# STRUCTURELLE DETERMINANTEN DER INTERAKTION EINWÄRTSGLEICHRICHTENDER KALIUMKANAL-PROTEINE MIT PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHAT

**DISSERTATION**

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## ZUSAMMENFASSUNG

Einwärtsgleichrichter-Kaliumkanäle (Kir-Kanäle) haben in Säugerzellen die Aufgabe, das Ruhepotential der Zellmembran zu stabilisieren sowie den Transport von Kalium-Ionen über die Membran zu vermitteln. Verschiedene Mutationen in Kir-Genen führen beim Menschen zu erblichen Erkrankungen und unterstreichen damit die physiologische Bedeutung dieser Kanal-Familie. Allen Kir-Kanälen gemeinsam ist eine tetramere Grundstruktur mit zwei Transmembransegmenten und einer Porenschleife in jeder Untereinheit. Einige Vertreter der Kir-Familie sind konstitutiv aktiv, während andere durch spezifische Liganden wie Protonen, ATP oder $G_{\beta\gamma}$-Untereinheiten der trimeren G-Proteine reguliert werden. Man nimmt an, dass diese Regulatoren mit den zytoplasmatischen, amino- und carboxyterminalen Domänen der Kanäle interagieren und dadurch Konformationsänderungen auslösen, die zum Öffnen oder Schließen der Pore führen.

Allen Kir-Kanälen gemeinsam ist eine erhöhte Offenwahrscheinlichkeit in Gegenwart des Membranlipids Phosphatidylinositol-4,5-Bisphosphat ($PI(4,5)P_2$). Dabei unterscheiden sich die verschiedenen Mitglieder der Familie in ihrer Affinität und Spezifität für Phosphoinositide. Mutationsstudien legen nahe, dass für die Interaktion vorwiegend elektrostatische Wechselwirkungen zwischen dem negativ geladenen Lipid und konservierten positiven Ladungen in der C-terminalen Domäne der Kir-Kanäle verantwortlich sind.

Wir haben einen biochemischen Versuchsansatz zur Messung der Bindung zwischen gereinigten Kir-Proteinfragmenten und gemischten Liposomen entwickelt, der uns erlaubt, die Lipidbindung von Kir-Kanälen aus verschiedenen Subfamilien direkt miteinander zu vergleichen. Mit Hilfe dieses Versuchsansatzes konnten wir zeigen, dass sowohl die konstitutiv aktiven Kir2.1-Kanäle, wie auch die ATP-regulierten Kir6.2-Kanäle direkt nach dem zweiten Transmembransegment zwei Bindestellen enthalten, die unabhängig voneinander $PI(4,5)P_2$ binden können. Für Kir2.1 fanden wir, dass die Wechselwirkung eine starke elektrostatische Komponente hat, aber auch von der Proteinkonformation beeinflusst wird. Mutationen in der ersten Binderegion der Kir2.1-Proteins reduzieren die Affinität gegenüber $PI(4,5)P_2$, ohne dabei die Membranlokalisation des Proteins in Säugerzellen zu beeinflussen.

Ein konservierter Proteinabschnitt im hinteren C-Terminus von Kir2.1 und Kir6.2 zeigte keine unabhängige Bindung von $PI(4,5)P_2$. Da für Kir6.2 beschrieben wurde, dass diese Region wichtig für die Wirkung von $PI(4,5)P_2$ auf den Kanal ist, muss man

annehmen, dass hier eine indirekte Regulation über eine Interaktion mit anderen Proteinbereichen vorliegt.

Kir2.1 und die pH-regulierten Kir1.1-Kanäle zeigen beide hohe Affinität für $PI(4,5)P_2$ *in vivo*. Für beide Kanäle fanden wir eine zusätzliche $PI(4,5)P_2$-Binderegion im distalen C-Terminus, während die homologe Region in Kir6.2 keine Bindung zeigte. Zur Untersuchung der Bedeutung dieser distalen Region in Kir2.1 führten wir Mutationen ein, die die Bindung reduzierten. Die Auswirkung der Mutationen *in vivo* hing dabei von dem verwendeten Zellsystem ab, was wahrscheinlich auf unterschiedliche Gehalte an $PI(4,5)P_2$ in den Zellmembranen zurückzuführen ist.

Die distalen C-termini der $G_{\beta\gamma}$-aktivierten Kanäle Kir3.1 und Kir3.2 wiesen unterschiedliche Affinitäten gegenüber $PI(4,5)P_2$ auf. Während dieser Proteinbereich, der bei Kir3.2 eine Bindestelle für $G_{\beta\gamma}$ enthält, nicht zur $PI(4,5)P_2$-Bindung befähigt war, konnten wir in Kir3.1 eine $PI(4,5)P_2$-Bindestelle identifizieren, die mit der Bindestelle für $G_{\beta\gamma}$ überlappt. Dabei erhöhte die Bindung von $G_{\beta\gamma}$ im biochemischen Assay die Affinität für $PI(4,5)P_2$.

Sowohl bei Kir2.1, als auch bei Kir6.2 konnten wir zusätzlich zu den C-terminalen $PI(4,5)P_2$-Binderegionen auch eine Bindung an den Aminoterminus nachweisen. Dabei zeigte Kir6.2 deutlich höhere Affinität. Die Bindestelle, die auf die Region vor dem ersten Transmembransegment eingegrenzt wurde, trägt sehr wahrscheinlich zur Regulation von Kir6.2 bei. Hierauf weisen vergleichende elektrophysiologische und biochemische Messungen an Mutanten hin.

