin-induced channel activation. d) Average percent of activation by 1 μ M oxytocin in control (violet bar), and in the presence of 1 μ M Atosiban (green bar).

VIII.5 Project #1a: Deletion of G Protein-Coupled Receptor ICL3 versus G Protein

According to published data, the third intracellular loop (i3 loop) of the G Protein-Coupled Receptors plays a relevant role for the interaction and activation of the G proteins. It has been shown for the crystallization-optimized GPCRs that insertion of the T4L domain (or BRIL domain) in place of the i3 loop generates a steric hindrance that abolishes binding of the G proteins [Cherezov Science 2007]. This raises the question about the ability of GPCRs with deleted i3 loop to activate G proteins? In order to answer this question, we co-expressed G protein-activated channels (Kir3.4*) with two ICCRs based on receptors with deleted i3 loop (Δ i3): M2(Δ i3)-K-9-25 and β 2(Δ i3)-K Δ -62-25.

VIII.5.1 Functional properties of M2(Δ i3)-based ICCR

We investigated significance of the i3 loop in the G protein activation using M2(Δ i3)-K-9-25 ICCR, lacking M2 residues between 218 and 376. We co-injected oocytes with modified M2(Δ i3)- or wt M2-based ICCRs and the $G_{i/o}$ protein-activated channel Kir3.4. In our investigations we use mutated Kir3.4* (Kir3.4S143T) that forms functional homotetramers (naturally occurring Kir3.4 co-assembles with Kir3.1 subunits and creates heterotetramers) [Vivaudou J Biol Chem 1997].

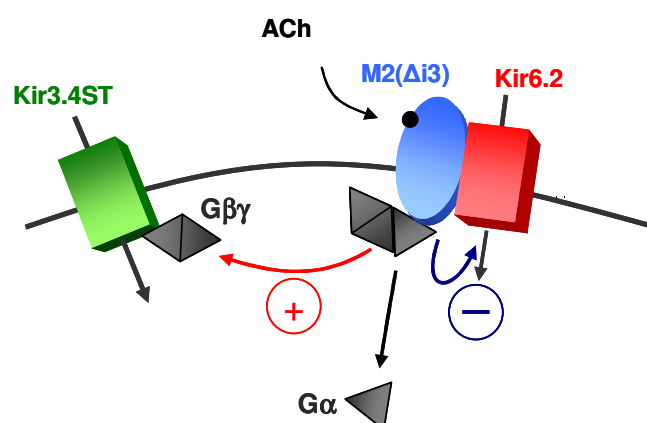


Figure 66. Schematic representation of events taking place in oocytes co-injected with M2(Δ i3)-K-9-25 ICCR and Kir3.4ST* channels upon agonist binding. ACh-bound receptor directly induces Kir6.2 channel inhibition and $G_{i/o}$ protein mediated activation of Kir3.4* channel present in plasma membrane.

Moreover, we performed additional controls injecting oocytes with M2(Δ i3)-K-9-25 alone or co-injecting non-fused M2 receptor with Kir3.4* channel.

First perceptible feature was a significant difference in the basal currents generated by the investigated constructs. When the M2 Δ i3-K-9-25 ICCR was expressed individually recorded basal currents were at the average level of 5 μ A, whereas co-expression of this construct (as well as wt M2-K-9-25 ICCR and unfused M2 receptor) with modified Kir3.4* channel resulted in a generation of a distinctive higher basal currents (Figure 67). Thus, we were sure that Kir3.4* channels are present in the plasma membrane of oocytes, and that the ligand-evoked changes in the current were predominantly generated by these channels.

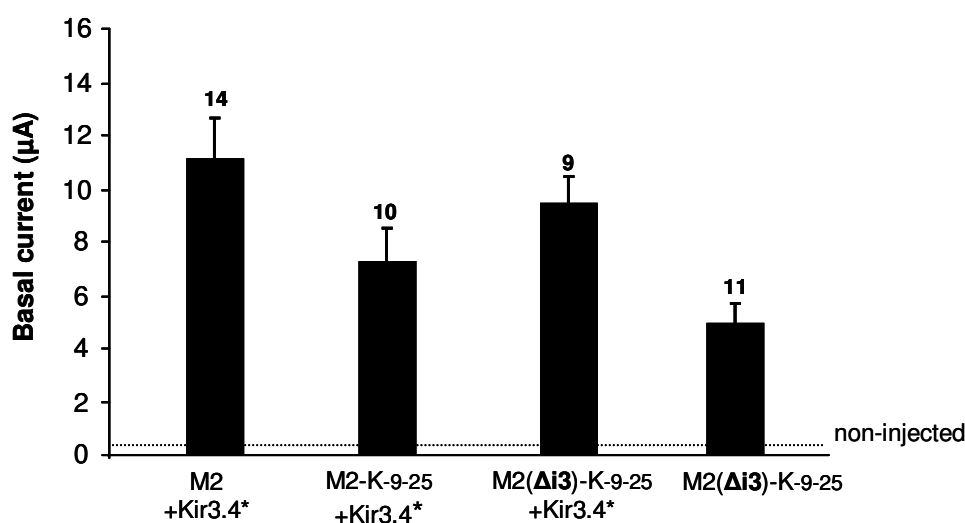


Figure 67. Basal currents generated by oocytes expressing indicated proteins.

Functional characterization was performed in the presence of 5 μ M acetylcholine (Figure 68). For a start we tested if fused in ICCR receptor maintained its capability to activate Kir3.4* channels through the $G_{i/o}$ proteins. Application of the agonist on oocytes co-injected with wt M2-K-9-25 and Kir3.4* channel resulted in generation of strong K^+ currents similar to these generated by Kir3.4* channel co-expressed with the non-fused M2 muscarinic receptor. These data proved clearly that the M2 receptor fused in ICCR can still activate $G_{i/o}$ proteins.

In the case of oocytes co-expressing M2-K-9-25 and Kir3.4*, two ACh-induced effects overlapped:

- a direct inhibition of the Kir6.2 in (fused in ICCR),
- an indirect activation of co-expressed Kir3.4* channel, through activation of the $G_{i/o}$ proteins by the M2 receptor,

Nevertheless inhibition of tKir6.2 was masked by the much larger G protein-dependent activation of Kir3.4* channels.

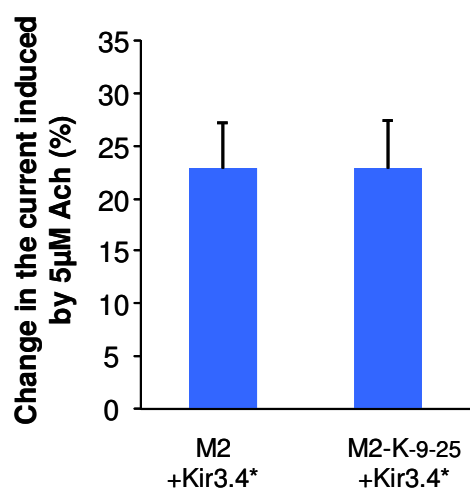


Figure 68. Functional characterization of M2 muscarinic receptor (non-fused or coupled with Kir6.2) co-expressed with Kir3.4* channel.

Application of agonist resulted in G protein-dependent activation of co-expressed with non-modified M2 receptor Kir3.4*.

Subsequently we tested functionality of the construct with deleted i3 loop, M2Δi3-K-9-25. Application of ACh on oocytes co-expressing modified ICCR(Δi3) and Kir3.4* triggered strong increased in the generated currents (Figure 69). The negative control, M2Δi3-K-9-25 construct expressed alone, demonstrated exactly opposite effect (inhibition of Kir6.2 channel).

These results show that, despite lack of the i3 loop, ligand-induced conformational changes of the M2 receptor were transmitted to cognate $G_{i/o}$ proteins, what in turn resulted in the activation of the Kir3.4* channels. Our finding stays in agreement with the previously published data ^[Liu PNAS 1995].

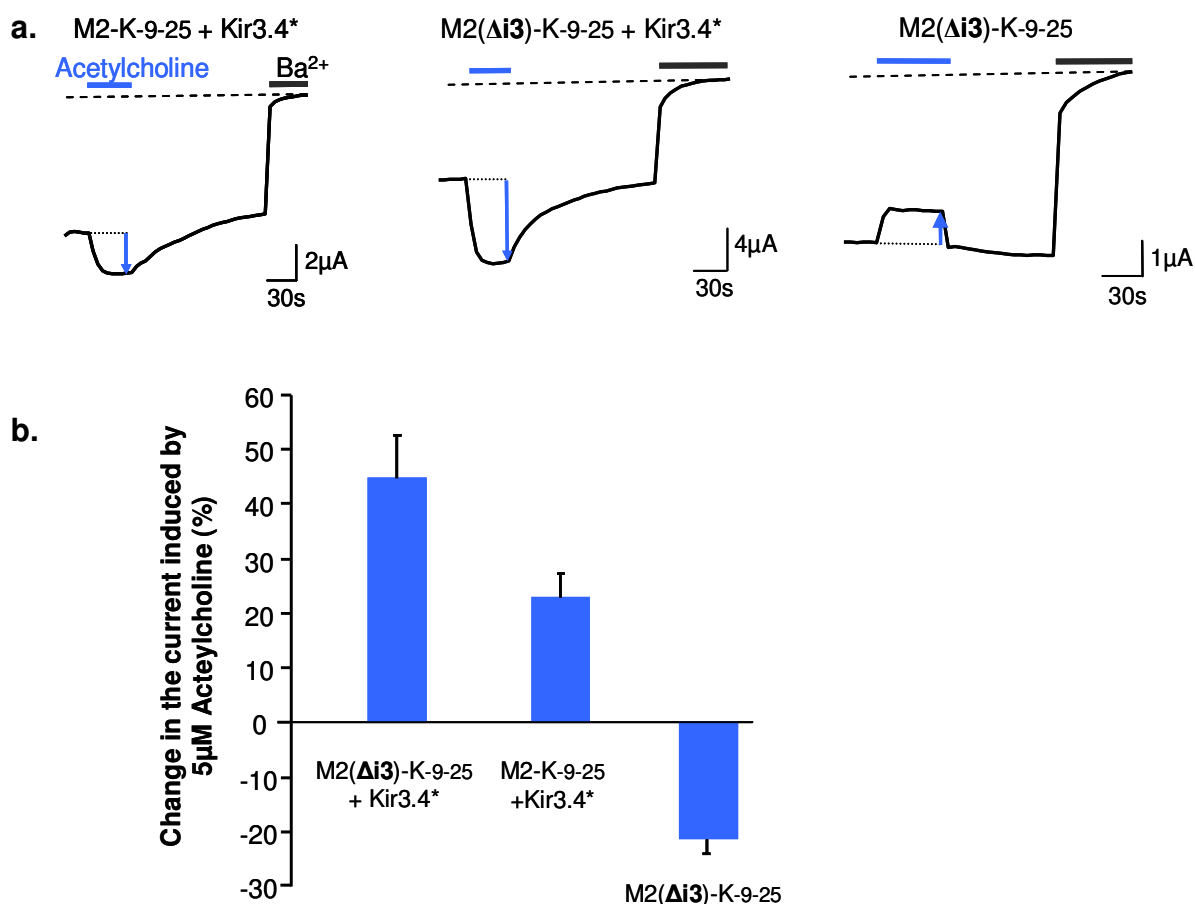


Figure 69. Functional characterization of M2(Δ i3) muscarinic receptor co-expressed with Kir3.4* channel.

a) Representative TEVC recordings from oocytes injected with indicated proteins. b) Changes in whole-cell current evoked by 5 μ M ACh. M2(Δ i3) ICCR expressed alone serves as control, revealing regulation of fused, Kir6.2 channel.

VIII.5.2 Proof-of-concept: functional test of G_s protein activation by β 2(Δ i3) receptor fused in ICCR.

Results obtained for the M2 muscarinic receptor encouraged us to examine another modified GPCR(Δ i3). In order to confirm results observed for the M2 we examined also functional properties of β 2-ICCR with deleted i3 loop (residues 231-262). Due to the reason that the level of the homologous expression for G_s proteins in *Xenopus* oocytes is very low and that β 2-AR is mainly coupled to these proteins, we co-injected the mRNA of $G\alpha_s$ subunit with β 2(Δ i3)-ICCR, and Kir3.4*. Like in the case of previously described M2 receptor, analogically for the β 2-AR, binding of the ligand to GPCR (fused in ICCR or non-fused) should result in activation of Kir3.4* channels through the $G\alpha_s$ proteins. As the negative control we used oocytes expressing the β 2(Δ i3)-KA-62-25 alone. As expected, the basal currents generated by oocytes co-injected with

$\beta 2(\Delta i3)$ -K Δ -62-25 and Kir3.4* channels were significantly higher than these generated by the ICCR alone (Figure 70).

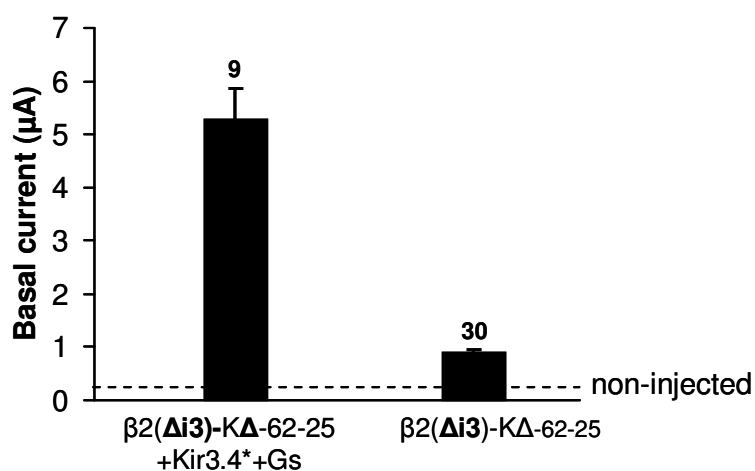


Figure 70. Basal currents generated by oocytes expressing $\beta 2(\Delta i3)$ -K Δ -62-25 alone, or co-expressed with Kir3.4* channel and $G\alpha_s$ protein.