Ausgehend von den beschriebenen Befunden können wir postulieren, dass $PI(4,5)P_2$-Bindregionen in der Nähe der Transmembrangänge von Kir-Kanälen vorwiegend der Stabilisierung der offenen Kanalkonformation dienen. Den weniger konservierten Bindestellen in den distalen C-Termini einiger Kir-Kanäle kommt dagegen eher eine modulatorische Funktion, wahrscheinlich im Zusammenspiel mit anderen Liganden, zu. Einige Mutationen, die zu erblichen Krankheitsbildern führen, liegen in Proteinregionen, die von uns als $PI(4,5)P_2$-Bindestellen identifiziert wurden. Möglicherweise liegt der Effekt solcher Mutationen in der Störung der Interaktion mit diesem Lipid, das für die Funktion der Kanäle von zentraler Bedeutung ist.

# STRUCTURAL DETERMINANTS FOR INTERACTION OF INWARDLY RECTIFYING POTASSIUM CHANNEL PROTEINS WITH PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE

# THESIS

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born on 30.07.1970 in Leningrad (St.Petersburg), Russia

**SUMMARY**

Mammalian inwardly rectifying potassium channels (Kir channels) are important in stabilising the resting membrane potential and for $K^+$ transport over the plasma membrane. Mutations in several Kir protein-coding genes lead to hereditary diseases in humans, that underscores the physiological significance of Kir channels. All pore-forming Kir channel complexes share the same structure of 4 subunits each consisting of two transmembrane segments connected by a pore loop. Members of some Kir channel subfamilies are constitutively active, others are specifically regulated by intracellular ligands like protons, ATP or $G_{\beta\gamma}$ subunits of trimeric G-proteins. These regulators are thought to interact with cytoplasmic N- and C-terminal domains of Kir channels, causing conformational changes that lead to opening or closing of the pore.

A common feature of all Kir channels is that their open probability is increased by the membrane phospholipid phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$). Members of different Kir channel subfamilies respond to $PI(4,5)P_2$ with various affinities and specificities. As suggested by mutational studies, $PI(4,5)P_2$ activates Kir channels interacting electrostatically with positively charged aminoacid residues in conserved regions of their C-terminal cytoplasmic domains.

We established a biochemical assay for the interaction of purified fragments of Kir cytoplasmic domains and mixed liposomes enabling us to compare directly Kir channel proteins from different subfamilies. Using this assay, we found that cytoplasmic C-terminal domains of constitutively active Kir2.1 and ATP-regulated Kir6.2 channel proteins contain two consecutive conserved regions proximal to the second transmembrane domain able to independently bind $PI(4,5)P_2$. For the Kir2.1 protein fragments this interaction includes a significant electrostatic component, but is possibly affected by other types of interactions or conformational constraints. Mutations reducing $PI(4,5)P_2$ affinity of the first Kir2.1 fragment inhibit Kir2.1 function without influencing the channel plasma membrane localisation as verified in a mammalian expression system.

Kir2.1 and Kir6.2 protein fragments containing the downstream conserved region of the cytoplasmic C-terminal domain did not bind $PI(4,5)P_2$ independently. As this region has been reported to play a role in the Kir6.2 channel's $PI(4,5)P_2$-response, we suggest that it is involved in $PI(4,5)P_2$ binding via interaction with other cytoplasmic regions.

The Kir2.1 and pH-regulated Kir1.1 channel proteins are both characterised by high $PI(4,5)P_2$ affinity *in vivo*. Both proteins contain $PI(4,5)P_2$ binding regions in their distal C-termini. The homologous region from the Kir6.2 protein does not substantially bind $PI(4,5)P_2$. For studying the role of this distal region in Kir2.1 channel, we introduced

mutations inhibiting its $PI(4,5)P_2$ binding . The influence of mutations on channel function depends on the expression system, possibly due to variations in the content of $PI(4,5)P_2$ in the plasma membrane of cells from various organisms.

The distal C-termini of the $G_{\beta\gamma}$-activated Kir3.1 and Kir3.2 channel proteins differ in their affinity towards $PI(4,5)P_2$. The Kir3.2 distal C-terminus, presumably containing the $G_{\beta\gamma}$ binding region, does not bind $PI(4,5)P_2$. In the distal C-terminus of the Kir3.1 channel protein, the $G_{\beta\gamma}$ interaction region overlaps with the $PI(4,5)P_2$-binding region. Binding to $G_{\beta\gamma}$ subunits in the biochemical assay increased $PI(4,5)P_2$ affinity of this region.

Both the Kir2.1 and Kir6.2 channel N-terminal cytoplasmic domains bind $PI(4,5)P_2$-containing liposomes. The Kir6.2 N-terminal domain has a higher affinity towards $PI(4,5)P_2$ and its $PI(4,5)P_2$-binding part can be narrowed down to the region of higher conservation proximal to the first transmembrane domain. Comparison of biochemical and physiological mutational analysis data suggests that this region might be part of the channel-$PI(4,5)P_2$ interaction site.

Based on the above observations, we suggest that the conserved proximal N- and C-terminal cytoplasmic regions of Kir channels stabilise the channel open conformation by interactions with $PI(4,5)P_2$ or other anionic phospholipids. The distal C-termini of some Kir channels contain less conserved $PI(4,5)P_2$-binding regions possibly with modulatory functions, interacting with other regulatory ligands. Several disease-causing mutations are located in Kir protein regions, identified in our study as $PI(4,5)P_2$-binding domains. These mutations may negatively affect Kir channel interactions with this phospholipid, that are crucial for channel activity and regulation.

**Contents**

# 1. Introduction.

## 1.1. Ion transport across cell membranes and potassium ion channels.

All biological membranes are composed of proteins associated with a lipid bilayer matrix. The ability of a lipid bilayer to partition two aqueous compartments is of fundamental importance for the plasma membranes that surround all cells and for the membranes that define the organelles of eukaryotic cells. Yet the nature of biological processes, which are characterised by steady-state fluxes of energy and material and by the ability to respond to changing external conditions, demands that certain molecules and ions pass through the lipid bilayer. Passage of water-soluble molecules and ions into and out of the cell across the surface membrane, or between different intracellular compartments, is mediated by *membrane transport proteins* known as ion channels, pumps, and transporters.

Pumps and transporters move a wide variety of ions and molecules across a membrane against an electro-chemical gradient, using the energy of ATP hydrolysis or by changing their conformation. Moving rate of molecules via pumps and transporters is about 10-$10^4$ molecules per second (Lodish et al., 1999). *Ion channels* transport specific types of ions down their electro-chemical gradients at a rate that is very high (up to $10^8$ ions per second), compared to pumps and transporters. They are considered to be gated pores whose opening and closing may be intrinsic or regulated by external factors. Ion channels are found in the membranes of all animal, plant and bacterial cells and play important roles in diverse cellular processes. The channel's response to a specific stimulus, called *gating*, is apparently a simple opening or closing of the pore. The open pore has the important property of *selective permeability*, allowing a flow of specific ions. Ion concentration gradients generated by active ion transport through ATP-driven pumps and the selective movement of ions through ion channels create a voltage across the plasma membrane, the *membrane potential* ($E_M$) (Lodish et al., 1999).

The membrane potential of resting excitable cell is usually about –70 mV. The equilibrium potential for $K^+$ ions ($E_K$ = -95mV) is not too far from the actual membrane potential, suggesting that there could be a number of open $K^+$-selective channels in the resting membrane. Indeed, open potassium channels stabilise the membrane potential, drawing it closer to the $K^+$ equilibrium potential and further from the firing threshold. In excitable cells, the roles of all types of $K^+$ channels are related to this stabilisation. For example, the voltage-gated $K^+$ channels underlie the electrical impulse generation in nerve,

muscle and endocrine cells and determine the length of the action potentials. They open upon membrane depolarisation, caused by the flow of $Na^+$ ions inside the cells via open voltage-gated $Na^+$ channels. This causes the increased efflux of $K^+$ from the cytosol that repolarises the plasma membrane to its resting potential. Most of the voltage-gated $K^+$ channel subunits have a characteristic structure of 6 transmembrane domains (Fig.1, left panel). The ion conduction pathway consists of a pore region (H5/P loop) between the S5 and S6 segments, with contributions of the S6 and S4-S5 linker. The H5 region dictates the $K^+$-selectivity. The S4 segment serves as a voltage sensor (Hille, 2001).

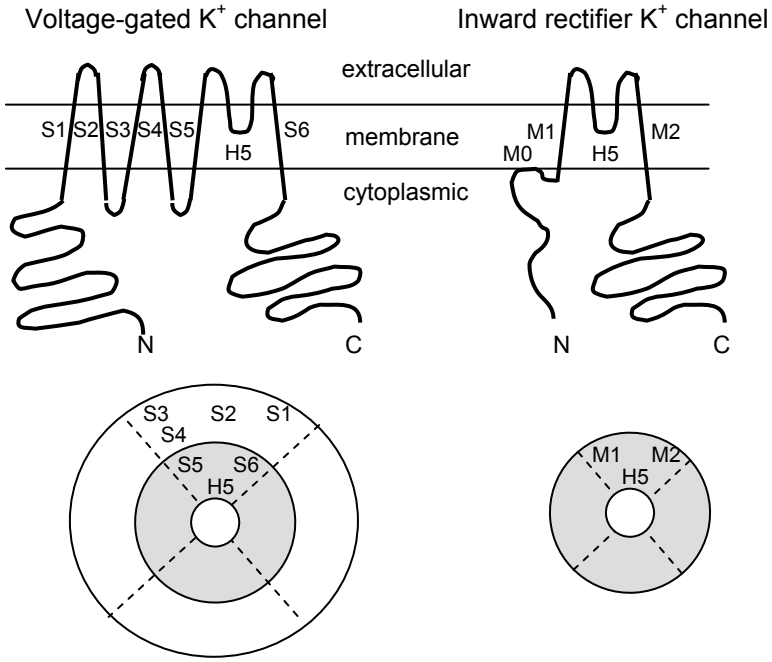


**Fig. 1.** Membrane topology proposed for voltage-gated $K^+$ channels (left) and inwardly rectifying $K^+$ channels (right). Lower diagram shows how four subunits might come together to form a channel. Common to both families is the pore-forming structure comprised of two transmembrane segments and the H5/P loop in between, in each of the four pore-forming subunits (modified from Kubo et al., 1993a).