In order to investigate the ability of $\beta 2(\Delta i3)$ receptor fused in ICCR to activate $G\alpha_s$ proteins we performed functional characterization using agonist, 0.5 μ M isoproterenol (Iso). As it has been shown previously, application of an agonist on oocytes expressing individually $\beta 2(\Delta i3)$ -K Δ -62-25 ICCR, resulted in significant inhibition of the K^+ current generated by Kir6.2 channel (Figure 56). In the case of $\beta 2(\Delta i3)$ -K Δ -62-25 co-expressed with Kir3.4* and $G\alpha_s$, we observed an activated potassium current, clearly proving that deletion of the i3 loop did not alter interaction of the modified $\beta 2(\Delta i3)$ -AR with the cognate G_s proteins. Thus, using a G protein-activated channel as a reporter of $G_{i/o}$ and G_s protein activation, we corroborated that the 3rd intracellular loop is not essential for the activation of the G proteins.

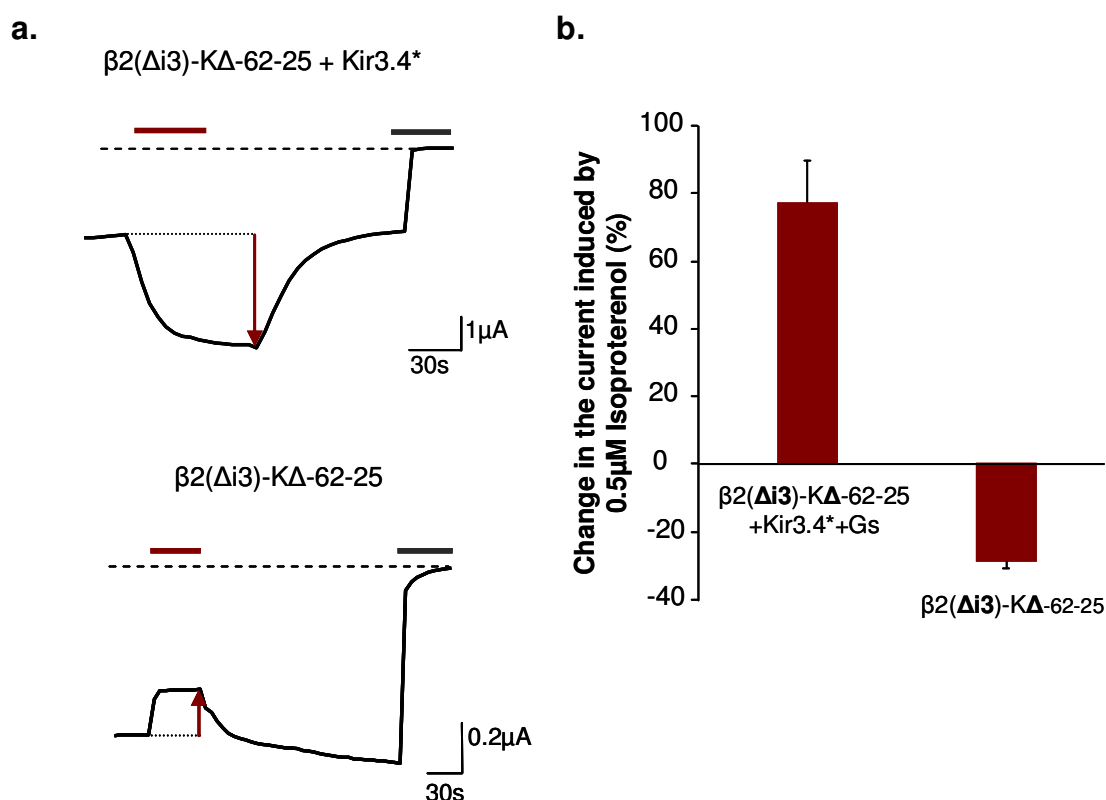


Figure 71. Functional characterization of $\beta 2(\Delta i 3)$ -adrenergic receptor co-expressed with Kir3.4ST* channel.

a) Representative TEVC recordings from oocytes injected with indicated proteins. b) Changes in whole-cell current evoked by 0.5μM Iso. $\beta 2(\Delta i 3)\text{-K}\Delta\text{-62-25}$ ICCR expressed alone serves as a control of direct Kir6.2 channel modulation

IX Project #2: Cholesterol-dependence for GPCR function.

It is well known that steroids present in lipid bilayers can act on structural and functional properties of membrane proteins. This effect has been proposed to occur through three possible modes: i) alterations of the physico-chemical properties of the lipid bilayer, ii) specific and localized molecular interactions between cholesterol, and membrane proteins, or iii) by the combination of both ^[Chini J Mol Endocrinol 2009]. Importantly, this modulatory effect of cholesterol has been reported as well for many members of the GPCR family, such as oxytocin, galanin, neuronal serotonin_{1A} and rhodopsin receptor. It has been proved that cholesterol-receptor interactions play important role in modulation of ligand-binding affinity of these GPCRs ^{[Klein Biochemistry 1995] [Pang Biochemistry 1999] [Pucadyil Prog Lipid Res 2006] [Albert Biochim Biophys Acta 1996]}. Due to the reason, that Ion Channel-Coupled Receptors allow testing of GPCRs' functional properties, therefore we wanted to answer two questions regarding cholesterol-sensitivity:

- Are ICCRs sensitive enough to detect difference in GPCR activity evoked by changes in cholesterol composition of plasma membrane?
- Does modification of GPCRs by T4L insertion in the i3 loop affect the cholesterol-dependence of the receptors?

To investigate the utility of ICCR, and the influence of T4L domain on receptor sensitivity to cholesterol we used ICCR based on oxytocin receptor as a model. It has been published previously that function and physiological regulation of the OXTR system is strongly steroid-dependent. Cholesterol acts as a positive allosteric modulator of OXTR, and stabilizes the receptor in a high-affinity state for agonists and antagonists. Depletion of that lipid from the bilayer results in conversion of oxytocin receptor from a high-affinity state to a low-affinity state [Klein Biochemistry 1995][Gimpl Prog Brain Res 2002] [Reversi Am J Physiol Regul Integr Comp Physiol 2006]

The plasma membrane of *Xenopus* oocytes contains ~20% (mole/mole of total lipids) of cholesterol [Hill Am J Physiol Renal Physiol 2005]. To deplete this amount of endogenous cholesterol from plasma membrane we used the water-soluble lipid carriers: cyclodextrins. In general, all cyclodextrins exhibit a broad specificity for extracting different lipids from cells' membrane. However, the oligomer with seven residues (β -cyclodextrin) of methylated glucose, methyl- β -cyclodextrin (M β CD), displays a relatively higher affinity for cholesterol than for membrane phospholipids [Ohtani Eur J Biochem 1989].

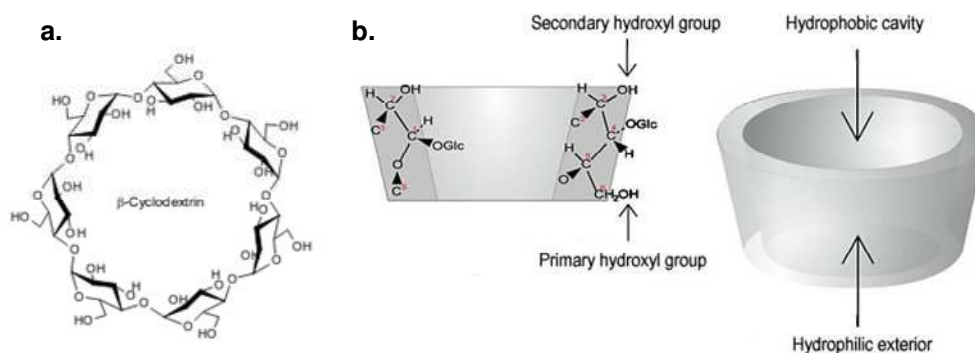


Figure 72. β -cyclodextrin - an efficient cholesterol-carrier.

a) The chemical structure of a β -cyclodextrin molecule (containing seven glucose residues). b) Cyclodextrins can trap a variety of hydrophobic compounds in its hydrophobic cavity. M β CD has methyl groups localized at the -OH positions [Pucadyil Prog Lip Res 2006]

Cyclodextrins are polar molecules, capable to trap cholesterol in their inner, hydrophobic cavity (Figure 72b). Due to their small dimensions, compared to liposomes and lipoprotein particles, they can easily reach the targeted membranes, and mediate efficient cholesterol transport. The close proximity between cyclodextrins and membranes allows spontaneous desorption of cholesterol from the lipid bilayer without any contact with the energetically unfavourable aqueous environment [Pucadyil Prog Lip Res 2006].

Based on previously reported protocols, I developed in the team the method for M β CD-based depletion and addition of cholesterol on living *Xenopus* oocytes. Functional characterization of ICCRs, in ‘cholesterol-modified’ oocytes, was performed using the manual TEVC technique.

IX.1 Cholesterol-sensitivity of the OXTR(T4L)-ICCR.

In order to determine the cholesterol-sensitivity of OXTR in ICCR, we investigated the functional properties of the OXTR(T4L)-ICCR in cholesterol rich (endogenous amount), and poor (depletion by M β CD) environments. Cholesterol-depletion was performed by 3 h incubation of oocytes with a relatively high (20mM) concentration of methyl- β -cyclodextrin. Controls were made by similar incubation in buffer only. Cholesterol-sensitivity was determined by the basal activity, and the response of the ICCR to oxytocin, using TEVC technique. The M β CD-treated oocytes were characterized with oxytocin, and compared to the control group incubated in buffer only.

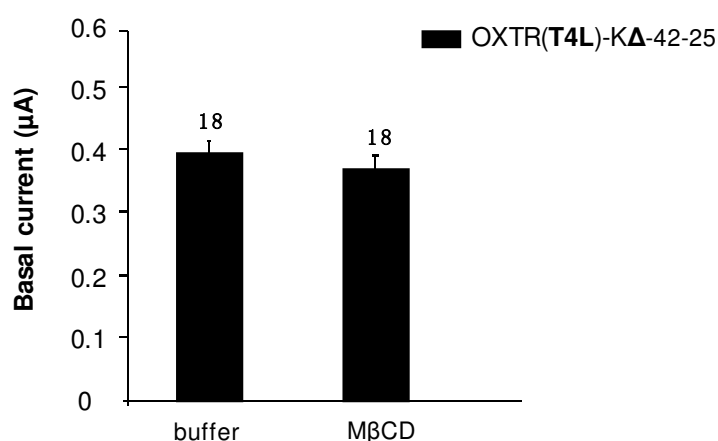


Figure 73. Whole-cell basal currents recorded from OXTR(T4L)-KA-42-25 expressing oocytes incubated in the control buffer and in 20 mM M β CD solution, respectively.

The first observation was no significant difference in the basal activity of the ICCR, between both tested groups. Average recorded basal currents were at the level $\sim 0,4 \mu\text{A}$ (Figure 73). These results stay in agreement with published data, which disclosed that cholesterol presence is crucial for the ligand binding affinity of the oxytocin receptor, but not for its basal activity [Klein Biochemistry 1995].

Characterization, with $1 \mu\text{M}$ oxytocin, revealed significant difference in the receptor function. In controls (incubation in buffer), the physiological level of cholesterol is maintained and resulted in a strong activation of the OXTR(T4L)-ICCR (Figure 74), similar to the activation in the absence of incubation (Figure 63). For oocytes depleted in cholesterol by incubation in 20mM M β CD, activation by oxytocin was drastically decreased indicating a loss-of receptor activity. Our results stay in agreement with the data published for the wild type receptor, obtained with radioligand binding assays [Klein Biochemistry 1995].

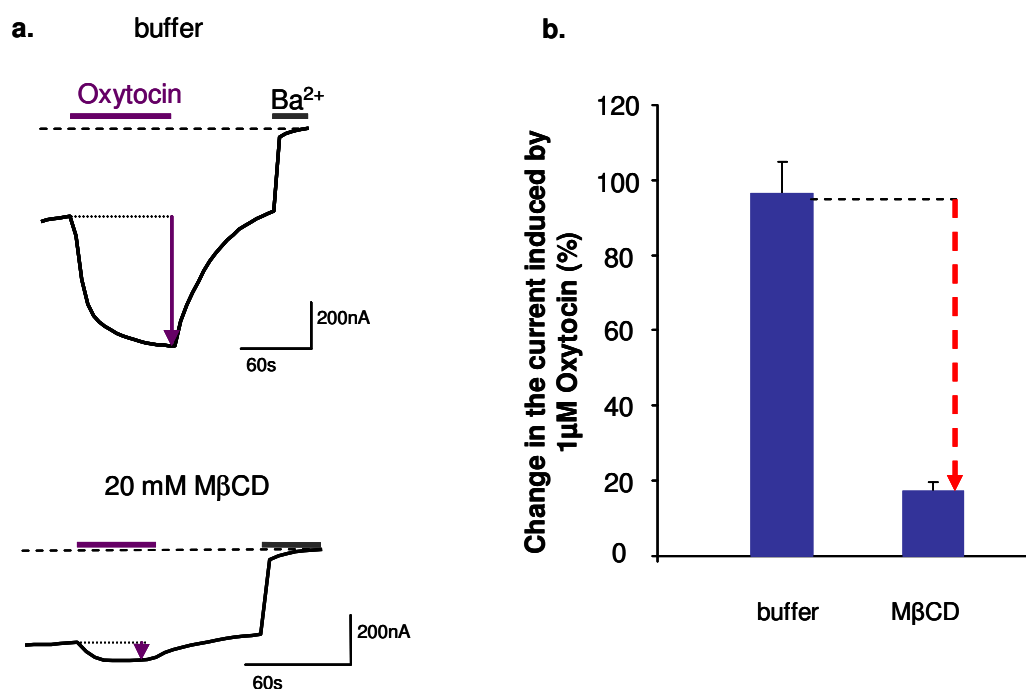


Figure 74. Sensitivity of the T4L-modified oxytocin receptor fused in the ICCR, to variation of the cholesterol level in the plasma membrane.

a) Representative TEVC recordings from *Xenopus* oocytes expressing OXTR(T4L)-K Δ -42-25 ICCR incubated in control buffer and cholesterol-complexing 20mM M β CD solution, respectively. b) Changes in whole-cell current evoked by agonist, $1\mu\text{M}$ oxytocin.

Even though fused with the channel, and modified by an insertion of the T4L domain, oxytocin receptor showed a great dependence of a binding function on the

cholesterol content in the plasma membrane. Presented data proved that incorporated, in place of the i3 loop, T4L domain did not disturb, or alter interaction between OXTR and its allosteric modulator. Moreover, receptor fused with the channel was able to retain its physiological properties. Consequently, these results answer the 2 questions of the project: ICCR is able to detect cholesterol-sensitivity and insertion of T4L in OXTR does not change this sensitivity.

In order to investigate if the loss of oxytocin-activation in M β CD-treated oocytes was reversible and specific to cholesterol, we examined also M β CD-treated oocytes re-incubated with cholesterol. After incubation with 20 mM M β CD, we transferred the oocytes into cholesterol solution. In order to solubilise the cholesterol in the aqueous buffer, we used again the M β CD, but as a vehicle. M β CD (20mM) was mixed with cholesterol (40mM) in ratio 1:8, ensuring saturation of all dextrin molecules with sterol. Incubation of oocytes with a cholesterol-M β CD mixture allowed us to replenish the plasma membrane in cholesterol.

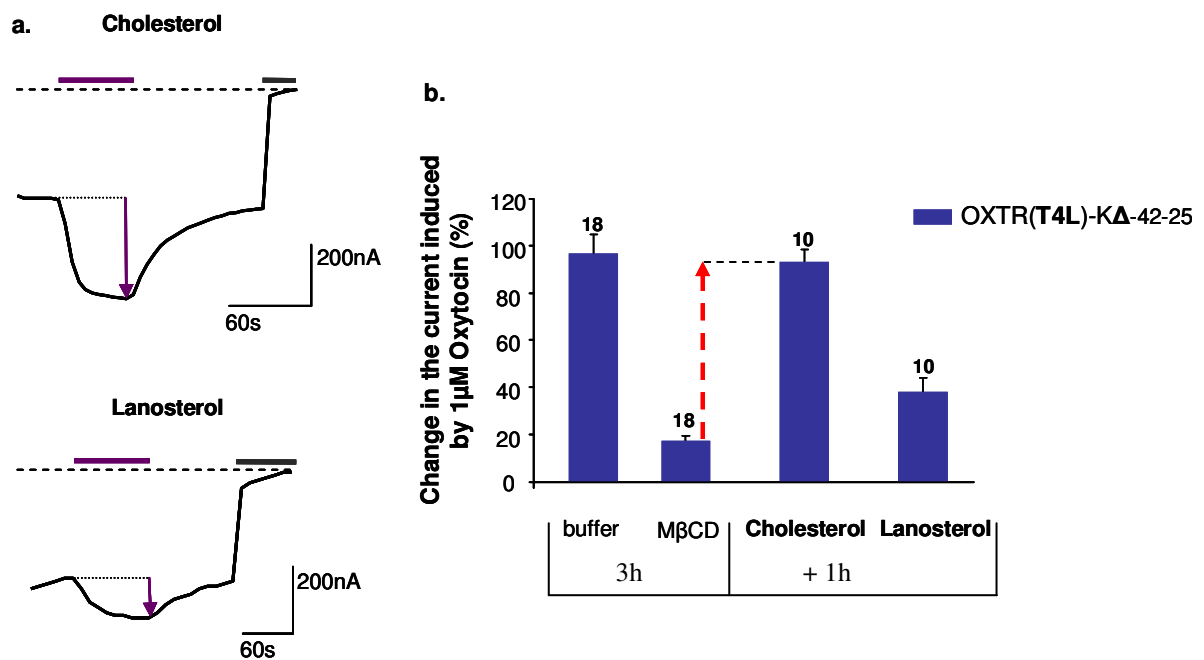


Figure 75. Functional characterization of OXTR(T4L)-KA-42-25 ICCR.

a) Representative TEVC recordings for OXTR(T4L)-KA-42-25 ICCR incubated in cholesterol and lanosterol solutions, respectively. b) Changes in whole-cell current evoked by 1µM oxytocin.

Upon oxytocin application on cells, incubated for 1 hour in cholesterol solution, we re-observed strong activation of the ICCR (Figure 75b). Cholesterol reintroduction

into plasma membrane resulted in a complete restoration of the OXTR(T4L) activity. Average change in the current, induced by oxytocin, was at the level of 93% for oocytes replenished in cholesterol, what did not significantly vary from 96% of activation observed for oocytes incubated in buffer only.

Another control for the specificity of cholesterol action was the reintroduction of a cholesterol-precursor molecule, the lanosterol. This steroid should not modulate the OXTR function, but is still able to change the physico-chemical properties of membrane. Used as a negative control, lanosterol should not reveal the specific capability of cholesterol to enhance the OXTR ligand binding properties. Surprisingly, a partial restoration of the ICCR activity (38%) was observed in presence of oxytocin. We suspect that partial regaining of oxytocin receptor function could originate from two sources:

- a high similarity in the structure of both steroids. It has been proved that oxytocin receptor binding function depends on the specific structural features of cholesterol molecule, especially on the presence of the 3 β -hydroxyl function and aliphatic tail. Lanosterol possess both, with a modified aliphatic tail. The same effect of partial restoration of receptor function has been shown as well for stigmasterol sharing these two features with cholesterol [Klein Biochemistry 1995].
- an incorporation of endogenous cholesterol molecules from intracellular stock into the plasma membrane during the lanosterol incubation.

IX.2 Kir6.2 - channel sensitive to cholesterol.

In ICCR, the oxytocin receptor is fused to Kir6.2 channel. Cholesterol is known also to modulate partially the Kir6.2 activity, but its role is quite controversial. While some studies have suggested that an activity of Kir6.2 channels is enhanced by the elevation of membrane cholesterol [Mathew Atherosclerosis 2001], other studies demonstrated the exactly opposite effect [Genda J Am Coll Cardiol 2002]. However, the most recent study on this topic have shown that Kir6.2 activity is suppressed by the elevation of membrane cholesterol, and enhanced by depletion of this sterol from plasma membrane [Rosenhouse-Dantsker Channels 2010], indicating that cholesterol acts as an inhibitor of Kir6.2 while it activates OXTR. In order to verify if obtained results for the OXTR(T4L)-ICCR were indeed due to cholesterol effect on the receptor and not directly on the channel, we performed the same experiments on Kir6.2 expressed alone (Kir6.2 Δ C36 = Kir6.2 Δ). In agreement with protocol established for the OXTR(T4L)-based ICCR, we incubated

oocytes injected with Kir6.2 Δ construct in a control buffer and 20 mM M β CD solution, and subsequently characterized its properties in the presence of 3mM azide (a cell metabolism inhibitor acting as an activator of Kir6.2). In agreement with data published by Rosenhouse-Dantsker et al., treatment of Kir6.2 Δ expressing oocytes with M β CD, resulted in an increased current compare to the current generated by the control group (Figure 76). Cholesterol-depletion by M β CD incubation induced an increase of Kir6.2 (52%) activation by azide compare to the control (38% activation).

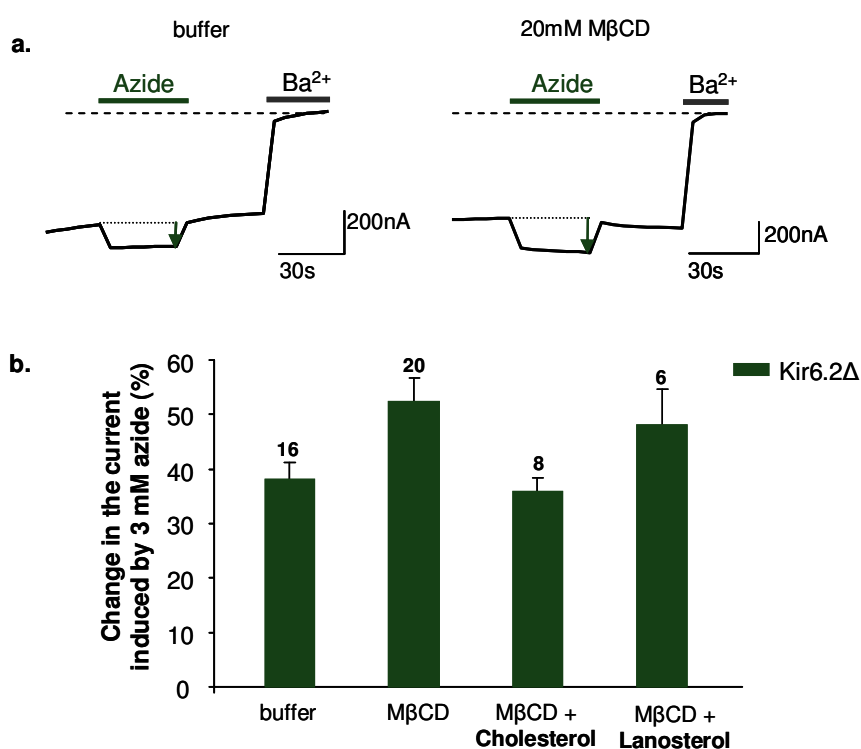


Figure 76. Effect of cholesterol content alterations on the Kir6.2 channel activity. Kir6.2 Δ refers to the C-terminal truncation mutant that renders these channels active/present as the homomers in the absence of SUR subunits [Tucker Nature 1997].

a) Representative TEVC recordings showing Kir6.2 Δ regulation upon 3 mM azide treatment, recorded from control and cholesterol-deficient oocytes treated with 20 mM M β CD. b) Average percent of Kir6.2 channel activation in the presence of 3mM azide.

Like for OXTR(T4L) ICCR, we investigated as well reversibility of observed phenomenon by the re-incubation of cholesterol-depleted oocytes in the solution containing inclusion cholesterol solubilized by M β CD. Upon functional characterization of oocytes incubated in cholesterol solution, application of 3mM azide induced only ~ 36% of the Kir6.2 channel activation. Obtained value was lower than one obtained for M β CD-treated oocytes (52%), but as expected, comparable with the buffer-incubated

group. Lanosterol did not induce the same inhibitory effect as cholesterol, thus corroborating strict specificity of Kir6.2 regulation by cholesterol.

Obtained results confirmed that cholesterol stands as a weak negative modulator of the channel function. Activity of Kir6.2 can be regulated in the reversible manner. Depletion of cholesterol enhanced channel activation, whereas restoration of that steroid to plasma membrane induced partial inhibition of channel. Importantly, cholesterol acts as a negative modulator of channel activity, whereas for oxytocin receptor modulation is positive. Thus, we proved that inhibition of ICCR by cholesterol depletion was directly due to the effect on the receptor, and not one of the channel functions.

IX.3 Specificity of observed phenomenon: ICCR based on M2-muscarinic receptor.

To ensure that cholesterol-sensitivity of the OXTR(T4L)-ICCR is receptor-specific, we tested another GPCR that has not been clearly proved to be directly modulated by steroids. For this purpose, we chose the M2-muscarinic receptor, which was already fused to Kir6.2 in wt and T4L version, and used it as a negative control. Crystal structures of the human M2, as well as M3 receptors, did not reveal any specific binding sites for cholesterol, which could act as an allosteric ligand [Haga Nature 2012][Kruse Nature 2012]. Nevertheless, in the literature we found published data claiming that M2 receptor ligand binding properties are modulated by cholesterol [Michal Eur J Pharmacol 2009]. However, similar experiments performed on opioid receptors revealed an effect of cholesterol on G protein signalling, and not directly on the receptors [Levitt J Biol Chem 2009]. Using ICCR as a direct, and G protein-independent reporter of GPCR activity, we tested the direct cholesterol-sensitivity of M2, suspecting that this receptor is not modulated by cholesterol. To investigate the controversial effect of the cholesterol on the M2 receptor we tested both: wt M2-K-9-25 and modified M2(T4L)-K Δ -9-25.

Recordings obtained from cholesterol-depleted oocytes, treated with 20 mM M β CD, did not display any significant change in the receptor activity. Upon agonist application (5 μ M Ach), on M β CD-treated oocytes expressing M2- and M2(T4L)-ICCR we observed strong inhibition of the ICCR, similar to the control group (Figure 77).

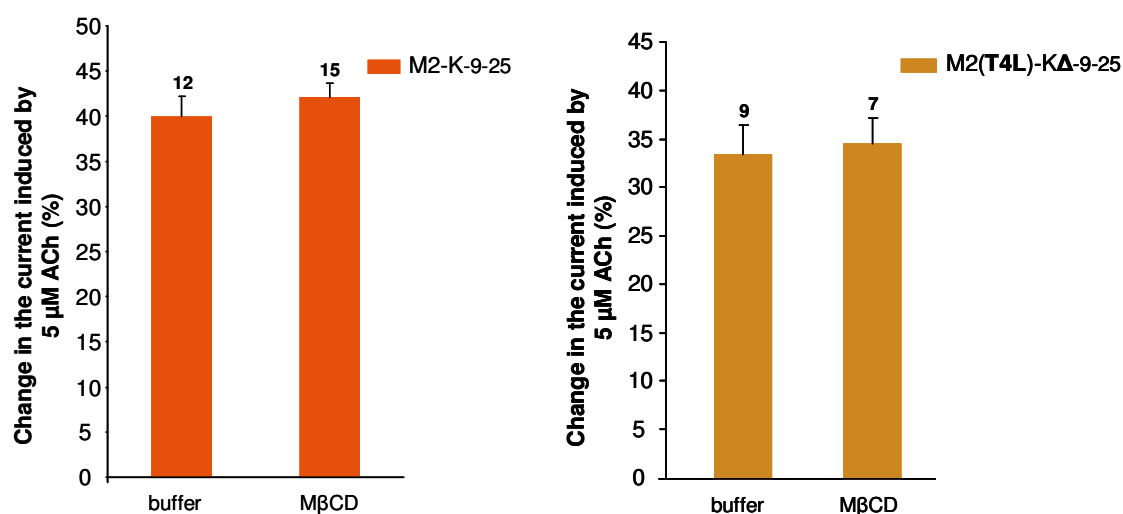


Figure 77. Effect of cholesterol on functional properties of the wt and T4L-modified M2-based ICCR. Depletion of the cholesterol from plasma membrane did not alter changes in whole-cell currents evoked by agonist, 5μM Ach.