When the membrane potential returns to a negative value, all voltage-gated $K^+$ and $Na^+$ channels close. The only open channels are these $K^+$ channels that open at membrane potentials near to, or more negative than, the resting potential, and allow $K^+$ ions to flow inwards but not outwards. These $K^+$-conducting channels belong to the family of *inwardly rectifying potassium channels* (Kir channels, Fig.1, right panel). Kir channels have only two transmembrane segments in each of the four $K^+$ channel subunits (M1 and M2 in Fig.1) connected with the pore loop. These subunits are homologous to the S5-H5-S6 pore section of voltage-gated $K^+$ channels (see Doupnik et al., 1995). Thus, Kir channels do not

possess an intrinsic voltage sensor, like the S4 segment in voltage-gated $K^+$ channels. Instead, they contain a presumably membrane-buried region M0 (Ho et al., 1993; Fig.1), with some homology to the S4 domain and containing several conserved charged and hydrophobic residues, which possibly stabilises the structure of the first transmembrane domain.

### 1.2. Inwardly rectifying potassium channels (Kir channels).

#### 1.2.1. Classification and electrophysiological properties.

Kir channels were first discovered in $K^+$-depolarised muscle by Katz in 1949. Their molecular nature was revealed in 1993, when the first two subunits (Kir1.1 and Kir2.1) were cloned (Ho et al., 1993; Kubo et al., 1993a,b). Since then a large number of Kir proteins have been identified. The current nomenclature of mammalian Kir channels includes 7 subfamilies (Table 1). The level of sequence identity among the Kir channel subfamilies is between 50% and 60%. Low areas of identity are found in the distal N- and C-termini as well as in the loop linking the M1 and P-region. The remaining Kir channel "core" shows relatively high levels of identity, especially in the P-region and in the proximal C-terminus (Doupnik et al., 1995).

Some unusual functional properties distinguish Kir channels from other known ion channels (see the typical Kir current curve in Fig.2):

(i) they open with steep voltage dependence on hyperpolarisation;

(ii) the voltage at which they open depends upon the concentration of extracellular $K^+$.

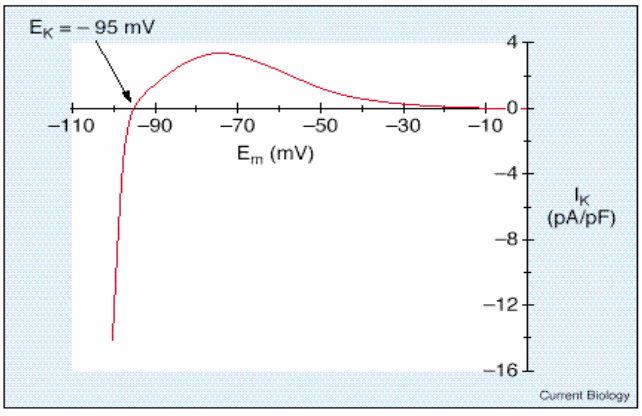


**Fig. 2.** Current-voltage relationship of the cardiac inward rectifier current ($I_{K1}$), as calculated from the Priebe-Beuckelmann model for electrical activity of human ventricular cells. Current density normalised to cell capacitance is plotted against potential at which the membrane is clamped for 200 ms from a holding potential of -70mV (from Jongsma & Wilders, 2001).

| Kir protein subfamily | Major expression site | Native channel multi-merisation | Associ-ated sub-units | Recti-fication | Regu-lation | PI(4,5)$P_2$ affinity | Associated diseases |
|---|---|---|---|---|---|---|---|
| **Kir1.x** | | | | | ATP | | |
| Kir1.1<br>Kir1.2*<br>Kir1.3* | kidney | homomers | SUR, CFTR | weak | pH<br>$CO_2$<br>PKA<br>tyrosine kinases | high | antenatal Bartter's syndrome |
| **Kir2.x** | | homomers, heteromers within subfamily or with Kir5.1 | | strong | | high | |
| Kir2.1 | heart, brain, skeletal muscle | | | | PKA | | Andersen's syndrome |
| Kir2.2 | | | | | | | |
| Kir2.3 | brain<br>kidney | | | | PKA<br>pH | | |
| **Kir3.x** | | | | inter-mediate | Gβγ<br>tyrosine kinases | weak | |
| Kir3.1 | heart, brain | no homomers | | | | | |
| Kir3.2 | brain | homomers, heteromeres with Kir3.1/3.3 | | | | | "weaver" mouse |
| Kir3.3 | brain | no homomers | | | | | |
| Kir3.4 | heart | homomers, heteromeres with Kir3.1/3.3 | | | | | |
| **Kir4.x**<br>Kir4.1 | brain | homomers, heteromers with Kir5.1 | | strong | pH<br>$CO_2$ | | |
| **Kir5.x**<br>Kir5.1 | brain, kidney | no homomers | | | | | |
| **Kir6.x** | | homomers | SUR | weak | ATP<br>PKA | | |
| Kir6.1 | ubiquitous | | | | | | |
| Kir6.2 | brain, heart, skeletal muscle, β-cells | | | | | | familial persistent hyperinsulinaemic hypoglycaemia of infancy |
| **Kir7.x**<br>Kir7.1 | brain, kidney | homomers | | weak | | | |

**Table 1.** Classification of mammalian Kir channel proteins and properties of Kir channels from different subfamilies (see references in the text).
* these isoforms are created by alternative splicing of Kir1.1 mRNA.