Our data clearly showed that wt and T4L-modified M2 muscarinic receptors, fused in ICCR, remained insensitive to cholesterol content modifications in the oocytes' plasma membranes.

X Project #3: Mapping of Kir6.2 gates regulated by N-terminus actuation

X.1 Problematic

K_{ATP} channels are composed of the Kir6.2 pore-forming subunit, which is physically and functionally associated with another membrane protein belonging to the ABC transporter family: the sulfonylurea receptor (SUR). In this octameric complex SUR controls the gating of Kir6.2 through the long-range propagation of the conformational changes from SUR-ligand binding sites to Kir6.2 gate(s) [Moreau J Mol Cell Cardiol 2005][Proks Prog Biophys Mol Biol 2009]. The precise map of the SUR/Kir6.2 interacting domains, the molecular mechanisms of this intermolecular gating, and the gate(s) remaining under control of SUR are still unknown. Crystallographic structures and functional characterizations of K^+ channels, demonstrated the presence of two gates in the transmembrane domains (the selectivity filter and the "A" gate/inner helix gate at the cytoplasmic interface), and a third

gate in the cytoplasmic domain of Kir channels (the G loop gate) [Proks EMBO Rep 2003][Hansen Nature 2011] [Whorton Cell 2011][Haider J Mol Cell Cardiol 2005]

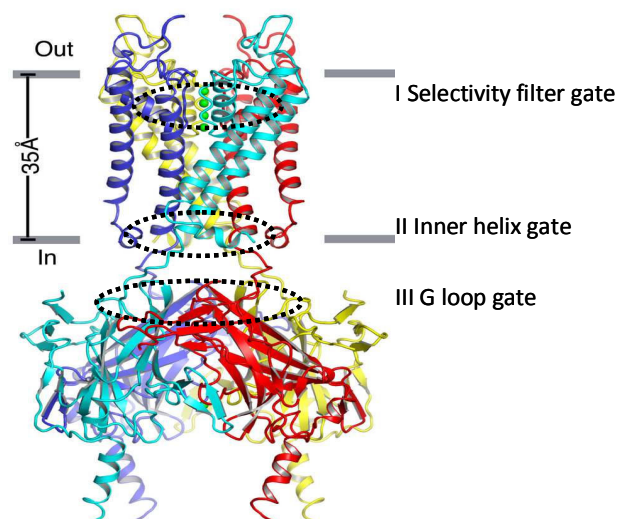


Figure 78. Structure of Kir2.2 channel with three gating regions marked with the dashed lines [Tao Science 2009]

Investigation of the gate(s) controlled by SUR is made difficult by the complex regulation of this channel. Indeed, the association of SUR with Kir6.2 modifies the ATP sensitivity of the channel, and consequently the gating. Therefore, straightforward interpretation of mutation phenotypes is limited by possible effects on the SUR/Kir6.2 association.

Due to this reason we have decided to use an artificial K_{ATP} channels, the Ion Channel-Coupled Receptors, in which Kir6.2 gating is regulated by the conformational changes of the fused G Protein-Coupled Receptor (but not through their physical interaction). This simplified model allowed us to rule out a modifying effect of ATP, occurring in a natural K_{ATP} . We used ICCRs as a tool to identify the Kir6.2 gate(s) directly regulated by an interaction of the channel N-terminus, with other regulatory subunits, such as SUR or fused GPCRs.

X.2 Applied approach.

Recently we developed a M2-based ICCR (M2-K-9-25), that inhibits the channel in presence of agonists (Figure 79). In order to identify the gate(s) regulated by the fused M2 receptor, we used a gate-scanning approach by site directed mutagenesis. This approach is based on the principle that in a multi-gate system like Kir6.2, closing of one

gate is enough to close the entire system. Consequently, mutations of gate-residues in short-chain residue should constitutively open the gates one by one. Identification of the mutants lacking the ligand-evoked inhibition will reveal the gate under control of the receptor.

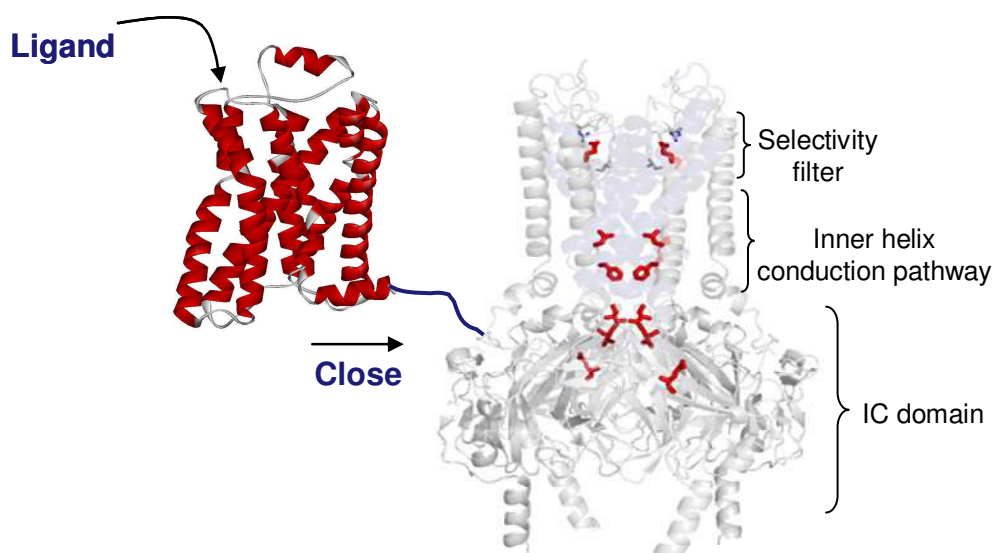


Figure 79. The principle of inhibited ICCR action.

Agonist application on GPCR (in red, PDB code: 2RH1 ^[Cherezov Science 2007]) fused to a model inward rectifier K⁺ channel (Kir6.2 model based on Kir2.2 in grey, PDB: 3SPI ^[Tao Science 2009]) induces channel closure. IC domain: intracellular domain.

Using the agonist-inhibited M2-K-9-25, we shortened the side-chains mutating one by one the known ‘gate residues’ in alanine, to constitutively open the gates.

Based on the published data for Kir6.2, and other representatives of the inward rectifier K⁺ channel family, we chose five residues revealing gating capability. These residues are located in three presumed ‘gating’ regions of Kir6.2 channel:

- the selectivity filter gate: E127A
- the “A”/inner helix gate: L164A and F168A
- the G loop gate: I296 and T297A

Moreover, we designed also a control mutant in the putative ATP-binding site: G334D, which impairs binding of ATP, without affecting the open probability of the channel ^[Drain PNAS 1998]. Consequently this residue is not a gate, but is involved in the stabilization of the channel in closed-state by ATP. Therefore, plausible inhibition of Kir6.2 by fused GPCR could be due to the effect on the action of ATP (and not directly to a gate). This ATP-binding site mutant was designed in order to answer this question.

X.3 Gating mutants

X.3.1 Kir6.2(E127A)

The first residue that we thought to be implicated in the intermolecular gating of Kir6.2 was Glu127 (E127) (Figure 80a), localized in the selectivity filter of the channel. This glutamate residue does not directly take a part in K⁺ ion conduction, but is a part of intricate hydrogen bonds and salt bridges network, which stabilizes the selectivity filter. It has been discovered in the homologous bacterial K⁺ channel, KcsA, that the E71 (equivalent to Kir6.2 E127) mutated in alanine (E71A), suppresses inactivation and constitutively opens the channel [Cheng PNAS 2011][Bhate PNAS 2012]. Moreover, electrophysiological studies on K_{ATP} argued existence of the ligand-modulated gate located in the region of selectivity filter [Proks EMBO Rep 2003].

Nevertheless, despite intensive efforts we could not detect any surface expression of the mutated M2-K(E127A)-9-25 ICCR. Basal currents produced by oocytes injected with mRNA encoding this construct were equivalent to these generated by non-injected ones (Figure 80b). We assumed that inserted mutation had a dramatic effect on the channel folding and resulted in a non-functional protein unable to conduct K⁺ ions and/or trapped in the endoplasmic reticulum.

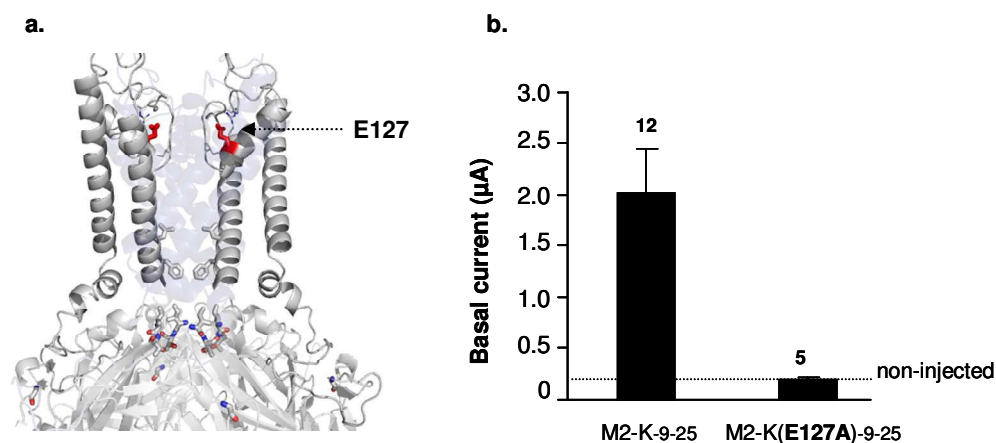


Figure 80. Selectivity filter gate: E127A mutant.

a) Homology model of Kir6.2, generated by using sequence alignment and crystallographic coordinates of Kir2.2 channel (PDB: 3SPI [Hansen Nature 2011]). For clarity two subunits are highlighted. Residue E127 is shown in red. b) Whole cell basal currents generated by oocytes injected with wt M2-K-9-25 and mutant M2-K(E127A)-9-25 ICCR.

Next mutants were created in the inner helix gate. Two residues were of particular interest: leucine 164 (L164) and phenylalanine 168 (F168). They have been proposed to form two hydrophobic seals that close the pore at the cytoplasm/membrane interface.

X.3.2 Kir6.2(L164A)

For M2-K(L164A)-9-25 ICCR the mutation did not disturb surface expression of the fusion protein. On the contrary, we found that basal currents generated by oocytes expressing this mutated ICCR were significantly higher than these produced by the wt M2-K-9-25 protein. We hypothesize that higher basal current recorded for this mutant indicates a “more” open channel as expected for a channel lacking a gate. Interestingly, it has been shown previously that mutation of this particular leucine residue into proline (L164P) causes permanent neonatal diabetes mellitus (PNDM). Functional studies of this mutant revealed, just like in the case of our M2-K(L164A)-9-25 ICCR large increase in resting whole-cell current.

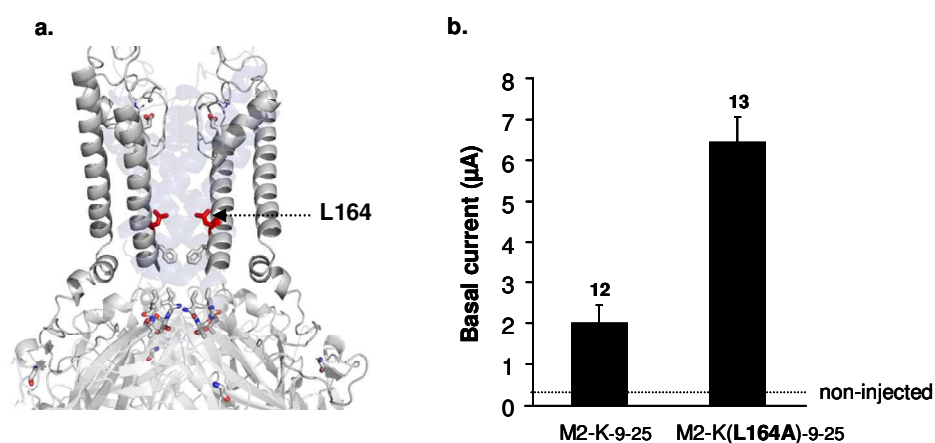


Figure 81. Inner helix gate (A gate): L164A mutant.

a) Kir6.2 model based on Kir2.2 channel structure (PDB: 3SPI). Residue L164 is shown in red. b) Whole-cell basal currents generated by oocytes injected with wt M2-K-9-25 and mutant M2-K(L164A)-9-25 ICCR.

However, during the application of agonist, 5µM ACh, M2 receptor was still able to close the channel (Figure 82), but the percentage of inhibition was much lower than for the wt M2-K-9-25 ICCR. The maximal channel inhibition was 50% of the basal current for M2-K-9-25 and only 12% for M2-K(L164A)-9-25. These results suggest that L164 constitutes probably a gate in Kir6.2, which is partially involved in the regulation by the GPCR.