In spite of their name, inward rectifiers do carry some outward current. Indeed, that is their usual physiological function, as the membrane potential of the animal cell rarely becomes more negative than $E_K$. In many cases, by conducting outward current in the voltage range a few millivolts positive to $E_K$, they maintain a resting potential near $E_K$, but once other depolarising influences act on the cell, the Kir channels close and $E_M$ is free to change. The range of membrane potentials over which Kir channels stabilise $E_M$ depends on the steepness of their gating. Steep inward rectifiers play a role in membrane potential

stabilisation, while weak inward rectifiers promote $K^+$ transport over the plasma membrane, like for example transepithelial $K^+$ transport in kidney by the Kir1.1 channels (Table 1; Hille 2001).

The "gating" that produces inward rectification is a result of voltage-dependent block by $Mg^{2+}$ ions, plugging the channel from the intracellular side during outward current flow. Steep inward rectification arises from a slower but similar block by multivalent organic cations (spermine or sperimidine) coming from the cytoplasm (see Nichols & Lopatin, 1999).

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 exist as heteromers in brain (Kir3.1/Kir3.2 and probably Kir3.2/Kir3.3) and heart (Kir3.1/Kir3.4). Kir3.1 and Kir3.3 are unable to form functional homomultimers, and are not transported to the plasma membrane, but stay in the cytoplasm. Although homomeric Kir3.x channels only produce very small macroscopic currents in heterologous expression systems, recent studies suggest that homomeric Kir3.4 and Kir3.2 channels might occur *in vivo*. Kir5.1 channels also do not form functional homomers, but can form heteromers with Kir4.1 and probably with Kir2.x subunits (see Reimann & Ashcroft, 1999; Yang et al., 2000 and references therein). Homotypic interactions in Kir channels are determined by the M2 domain, the proximal C-terminus (about first 100 aminoacids after the M2 domain) and possibly other cytoplasmic regions. Heterotypic interactions are also controlled by the M2 and the proximal C-terminus (Tinker et al., 1996; Tinker & Jan, 1999).

The Kir6.x channels coassemble with a regulatory subunit - a sulphonylurea receptor (SUR) to form an octameric (4:4) channel complex. SUR belongs to the ATP-binding cassette (ABC) transporter family, which also includes the cystic fibrosis transmembrane conductance regulator (CFTR) and the multidrug-resistance P-glycoprotein. It endows the channels with sensitivity to sulphonylureas, $K^+$ channel openers and the stimulatory effects of MgADP. Kir1.x might also couple in the same way to CFTR or SUR subunits. In the absence of SUR subunits, Kir6.x tetramers are almost not expressed in the plasma membrane. Partial complexes with fewer than eight subunits do not reach the cell membrane because of the exposure of an ER-retention/retrieval signal that is present both in Kir6.x and SUR subunits (Zerangue et al., 1999). Deleting at least the last 26 amino acids containing this ER retention/retrieval signal (RKR) therefore

permits functional expression of Kir6.2ΔC26 channels in the absence of SUR (reviewed by Reimann & Ashcroft, 1999).

### 1.2.2. Specific intracellular factors modulate different Kir channel subfamilies.

A characteristic of inward rectifiers is that they are subject to modulation by a variety of cytoplasmic agents. Physiologically important Kir channel subfamily-specific intracellular regulation is produced by protons, GTP-binding proteins (G-proteins) and adenine nucleotides (see Table 1 and Fig.3).

**Protons.**

Some Kir channels are inhibited by a decrease in intracellular pH. Intracellular acidification closes Kir1.1 and Kir4.1 channels rapidly and with steep pH dependence. Proton regulation is of particular importance in the kidney, as changes in internal pH influence Kir1.1 channel activity, and thereby transepithelial $K^+$ fluxes. Kir1.1 channels play a key role in $K^+$ recycling in the loop of Henle, a process which is important for the salt uptake from urine (see Ashcroft, 2000).

The pH-dependent gating of Kir1.1 results from formation of an intrasubunit triad by one lysine and one arginine residues in the N-terminus and one arginine residue in the C-terminus of the Kir1.1 protein (Schulte et al., 1999). In addition, a number of histidine residues in the cytoplasmic C-terminus are influencing the pH-gating of Kir1.1 channels (Chanchevalap et al., 2000). Phosphorylation of two C-terminal serine residues by PKA is necessary for the channel's open state stability and also shifts $pK_A$ for pH-gating of Kir1.x channels to more acidic values (Mac Gregor et al., 1998; Leipziger et al., 2000; Ali et al., 2001). The pH-dependent gating involves the movement of protein domains in both the N- and C-termini of Kir1.1 protein that probably trigger movements in the M1 and M2 domains, causing the channel to close (reviewed by Ruppersberg, 2000).

**G-proteins.**

Many hormones mediate their effects on cell excitability by activation of heterotrimeric G-proteins, which in some cases enhances the activity of members of the Kir3.x subfamily to stabilise the membrane at potential close to $E_K$. In brain neurones, this interaction plays a crucial role in regulating neuronal excitability, as it is involved in the generation of low synaptic potentials (see for review Mark & Herlitze, 2000). G-protein

regulation is also important in cardiac muscle as it mediates the slowing of the heart rate in response to acetylcholine (ACh). ACh binds to muscarinic receptors that mediate their effects by activation of G-proteins. The βγ-subunits of heterotrimeric G proteins dissociate from the α-subunit upon G-protien activation and interact directly with the cardiac G-protein gated Kir channels (Kir3.1/Kir3.4 heteromers, or $K_{ACh}$) (see Ashcroft, 2000).