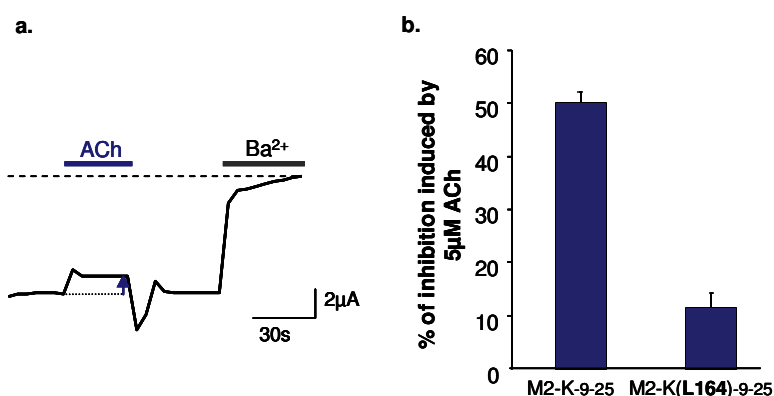


Figure 82. Functional properties of M2-K(L164A)-9-25 ICCR.

a) Representative TEVC recording of L164A mutant. b) Average change in the current induced by applied 5 μM ACh.

X.3.3 Kir6.2(F168A)

The next investigated residue along the ion conduction pathway is the phenylalanine 168 (F168), which is located in the narrowest region of the pore, just at the lipid/cytoplasm interface (below L164 in the tertiary structure). Like observed for the L164A mutant, mutation of F168 in alanine did not disturb surface expression of the M2-K(F168A)-9-25 ICCR. Surprisingly, the basal current recorded from the mutant F168A did not vary significantly from the wt ICCR (Figure 83).

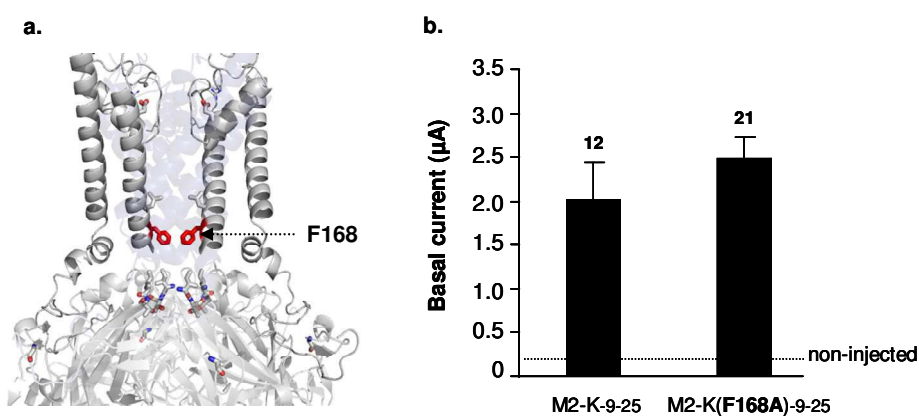


Figure 83. Inner helix gate: F168A mutant.

a) Residue F168 is shown in red. b) Whole cell basal currents generated by oocytes injected with indicated proteins.

Despite no difference in generated basal currents, application of agonist, on M2-K(F168A)-9-25 ICCR induced a dramatic loss of ligand-evoked inhibition of the channel

(Figure 84). Average inhibition induced by ACh was only 6% compared to the basal current. This striking result indicated that M2 receptor was not anymore able to close Kir6.2, suggesting that F168 constitutes a gate regulated by the fused GPCR.

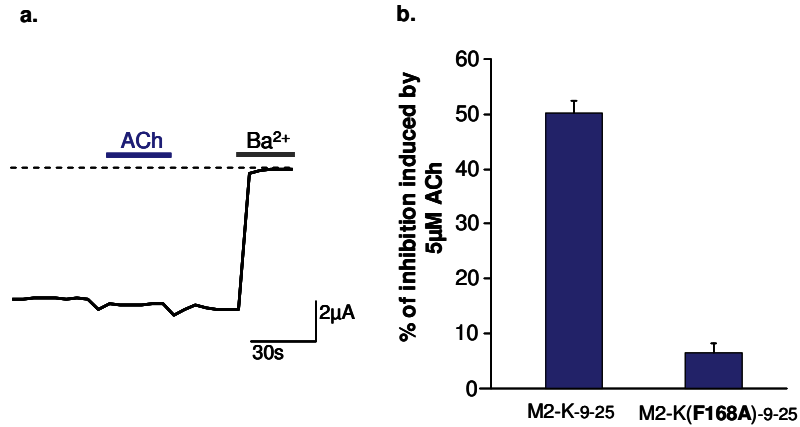


Figure 84. Functional characterization of M2-K(F168A)-K-9-25.

a) Representative TEVC recording of mutant protein. b) Average change in the current induced by applied 5 μM ACh.

The next two residues that we studied were I296 and T297, located in the narrowest constriction of the cytoplasmic domain of Kir6.2, at the apex of the tetramer.

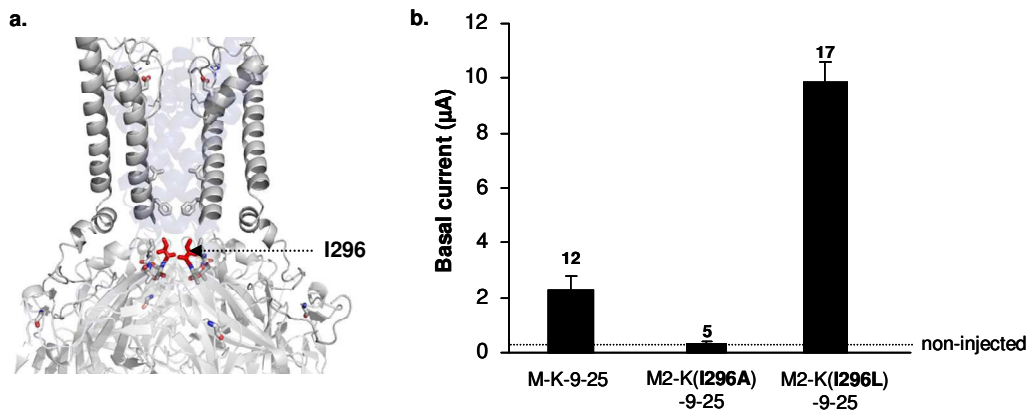


Figure 85. G loop gate: I296A/L mutants.

a) Residue I296 is shown in red. b) Whole cell basal currents generated by oocytes injected with wt M2-K-9-25 and mutants: M2-K(I296A)-9-25 and M2-K(I296L)-9-25 ICCRs.

X.3.4 Kir6.2(I296A)

Isoleucine 296 lies in the ion conduction pathway, and is distant from the putative ATP-binding site. It is located $\sim 7\text{\AA}$ below the plane of the membrane in a PIP2-bound model, and forms a hydrophobic ring that defines the narrowest part of the cytosolic pore proposed to be implicated in the Kir6.2 gating [Proks EMBO 2005]. Unfortunately, likewise for E127A mutant we could not detect any basal activity of M2-K(I296A)-9-25 ICCR. The whole-cell basal currents generated by M2-K(I296A)-9-25 were equivalent to these recorded from the negative control of non-injected oocytes. These results suggest a critical role of the I296 and E127 residues in the structure and the function of Kir6.2 limiting their study by drastic modifications of their side-chain.

X.3.5 Kir6.2(I296L)

We decided to investigate another well characterized pathophysiological mutant based on isoleucine substitution into leucine (I296L). It has been shown that Kir6.2I296L mutation causes a triad of symptoms called DEND syndrome for Developmental delay, Epilepsy and Neonatal Diabetes.

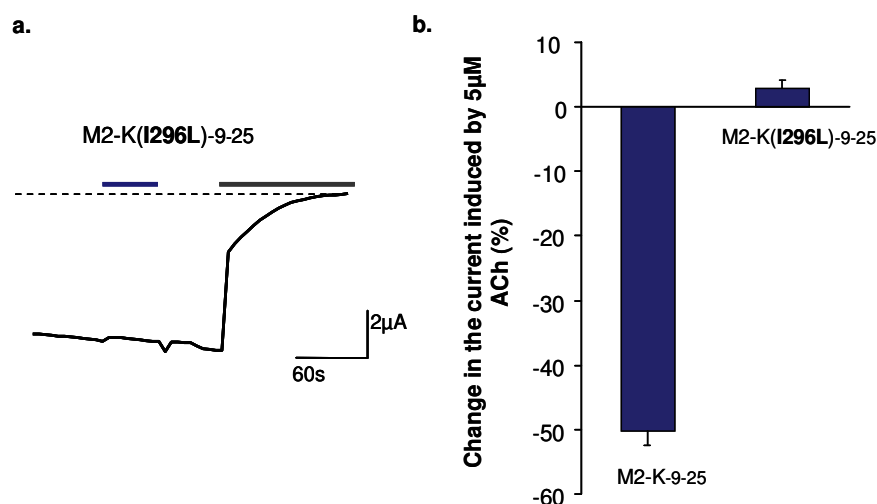


Figure 86. Functional characterization of M2-K(I296L)-K-9-25 ICCR.

a) Representative TEVC recording of mutant protein. b) Average change in the current induced by applied 5 μM ACh.

Whole-cell basal currents generated by M2-K(I296L)-9-25 were finally detectable, and more interestingly, these currents were larger than for the wild type (Figure 85). Likewise for the L164A mutant, the increase in the channel basal activity is

interpreted as a more frequently open conformation of the channel due to impairment of the I296 gate.

Functional characterization by application of 5 μ M ACh revealed a complete loss of channel regulation by the fused M2 receptor (Figure 86). This lack of M2-evoked modulation points I296 as a physical gate under control of the fused receptor. Interpretation of the effects of I296L mutation is puzzling as the side-chain modification is minor compared to alanine mutation. Indeed, the leucine side chain is similar in terms of length and hydrophobicity to the isoleucine side chain. So in fact I296L mutation did not shorten the side chain but it has been shown to increase the open probability P_o , from 53% to 80% [Proks EMBO Rep 2005].

X.3.6 Kir6.2(T297A)

The last examined, ‘gating’ residue was threonine 297, lying just below the I296 residue. Its hydroxyl group lies in the cytosolic pore and exposes the oxygen atoms to K^+ ions, like it takes place in the selectivity filter. This architecture suggests that K^+ ions could interact with T297 that could act as a gate. The mutation in alanine, T297A, did not change the basal current (Figure 87), as the amplitude of the average currents ($\sim 2 \mu$ A) was comparable to the wt ICCR.

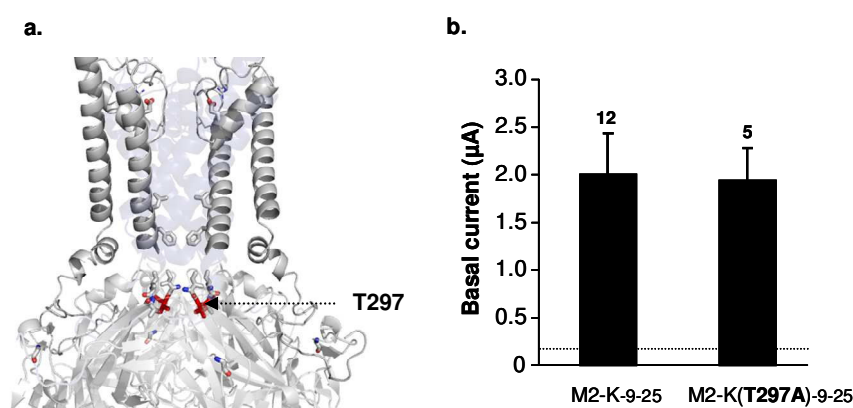


Figure 87. G loop gate: T297A mutant.

a) Residue T297 is shown in red. b) Whole cell basal currents generated by oocytes injected with indicated proteins.

Application of the ACh agonist on the M2-K(T297A)-9-25 mutant induced a strong, inhibition of the channel ($\sim 45\%$) similar to the inhibition observed for the wt M2-K-9-25 ICCR. Thus, despite very close spatial proximity to I296, mutation of T297 in

alanine did not abolish the inhibition of Kir6.2 by M2, indicating that this residue is not involved in this gating mechanism.

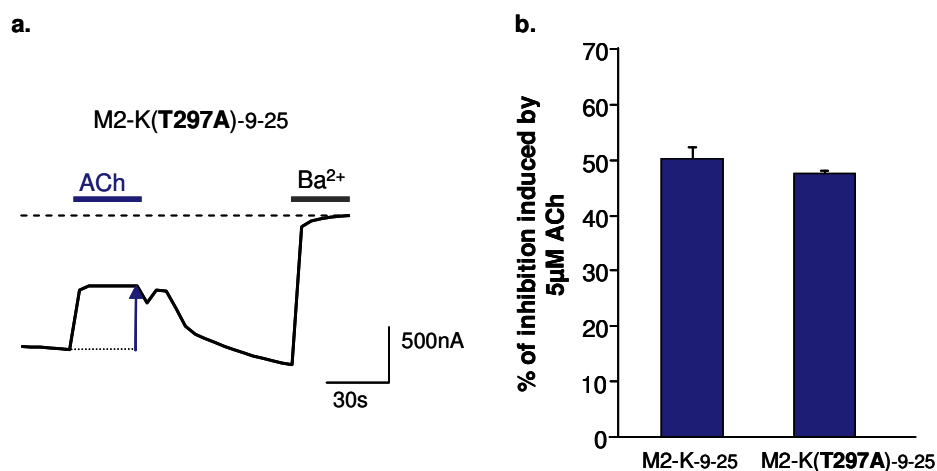


Figure 88. Functional characterization of M2-K(T297A)-K-9-25 ICCR.

a) Representative TEVC recording of mutant protein. b) Average change in the current induced by applied 5 μM ACh.