Purified fragments of Kir3.1, 3.2, 3.3 and 3.4 proteins C-and N-termini were shown to directly interact with $G_{\beta\gamma}$ dimers *in vitro* (see for review Reuveny & Jan, 1999). Further studies narrowed down the $G_{\beta\gamma}$-binding region in the Kir3.1 and Kir3.4 N-termini to about 50 aminoacid residues proximal to the M1 domain. Kir3.1 C-terminal fragments interacting with $G_{\beta\gamma}$ or conferring the $G_{\beta\gamma}$ sensitivity to other channels (like Kir2.1) are all overlapping in the region completing the part of the cytoplasmic C-terminus conserved in Kir3.x subfamily proteins. The overlapping region from Kir3.4 is important for the $G_{\beta\gamma}$-mediated agonist-induced activation of Kir3.4 homomeric channels. A number of aminoacid residues in the N- and C-termini have been identified as important for $G_{\beta\gamma}$-mediated current activation and binding of $G_{\beta\gamma}$ subunits *in vitro* (Huang et al., 1995; Kunkel & Peralta, 1995; Slesinger et al., 1995; Huang et al., 1997; He et al., 1999; He et al., 2002; Fig.3).

The Kir3.1 protein posesses a unique distal C-terminal region, that has been tested for a role in channel-$G_{\beta\gamma}$ interaction with controversal results. A fragment of this distal region was shown to bind purified $G_{\beta\gamma}$ subunits *in vitro* and to be responsible for the stronger $G_{\beta\gamma}$ binding to the C-terminal domain of Kir3.1 protein in comparison to the C-termini of Kir3.2-3.4 proteins (Huang et al., 1997), but was found to be $G_{\beta\gamma}$-insensitive in other studies (Kunkel & Peralta, 1995; Slesinger et al., 1995). Also, this region was found to be not necessary for $G_{\beta\gamma}$-activation of the Kir3.1/Kir3.4 channels, but instead to determine the intracellular retention of the Kir3.1 protein (Krapivinsky et al., 1998; Kennedy et al., 1999). A part of it is necessary for the Kir3.1 protein phosphorylation by serine/threonine kinases, that renders the Kir3.1/Kir3.4 channels competent for activation by $G_{\beta\gamma}$ subunits. However, this region is not phosphorylated itself, probably being an inhibitor of the unphosphorylated channel (Medina et al., 2000). This could mean that, if the $G_{\beta\gamma}$ binding to the unique C-terminus of Kir3.1 protein really occurs *in vivo*, it would probably take place at some specific conditions.

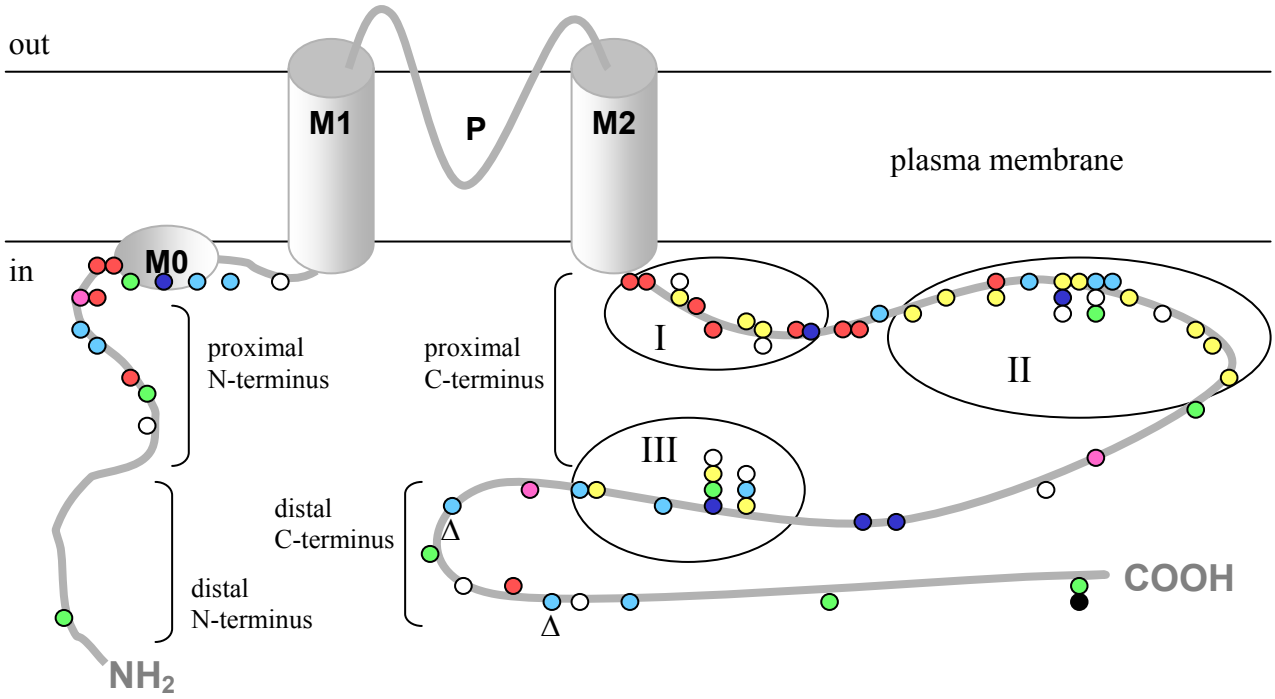


**Fig. 3.** Involvement of the cytoplasmic domains of Kir proteins in Kir channel regulation. M1, M2 - putative transmembrane domains, P- pore region, M0 - putative membrane-buried domain. The coloured circles indicate the sites identified in cytoplasmic domains of Kir proteins responsible for (see references in the text):

- ○ pH
- ● Phosphorylation (Rogalski et al., 2000; Sterling et al., 2002 and references in the text).
- ● PDZ protein binding
- ● ATP-inhibition
- ● $G_{\beta\gamma}$ -activation
- ● $PI(4,5)P_2$ -activation

  **I**, **II**, **III** - regions with concentrated aminoacid residues important for Kir channels' activation by $PI(4,5)P_2$.
- ● Bartter's syndrome

  Δ - frameshifts leading to Kir1.x truncation (N-terminal frameshifts are omitted)
- ● Andersen's syndrome

**Adenine nucleotides.**

Potassium channels which are inhibited by elevation of intracellular ATP are generally known as ATP-sensitive $K^+$ channels ($K_{ATP}$ channels). Since they are sensitive to intracellular ATP levels, they serve to couple cell metabolism to cell excitability. $K_{ATP}$ channels in pancreatic β-cells and cardiac muscle are formed by Kir6.x/SUR proteins, and the epithelial $K_{ATP}$ channels are encoded by members of the Kir1.x subfamily. The ATP-

regulation of Kir6.2/SUR channels in pancreatic β-cells plays a key role in glucose-stimulated insulin secretion. Another action of adenine nucleotides on $K_{ATP}$ channels is upregulation by ADP which is mediated by SUR subunits (see Ashcroft, 2000).

The regulatory ATP target site of Kir6.x/SUR channels has been proposed to locate on the Kir6.x subunit, since channels expressed in the absence of SUR remained dependent on ATP. Indeed, aminoacid residues in the proximal and distal cytoplasmic C-terminal region, and also in the cytoplasmic N-terminus have been shown to be important for ATP-response of the Kir6.2/SUR or Kir6.2CΔ26 channels (Tucker et al., 1998; Drain et al., 1998; Lin et al., 2000; Shyng et al., 2000b). Some of these mutations (especially in the proximal C-terminus) seem to render their effect via increasing the open probability ($P_O$) of the channels or abolishing the inhibition gate closure. Aminoacid residues that do not influence $P_O$ could be candidates for direct ATP binding. Such residues are found in the beginning of the M0 domain, in the proximal C-terminus (region II in Fig.3) and in the distal C-terminus (Tucker et al., 1998; Drain et al., 1998).

### 1.3. Regulation of Kir channels by $PI(4,5)P_2$.

For a long time membrane phospholipids have been viewed as a passive environment for the membrane proteins. However, in the last decade the membrane phospholipids have been found to play an important role in diverse cellular processes and also in regulation of membrane proteins. A group of these biologically active membrane phospholipids is represented by polyphosphoinositides (PIPs) - phosphorylated derivatives of PI (Fig.3). One of the phosphoinositides, $PI(4,5)P_2$, has been found to regulate a number of membrane ion transport proteins, including the members of the Kir channel family (see for review Hilgemann et al., 2001).

#### 1.3.1. $PI(4,5)P_2$: functions and localisation.

$PI(4,5)P_2$ is localised to the cytoplasmic face of cellular membranes. The major pathway for $PI(4,5)P_2$ synthesis is by phosphorylation of PI (synthesised at the ER) by a PI4-kinase to PI(4)P followed by phosphorylation by a PI4P(5)-kinase (Fig.4). These kinases have been found to express in several subcellular compartments, including ER, Golgi, cytoskeleton and nucleus. However, the highest activity of these enzymes is associated with the plasma membrane (see Tolias & Carpenter, 2000).