X.3.7 Kir6.2(G334D)

Last mutant we examined was M2-K(G334D). The glycine in position 334 is exposed to the surface of the cytoplasmic domain of Kir6.2 and not in the ion translocation pathway. This residue is not a gate but contributes to the Kir6.2 gating by interacting with ATP molecules. ATP binding on the cytoplasmic domain of Kir6.2 stabilizes its close state. To test the hypothesis that M2 does not close Kir6.2 by acting on stabilization of ATP binding, we mutated G334 in aspartate. Kir6.2G334D has been shown directly impair ATP binding without affecting the open probability of the channel, and consequently, without affecting the channel gates. As expected for an ATP-insensitive Kir6.2 channel, the basal current of M2-K(G334D)-9-25 was higher than the wt ICCR (Figure 89b).

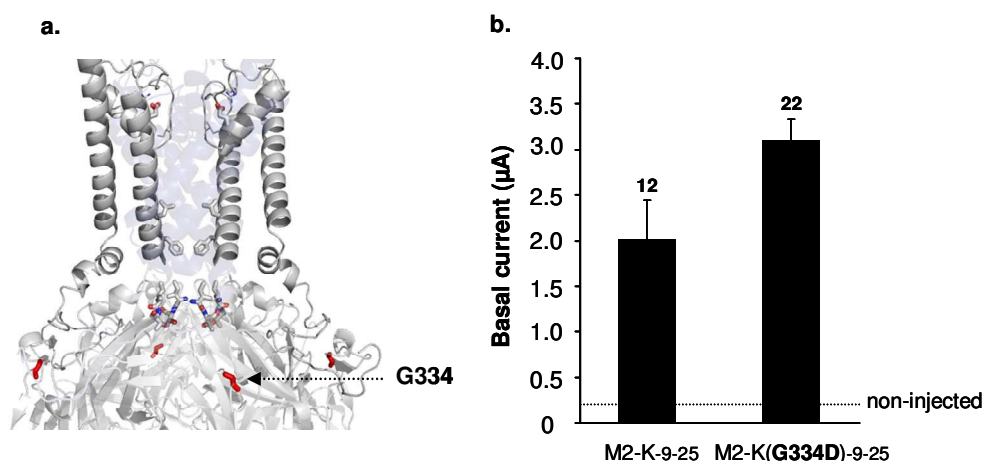


Figure 89. Putative ATP-binding site: G334D mutant.

a) Residue G334 is shown in red. b) Whole cell basal currents generated by oocytes injected with indicated proteins.

Application of ACh on M2-K(G334D)-9-25 mutant resulted in similar inhibition of the channel (~43%) compared with the wt ICCR (Figure 90). Thus, the inhibition of Kir6.2 by M2 receptor is independent of ATP binding. This finding indicates that the GPCR is able to stabilize the close state of Kir6.2 in absence of ATP reinforcing the hypothesis of direct actuation of gate residues by M2 conformational changes.

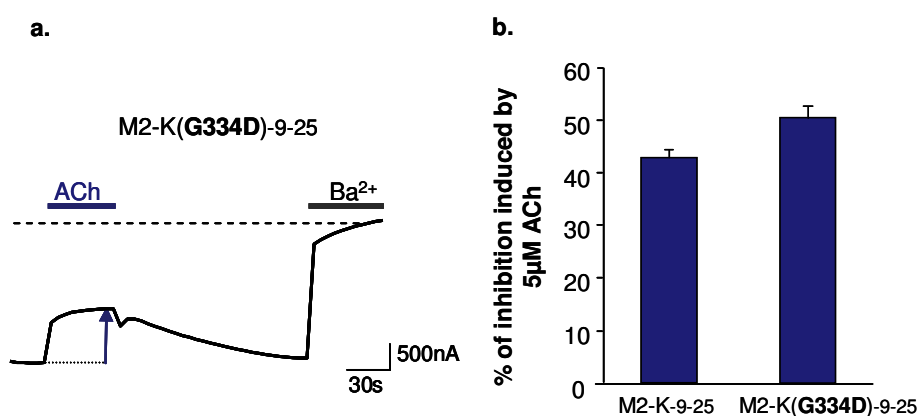


Figure 90. Functional characterization of M2-K(G334D)-K-9-25 ICCR.

a) Representative TEVC recording of mutant protein. b) Average change in the current evoked by 5µM ACh.

DISCUSSION

I Ion Channel -Coupled Receptors as a tool for functional assessment of G Protein-Uncoupled Receptors.

In the cellular environment binding of a ligand to a GPCR promotes its interaction with heterotrimeric G proteins. This interaction results in a functional dissociation of G proteins into $G\alpha$ -GTP subunit and $G\beta\gamma$ subunits. The separated $G\alpha$ and $G\beta\gamma$ subunits modulate the activity of different cellular effectors (channels, kinases or enzymes). Thus, taking the advantage of this interaction, functional characterization of wt receptors is performed mostly with standard, G protein-based, assays. Conventionally, these assays rely on detecting the activation of downstream cellular pathways through the subsequent production of the intracellular second messengers such as: Ca^{2+} , cyclic AMP, and inositol trisphosphate (IP_3). Although these assays have been the mainstay of GPCR drug discovery, they generate false positives due to the promiscuous nature of second messenger signalling, and they are not universal, as the specificity of G protein coupling to GPCRs requires the use of dedicated functional assays. More recently, assays have been designed to detect also binding of the β -arrestins to activated GPCRs.

Nevertheless, situation changes when GPCR is not any more able to active cognate G proteins, like in a case of the engineered receptors, optimized for crystallographic studies. Insertion of soluble domains (T4L or BRIL) in place of the third intracellular loop disrupts interaction between modified-GPCRs and G proteins, thus impeding utilization of standard G protein-based assays [Cherezov Science 2007] [Wu Science 2010] [Liu Science 2012]. Due to this reason functional assessment of engineered receptors is carried out by radioligand binding and competition assays. Utilization of radioligands allows attesting the proper folding of the ligand binding sites by comparison of affinities with the wt receptor. The limitation of this method is the restriction of the results to the binding site area without monitoring of the global conformational changes of the receptors. Only in the single case of the β_2 -adrenergic receptor, the functional characterization of the $\beta_2AR(T4L)$ was completed by fluorescence spectroscopy of the bimane-labeled Cys2656.27, demonstrating ligand-induced conformational changes of the cytoplasmic end of helix VI (similar to these observed with the wild-type β_2 -adrenergic receptor) [Rosenbaum Science 2007]. This method has not been used with other crystallized GPCRs. In the case of insertion of the T4L domain in the i3 loop of receptor, it is necessary to perform adjustments of the fusion protein insertion sites in order to

obtain a sufficient crystallographic resolution. These manipulations potentially affect receptor dynamics and lead to partially active or inactive states ^[Kruse Nature 2012].

Few functional assays are already available for the characterization of these modified G protein-uncoupled receptors, so an obvious question crosses our mind: what would be the contribution of the Ion Channel-Coupled Receptors to that field?

ICCR technology allows us to monitor, during a single experiment, global conformational changes evoked by a ligand binding. As it was mentioned before, channel acts as a direct reporter of the agonists- and antagonists-induced conformational changes of the GPCR ^{[Moreau Nat Nanotechnol 2008][Caro PLoS ONE 2011][Caro PLoS ONE 2012]}. We proved with the T4L-ICCRs based on: β 2-adrenergic and M2 muscarinic receptor, that inserted modification did not alter functional properties compared to the wt fusion proteins. The signal amplitude was correlated with the concentration of ligands, and enabled estimation of apparent affinities by concentration-response measurements on single cells. For β 2-based ICCR we obtained EC_{50} value ~200 nM, which is comparable with values obtained from other functional studies based on competitive radioligand binding and fluorescence spectroscopy ^{[Yao Nat Chem Biol 2006][Baker Br J Pharmacol Biol 2010]}. Strikingly, ICCRs(T4L) showed a lower agonist-efficacy compared to the wt ICCRs. The same effect was observed for the control without i3 loop [ICCR(Δ i3)], suggesting that the i3 loop is required to stabilize the receptor in a fully active state.

Although the replacement of i3 loop with T4L domain has already proven its utility in GPCR crystallization, in some cases the T4L domain had unfavourable effect on a protein expression or stability ^[Chun Structure 2012]. This was the case in attempts to crystallize the nociceptin receptor (NOP) with T4L domain. Thus, new fusion partner was searched to improve the success rate and the crystallographic resolution ^[Thomson Nature 2012]. The criteria used to find a better soluble domain was: distance constraints between the N- and C-termini of a fusion partner to be separated by 6-14 Å (the optimal distance between the intracellular ends of V and VI), as found in existing GPCRs structure with T4L domain. Other requirements were: small size, high stability and solubility. Five best matching domains: T4L fragment, flavodoxin, xylanase, rubredoxin and cytochrome b562RIL, were tested (Figure 91). Of these five considered domains thermostabilized apocytochrome b562RIL (BRIL) showed great utility in the crystallization of receptors. Few latest structures of GPCRs (nociceptin, smoothed and serotonin receptors) were obtained with the BRIL domain in place of the i3 loop ^{[Thomson Nature 2012][Wang Nature 2013][Wacker Science 2013]}. Having demonstrated that T4L-modified GPCR can be functionally characterized by ICCR technology, this concept should be also suitable for the characterization of BRIL-modified GPCRs. Moreover, structures of 11

GPCRs published in 2012 were obtained with the T4L-strategy (shingosine 1-phosphate, M2, M3, κ -opioid, μ -opioid, δ -opioid, nociceptin, neurotensin NTS1 and the protease-activated PAR1 receptors).

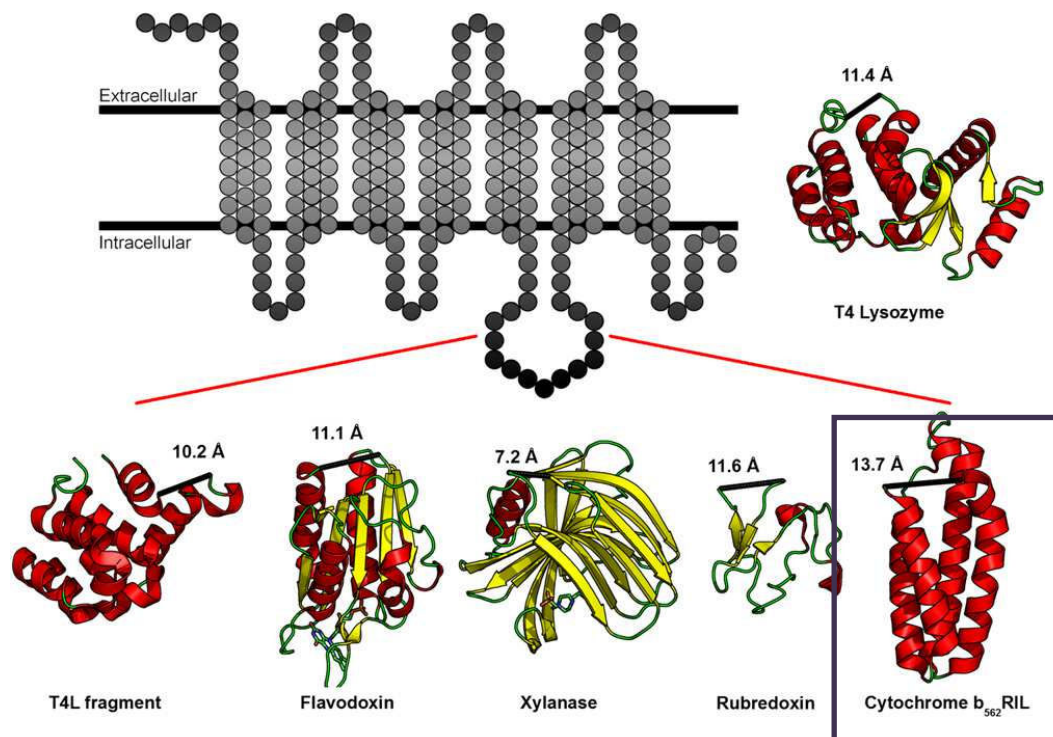


Figure 91. Five domains selected for fusion into third intracellular loop to facilitate GPCRs crystallization attempts.

Thermostabilized apocytochrome b562RIL (BRIL) reveal the best property for the crystallization of GPCRs [Chun Structure 2012].

Thus, in our opinion, several features make ICCR a potent tool in a functional assessment of the engineered GPCRs: i) signal is measured by standard real-time electrophysiological recording in cells or cell-free environments, ii) the absence of radiolabelling, iii) no biochemical assays requiring protein purification. Worked performed on the T4L-modified ICCRs has been enclosed in the article submitted to the ‘Structure’ journal.

During our investigation on the T4L-modified ICCR we found as well that T4L insertion resulted in loss-of surface expression when the C-terminus of Kir6.2 channel was intact (Figure 92). We observed this phenomenon for the all tested ICCRs based on M2(T4L), β 2(T4L) and OXT(T4L) receptor. We assumed that the insertion of T4L disrupts an interaction with a partner protein, which masks the Kir6.2 ER retention signal. The absence of interaction with this partner in GPCR(T4L)-ICCR leads to constant exposition of the retention signal and trapping of the ICCR in the endoplasmic reticulum [Tucker Nature 1997]. This is confirmed by the surface expression of ICCRs [wt and GPCR(Δ i3)] that are still able to

interact with partner proteins such as G proteins. Moreover, removal of this ER retention signal by truncation of the 36 C-terminal residues of Kir6.2 was required for surface expression of ICCR(T4L), but not for surface expression of ICCR still able to interact with G proteins [Moreau Nat Nanotech 2008].

Postulating that G proteins are the determinants involved in a masking of the Kir6.2 ER retention signal (however until now there is no straightforward evidence), we think that ICCR could be a useful tool to discriminate the effect of mutations in GPCRs that affect G proteins activation and no G protein interaction (Figure 92). Thus, if an engineered GPCR is not able to interact with G proteins, for instance due to physical hindrance created by a fusion of the T4L or BRIL domains, it will result in loss-of surface expression (but not in the function) of the receptor. On the second hand, when only the activation step of G proteins is altered, throughout particular mutations, then ICCR will be still able to reach surface of the plasma membrane, and the global conformation changes of the receptor could be affected and detected in ICCRs.

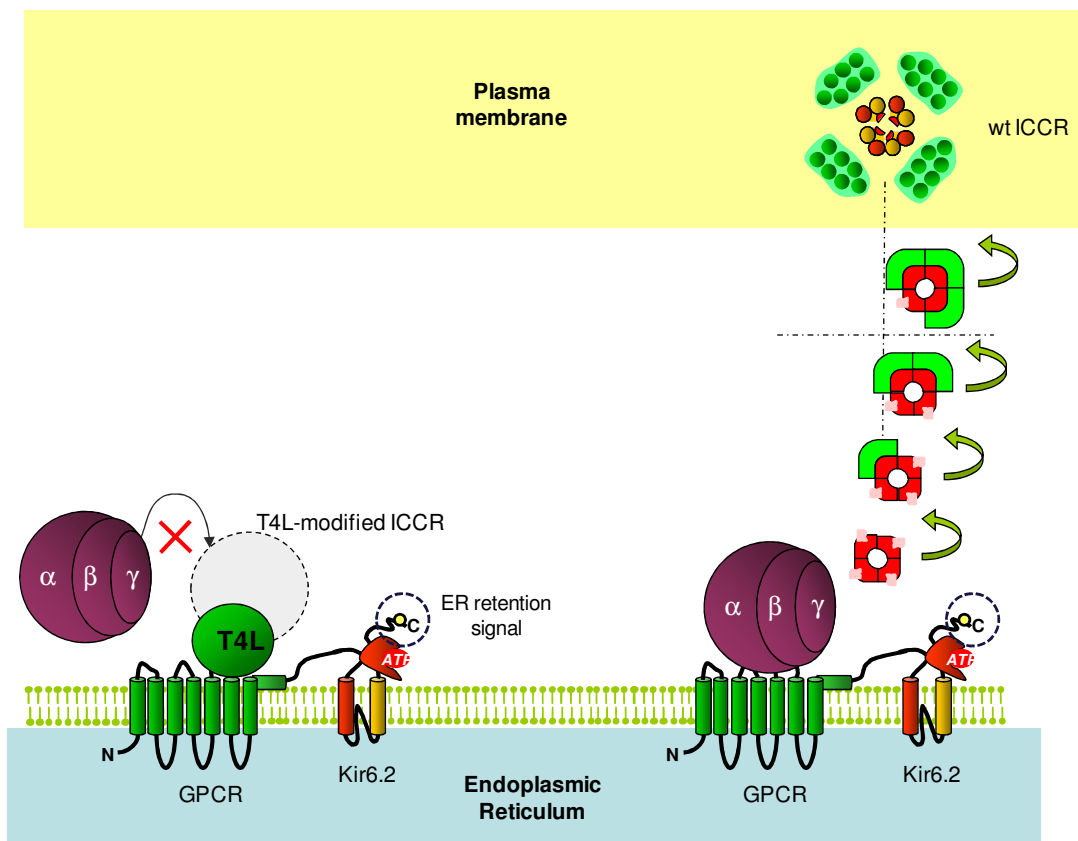


Figure 92. Comparison of events undergoing in the endoplasmic reticulum between the T4L-modified and wild type ICCR.

Presence of Kir6.2 ER retention signal results in a disrupted surface expression of the ICCR(T4L), whereas wt protein reaches the plasma membrane [Channels Group].

Due to the reason that we created ICCR based on T4L-modified oxytocin receptor which functional properties, and physiological regulation has been proven to be cholesterol-dependent, we examined the utility of ICCR in a detection of the cholesterol-dependence of the receptor [Klein Biochemistry 1995] [Gimpl Eur J Biochem 2000] [Reversi Am J Physiol Regul Integr Comp Physiol 2006]. It is well known that cholesterol plays a leading role in a regulation of the activity of numerous membrane proteins. G Protein-Coupled Receptors do not make an exception [Gimpl Biochemistry 1997][Pucadyil Prog Lip Res 2006][Chini J Mol Endocrinol 2009]. Nowadays assessment of cholesterol influence on GPCR functional properties is performed for the most part with radioligand binding or G protein-based assays. Utilization of radioligand allows attesting the cholesterol influence on ligand binding affinities of receptors in cholesterol rich- and poor environments. On the second hand, standard G protein-based assays allow to detect the cholesterol-influence through the scope of second messenger levels (Ca^{2+} , IP_3). However observed differences can be due to cholesterol effect on GPCR activity, as well as on effector proteins. Thus, reliability of data obtained with the G protein-based assays is limited due to indirect measurements. With results obtained for the OXTR(T4L)-ICCR we proved that our tool allows direct detection of cholesterol-dependence by comparison of the receptor activities in the cholesterol naturally rich and artificially depleted cell membrane. Moreover insertion of the T4L domain does not altered sensitivity of a modified receptor to cholesterol.

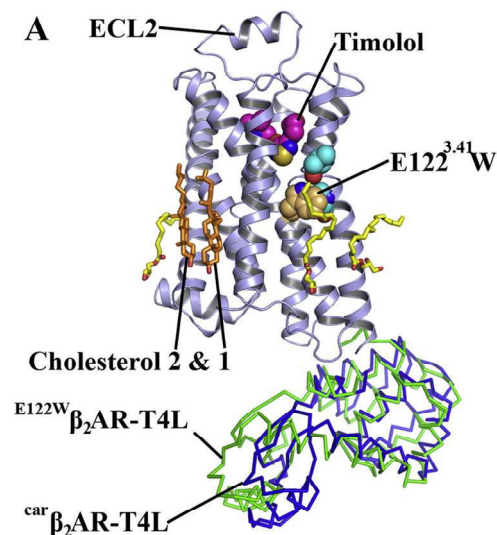


Figure 93. Crystal structure of an engineered β_2 adrenergic receptor with established two specific cholesterol binding sites [Hanson Science 2008].

We wanted to ensure that the cholesterol-dependence of OXTR(T4L)-ICCR was receptor-specific. Thus, we used another ICCR based on the wt/T4L-modified M2 muscarinic

receptor, as a negative control. Crystallographic studies of the M2 receptor did not identify any specific cholesterol binding sites ^[Haga Nature 2012]. Modifications of the cholesterol content of the plasma membrane did not change the activity of M2-based ICCR, neither for wild type, nor for the T4L-engineered protein. Even though our results match with the crystallographic data, there are studies which claim that membrane cholesterol content influence the ligand binding properties of the M2 receptor, and the efficacy of signal transduction through G proteins. It has been proposed by Colozo et al. that cholesterol may bind to muscarinic M2 receptors and influence its properties in a fashion similar to that of allosteric modulators ^[Colozo Biochem Pharmacol 2007]. Michal et al. reported that modification of the cell membrane cholesterol content using M β CD in intact mammalian cells (CHO) leads to various alterations in both, the potency and efficacy of carbachol (an ACh analogue) in signalling through different subclasses of G proteins ^[Michal Eur J Pharmacol 2010]. However, in our opinion, an assessment of the cholesterol-dependence by measurement of the second messenger responses can create false-positive responses due to an indirect effect on effector proteins.

II Investigation of the Kir6.2 channel gates controlled by regulatory membrane proteins.

The gating of the inward rectifiers K⁺ channels (Kir channels) is known to be controlled by diverse factors: some are regulated by G proteins, others by intracellular nucleosides, pH or Na⁺ ions (allosteric ligand), whereas all eukaryotic Kir channels are stimulated by phosphatidylinositol 4,5-bisphosphate (PIP₂) ^{[Baukrowitz Science 1998] [Shyng Science 1998] [Rohács J Biol Chem 1999]}. However, the molecular mechanisms underlying the control of gating process by regulatory proteins are still unknown. In order to broaden our knowledge about this phenomenon we investigated the gating of the Kir6.2 channel fused to the M2 muscarinic receptor.

Like in the case of other Kir channels, several cytosolic factors (MgATP, H⁺) and membrane lipids (PIP₂, long chain acyl CoAs) regulate Kir6.2 gating (opening and closing) ^{[Baukrowitz Science 1998][Shyng Science 1998][Davies Nature 1990]}. On the second hand, Kir6.2 constitutes specific representative of K⁺ inward rectifiers. Physiologically it co-assembles with the SUR receptor regulatory subunit that contributes to the regulation of the channel gating through long-range propagation of conformational changes from SUR-ligand binding sites to Kir6.2 gate(s). Binding of sulphonylureas or K⁺ channel openers to SUR subunit results in Kir6.2 channel closure or opening, respectively ^{[Babenko Annu Rev Physiol 1998] [Moreau J Mol Cell Cardiol 2005]}.

However, to identify the gate(s) which is directly governed by SUR is very challenging. The reason is that besides direct regulation of Kir6.2 gating, the physical interaction with the receptor enhances as well its sensitivity to ATP, and affects its activity. In order to identify gate(s) controlled directly by the receptor we decided at first place to exclude the factor of modified ATP-sensitivity. Thus we simplified the system by using Ion Channel-Coupled Receptors [Moreau Nat Nanotechnol 2008][Caro PLOS One 2011][Caro PLOS One 2012]. In ICCRs Kir6.2 is directly fused to GPCR, which does not modify Kir6.2 ATP-sensitivity. Thanks to that we could directly interpret mutation phenotypes and identify individual residues being of importance for receptor-controlled Kir6.2 gating.

As mentioned it has been proposed that K⁺ ions conduction might be regulated by three gates localised in:

- the selectivity filter [Proks EMBO Rep 2003][Cordero-Morales Nat Struc Mol Biol 2006][Cheng PNAS 2011]
- the bundle crossing region of the transmembrane domains (TMD) [Doyle Science 1998][Jiang Nature 2002]
- the apex of the cytoplasmic domain (CTD) [Pegan Nat Neurosci 2005][Nishida EMBO J 2007]

We investigated all these regions in order to check if some resident residues proposed to be implicated in Kir channels gating are directly regulated by fused GPCR, and possibly by SUR protein. We started our investigation with a residue present in the selectivity filter of Kir6.2 – glutamate 127 (E127). Several pieces of evidence point the selectivity filter as the region that can also act as a gate in K⁺ channels. Proks et al. claimed that intracellular ligand-regulated gate of Kir6.2 is localized in, or just above the selectivity filter [Proks EMBO Rep 2003]. Nevertheless, they did not propose any individual residues that might act as a gate.

More recent studies with a bacterial homolog, KcsA channel, also promote the hypothesis of the selectivity filter as a gate. It has been shown that despite the selectivity filter acclaimed as highly rigid, it undergoes larger conformational excursions than previously estimated [Cordero-Morales Nat Struc Mol Biol 2006][Cheng PNAS 2011]. Cordero-Morales et al. argued that KcsA E71 (Kir6.2 E127) constitutes part of an intricate hydrogen bond network, and can function as a gate. They demonstrated that mutation to alanine (E71A) affected the selectivity filter toward a constitutively open state of KcsA. However, in our trials of equivalent mutation in Kir6.2, it was impossible to determine if E127 constitutes a gate, as we did not observe any macroscopic currents generated by E127A mutant. We assumed that this mutation could trigger strong structural rearrangements which, in contrast to KcsA, resulted in nonconductive channel (or incorrectly folded ICCR trapped in the ER). This finding was very

surprising taking into account that the surface expression of the homologous mutation E71A in KcsA was not altered [Cordero-Morales Nat Struc Mol Biol 2006].

The second region which is considered to serve as a gate in K⁺ channel is the helix bundle-crossing. The inner helix gate has been proposed to be formed by inner helices of the TMs, just inside the membrane, above the level of the interfacial helix. Crystallographic studies of eukaryotic homologous channels, Kir3.2 (GIRK2) and Kir2.2, revealed meaningful motions occurring in this region upon binding of specific ligands [Whorton Cell 2011] [Hansen Nature 2011]. Although crystal structures suggest that this region is important for channel closure, the presence of a functional gate in the Kir channel bundle-crossing has been frequently questioned [Proks EMBO Rep 2003] [Clarke Cell 2010].

We chose two residues, we believed to play primary role in the gating of this region: leucine 164 and phenylalanine 168 (Figure 94). These residues form two constrictions in the ion permeation pathway. Loussouarn et al. performed functional studies in order to identify pore-lining residues using cysteine-scanning mutagenesis, and subsequent ability of intracellular Cd²⁺ to block the channel. The assumption was that Cd²⁺ block requires coordination of four cysteines that face the pore, and lay only few Å apart. Highest affinity block was observed for these two residues, forming two hydrophobic seals that close off the pore at the cytoplasm interface [Loussouarn J Biol Chem 2000] [Haider J Mol Cell Cardiol 2005].

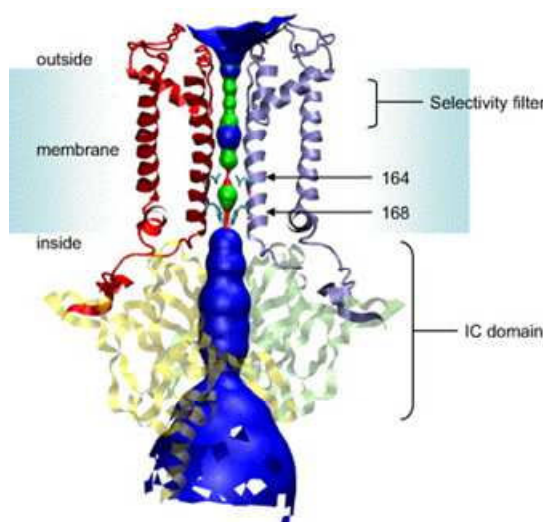


Figure 94. HOLE plot of the permeation pathway of Kir6.2 channel.

Residues of interest, leucine 164 and phenylalanine 168, create a ring that lies in the plane of the membrane, at the level of helix-bundle crossing [Haider J Mol Cell Cardiol 2005].

Moreover, there are several functional studies corroborating crystallographic data. It has been discovered that first tested residue from inner helix gate, L164, remarkably

contributes to a proper function of the channel. Tammaro et al. reported that naturally occurring physiopathological mutation in K_{ATP} channels, L164P, induces a marked enhancement in the channel activity. Macroscopic whole-cell currents generated by the mutant were greatly higher, when compared to wt protein. What is more interesting, they proved that these currents were unaffected by sulphonylureas (4% block), drugs which bound to SUR inhibit K_{ATP} channel activity [Tammaro Diabetologia 2008]. This could suggest disrupted functional interaction between SUR and Kir6.2. These findings are similar to our data demonstrating as well significant increase in whole-cell currents for mutated M2-K(L164A)-9-25 ICCR (3-fold larger than ones generated by wt proteins). However, despite enhanced basal currents, fused GPCR was still able to partially modulate channel activity. Intriguingly, modulation was significantly lower (10% inhibition of basal currents), than in the case of wt ICCR (50% inhibition). In our opinion significant change in generated basal whole-cell currents, and 80% loss of channel modulation, suggest implication of leucin 164 in M2 receptor-controlled gating of Kir6.2 channel. In contradiction to our observations, there are studies suggesting that L164 has no great importance for gating process. Khurana et al. also undertook a mutagenic approach to investigate the functional importance of residues present in the bundle-crossing region, and inner cavity of Kir6.2 channel. They substituted pore-lining residues with glutamate (possessing protonatable side chain), thus introducing pH-dependent motions (mutual repulsions of deprotonated carboxylates) in the helix-bundle crossing. Results obtained for L164E mutant did not reveal any activation of channel at alkaline pH, suggesting minor or none contribution of this residue into channel gating [Khurana J Biol Chem 2011]. Puzzling M2-K(L164A) mutant, as it was mentioned above, was partially affected suggesting that this residue has a partial role in the regulation by M2 receptor. Interestingly, for M2-K(F168A)-9-25 mutant we observed a complete loss-of ACh-evoked inhibition of Kir6.2. In contrast to L164, F168A was insensitive to ACh-modulation. This result clearly proved that unquestionably F168 serves as the gate controlled by the M2 receptor. Strikingly, whole-cell basal currents recorded from F168A mutant did not vary significantly from these generated by wt protein. Theoretically, we should expect much higher basal current due to 'loss-of' a gate. Nevertheless, one possible explanation could be a higher open probability of the mutant, but a lower surface expression. In the case of whole-cell currents it is very difficult to distinguish between the level of surface expression, and a basal activity of expressed protein. Obtained results stay in agreement with previous studies considering the phenylalanine 168 as a gate [Rojas Biochim Biophys Acta 2007] [Khurana J Biol Chem 2011]. Rojas et al. proposed that F168 gate has not only a structural existence, but is also a functional entity. They investigated the role of F168 in

Kir6.2 gating using mutagenesis approach based on replacing this residue with amino acids having side chains of different size and hydrophobicity. They have demonstrated that bulky, aromatic phenylalanine residue not only may occlude the ion conduction pathway in the closed state, but can also act as the facilitator (which lowers the energy barrier necessary for the gating transition) in the Kir6.2 channel gating [Rojas Biochim Biophys Acta 2007]. The pointing role of F168 in Kir6.2 channel gating was as well supported by previous investigations of Khurana et al., who excluded a possible role of L164 in this process. They proposed that possible conformational changes in the region of F168 can promote channel opening, and are functionally coupled to the intrinsic ATP-binding site [Khurana J Biol Chem 2011]. In our opinion results for both tested mutants [M2-K(F168A)-9-25 and M2-K(L164A)-9-25] suggest that they could stand as one gate controlled by the fused GPCR, due to their close spatial proximity in predicted secondary structures.

The last gate proposed to function in K^+ channels is formed by the G loop (G loop gate) at the apex of the CTD. It is localized just outside the membrane, below the level of the interfacial helix. Due to the reason that crystallographic, and functional studies of other members (prokaryotic and eukaryotic) of K^+ inward rectifiers family suggest that this region of channel stands as a gate, we have decided to investigate its involvement in Kir6.2 gating [Pegan Nat Neurosci 2005] [Whorton Cell 2011] [Hansen Nature 2011]. The isoleucine 296 creates the narrowest section of the cytoplasmic ion permeation pathway (Figure 95). Significance of this residue is emphasized by the fact its mutation into alanine (M2-K(I296A)-9-25) did not produced detectable currents, probably due to a non-functional channel. M2-K(I296L)-9-25 ICCR was well-expressed, with a significant increase in the whole-cell basal currents and was insensitive to M2 regulation. Thus, the I269L mutation abolished the regulation of Kir6.2 by M2 receptor. Moreover, Proks et al. reported that naturally occurring I296L physiopathological mutation in K_{ATP} channels, alike L164P, induces a marked enhancement in the channel activity. Resting whole-cell currents generated by the mutant were greatly higher when compared to the wt channel, and no regulation by azide (metabolic inhibitor) was observed, suggesting that the channel is constitutively open. Importantly, these currents were unaffected by the blockers, sulphonylureas [Proks EMBO Rep 2005]. Our results obtained for the artificial M2-K(I296L) ICCR thoroughly corroborate with data concerning natural K_{ATP} channels. They strongly suggest that I296 serves as the functional gate under control of the fused receptor. Furthermore, high similarity in I296L mutation-evoked effect on artificial ICCR, and natural K_{ATP} channel encourages us to speculate that possibly the same gate in K_{ATP} channels could be controlled by the natural partner of Kir6.2, the SUR receptor.

Interestingly, mutation of the second investigated residue in the cytoplasmic apex of Kir6.2, T297A, that lies just below I296 in the predicted secondary structure did not induce any significant change, neither in basal current, nor in regulation induced by applied ligand. This finding clearly proved that creation of physical constriction in the ion pathway does not systematically create a functional gate. Thus, the M2-K(T297A)-9-25 mutant did not alter functional properties of Kir6.2.

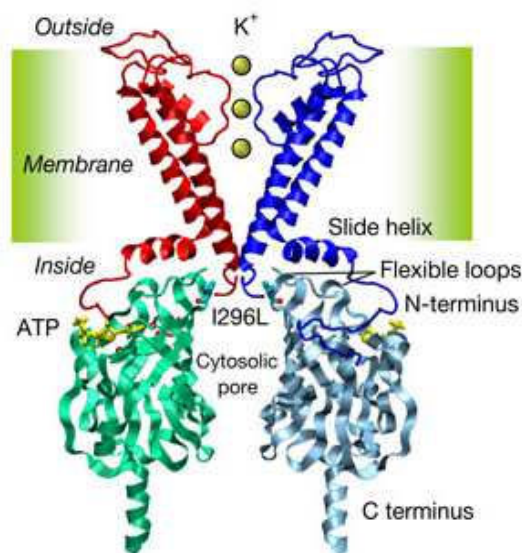


Figure 95. Location of I296 in Kir6.2 channel.

Molecular model of Kir6.2 viewed from side. For clarity, each subunit is shown in different colour, and only two transmembrane domain (TMs) and two cytosolic domains (CTDs) are illustrated [Proks EMBO Rep 2005].

In contrast to voltage-dependent and other ligand-gated K^+ channels, it has been clearly shown for Kir channels (GIRK2) that they contain two regulated gates. Our results obtained with Kir6.2 channel suggest as well the existence of two gates under control of the fused M2 receptor.

However beside recognition of residues implicated in GPCR-controlled gating of Kir6.2 channel we also wanted to understand the molecular mechanisms of a signal transmission from ligand binding site in GPCR, to Kir6.2 gate(s). In ICCR Kir6.2 channel is directly fused by its N-terminus to C-terminus of GPCR, created by helix VIII. It has been previously shown that length of the linker between fused proteins plays an important role in ligand-dependent modulation of ICCR [Moreau Nat Nanotechnol 2008][Caro PloS ONE 2011][Caro PloS ONE 2012]. Due to this reason we suspected that functional communication between Kir6.2 and fused GPCR engages the motions of the helix VIII. Recent progress in GPCR crystallographic studies disclosed molecular basis of ligand-induced receptor conformational changes. Intriguingly, comparison of active (agonist-bound) and inactive structures of GPCR, for

example of β_2 adrenergic receptor, revealed significant structural changes for the transmembrane domains TM5 and TM6 (but not for helix VIII) [Cherezov Science 2007][Rasmussen Nature 2011]. The biggest changes were especially observed in the intracellular part of the TM5 and TM6. Nevertheless, GPCR activation dynamics are characterized by a complex equilibrium among multiple conformational states. Due to the reason that crystallographic data give a highly detailed, but ‘frozen’ picture of the lowest energy receptor state, we cannot conclude if motions of those TM helices play a substantial role in Kir6.2-GPCR functional communication. In our opinion linker region, created by the C-ter of GPCR and the N-ter of Kir6.2, is crucial for the stabilization of the closed state of Kir6.2 upon binding of the specific ligand to GPCR. Moreover, it has been shown as well for wild-type K_{ATP} channel that the N-ter of Kir6.2 interacts with the C-ter of SUR creating a high affinity binding site for sulphonylureas. Thus, the N-terminus of channel appears to be crucial for transduction of the signal from the GPCR or SUR.

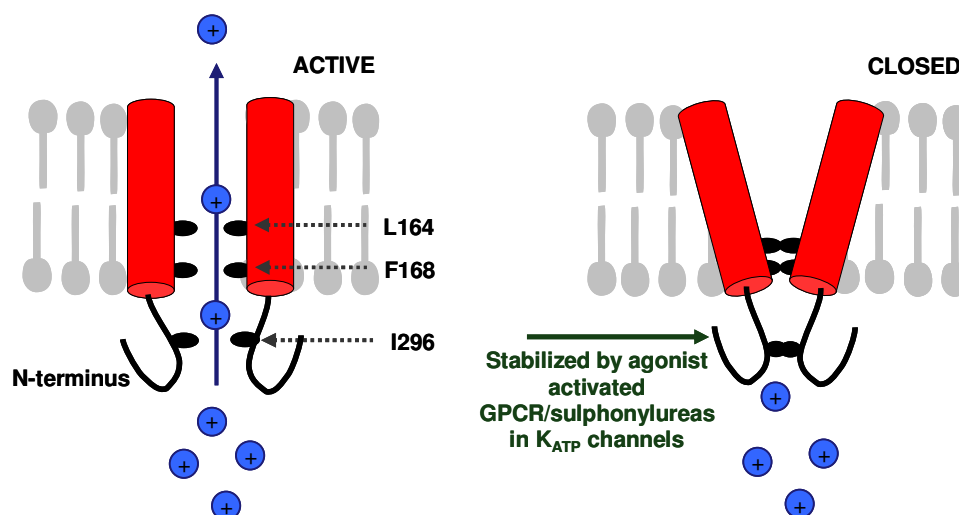


Figure 96. Representation of Kir6.2 channel gating mechanism.

a) Kir6.2 channel fluctuates between open and close conformations. In open state it can conduct K⁺ ions. b) When it is fused in ICCR, ligand-induced conformational changes in GPCR stabilizes the closed state of the channel (all three gating residues create hydrophobic seals that prevent ion conduction through the pore) [Channels Group].

We believe that our findings could be helpful in understanding the molecular mechanism of the gating phenomenon occurring in natural K_{ATP} channels. To our knowledge there is no structural data about this channel, and the precise map of the SUR/Kir6.2 interacting domains remains unknown. Thus, results obtained with an artificial ICCR could assist with comprehension of mechanisms underlying the gating process in K_{ATP} channel.

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Title: Development of the Ion Channel-Coupled Receptor technology in structure-function studies of G protein-coupled receptors and Kir6.2 channel.

Abstract

Ion Channel-Coupled Receptors (ICCRs) are artificial ion channels created by the fusion of a G protein-coupled receptor to a Kir6.2 channel. In this concept, the channel acts a direct reporter of the conformational changes of the GPCRs, allowing the detection by simple current recordings of agonists and antagonists binding in concentration-dependent manner.

The signal being directly correlated to the receptor activity, independently of G protein signalling pathways, we exploited this advantage to extend the field of applications of ICCRs during this thesis. We developed 4 applications: 1) the functional characterization of the optimized GPCRs for crystallization by insertion of the T4 phage lysozyme domain in the ICL3 loop; 2) the detection of a cholesterol-dependence of the GPCRs; 3) the detection of the so-called "biased ligands" to simplify their screening; and 4) the functional mapping of the Kir6.2 channel gates under control of membrane proteins interaction with the N-terminus domain.

Titre: Developpement de la technologie des Récepteurs Couplés à un Canal Ionique pour des études structure-fonction des Récepteurs Couplés aux Protéines G et du canal Kir6.2.

Résumé

Les Récepteurs Couplés à un Canal Ionique (ICCRs) sont des canaux ioniques artificiels créés par fusion d'un Récepteur Couplé aux Protéines G (RCPG) au canal ionique Kir6.2. Dans ce concept, le canal agit comme un rapporteur direct des changements conformationnels des RCPGs permettant de détecter par simple mesure de courant, la fixation d'agonistes et d'antagonistes proportionnellement à leur concentration.

Le signal induit étant directement corrélé à l'activité du récepteur, indépendamment des voies de signalisation des protéines G, nous avons exploité cet avantage pour étendre le champ d'applications des ICCRs au cours de cette thèse. Nous avons développé quatre applications qui sont: 1) la caractérisation fonctionnelle des RCPG optimisés pour la cristallisation par insertion de domaine du lysozyme du phage T4 dans la boucle ICL3; 2) la détection de la dépendance des RCPGs au cholestérol; 3) la détection de ligands dits "biaisés" pour faciliter leur criblage; et 4) la cartographie fonctionnelle des portes du canal Kir6.2 régulées par des protéines membranaires interagissant par le domaine N-terminal.

Discipline:

Biologie structurale et biologie moléculaire

Mots Clés:

GPCR, canal KATP, biocapteur, électrophysiologie (patch-clamp, double micro-électrodes), ingénierie moléculaire

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