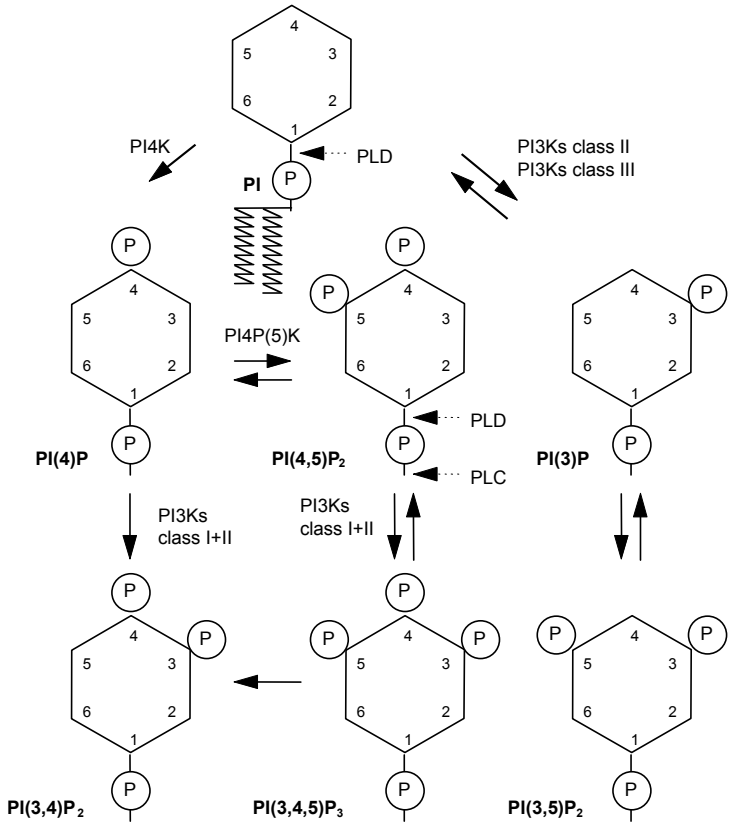


**Fig. 4.** Phosphoinositides. The inositol ring of phosphatidylinositol (PI) can be phosphorylated in the 3-, 4- and 5- positions, giving rise to seven different phosphoinositides (PI(5)P, whose function is unknown, is omitted from the figure). The most important phosphorylation and dephosphorylation pathways, and also the phospholipase cleavage sites are indicated (after Simonsen et al., 2001).

$PI(4,5)P_2$ is a substrate for numerous enzymes, giving rise to important second messengers - $IP_3$, DAG, $PI(3,4,5)P_3$ or PA (Fig.4, 5). In addition, it is itself a powerful signal regulating the activity of numerous enzymes and structural proteins through direct interaction with $PI(4,5)P_2$-binding domains and regions (Fig.5). The ability of $PI(4,5)P_2$ to participate in so many diverse cellular processes suggests that functionally distinct $PI(4,5)P_2$ pools exist in cells (for example, in the nucleus and the cytoskeleton). It is not clear, however, how these pools are spatially and temporally regulated (see Tolias & Carpenter, 2000).

One of the mechanisms by which $PI(4,5)P_2$ can regulate spatially localised membrane events is the localisation of $PI(4,5)P_2$ synthesis to specific membrane domains. Several studies suggest that $PI(4,5)P_2$ is not diffusely distributed in the membrane, but localises to raft-like structures at which the PIPs content can be as much as 50% (see for review Janmey et al., 1999; Martin, 2001; Caroni, 2001). Lipid rafts are mostly detergent-

resistant plasma membrane microdomains with a specific lipid and protein composition (see Galbiati et al., 2001). $PI(4,5)P_2$ clusters and rafts play central roles in recruiting and modulating of key components that regulate the actin cytoskeleton and the endocytic machinery. The high local concentration of $PI(4,5)P_2$ may be significant in relation to finding that several proteins require in the order of 10% mole fraction of $PI(4,5)P_2$ in a PC membrane in order to bind the lipid (Janmey et al., 1999).

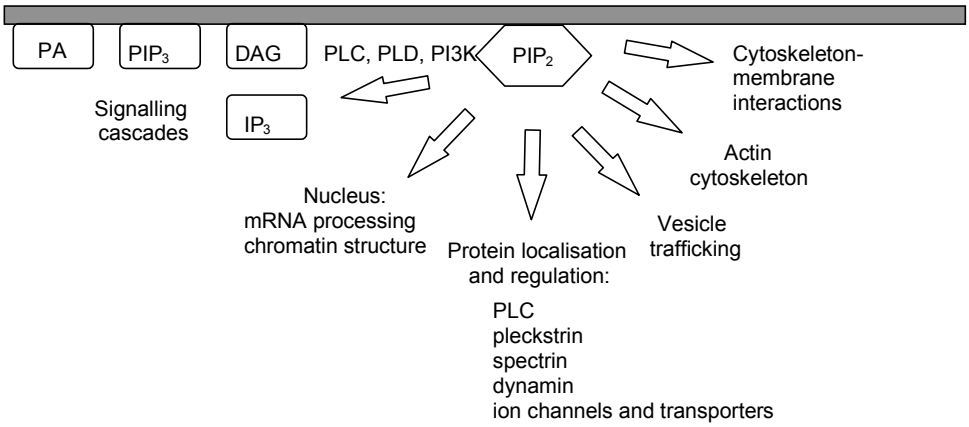


**Fig. 5.** Cellular roles of $PI(4,5)P_2$. $PIP_2$ - $PI(4,5)P_2$; $PIP_3$ - $PI(3,4,5)P_3$ (modified from Nebl et al., 2000; Tolias & Carpenter, 2000)

To date, there is no direct evidence on co-localisation of $PI(4,5)P_2$-regulated ion channels and transporters, including Kir channels, to $PI(4,5)P_2$-containing rafts. However, the fact that in isolated cells the function of Kir channels can be modulated by $PI(4,5)P_2$ depletion via activation of PLC-coupled receptors (see below) suggest that they interact with $PI(4,5)P_2$-pools available for PLC. PLC-stimulation leading to $PI(4,5)P_2$ cleavage also affects other cellular processes, that are thought to be mediated by clustered $PI(4,5)P_2$, like actin cytoskeleton regulation and phagocytosis (see Nebl et al., 2000; Simonsen et al., 2001). Thus, it seems likely that the proteins requiring $PI(4,5)P_2$ for regulation of their functions, like Kir channes, would also be driven to the regions with locally increased $PI(4,5)P_2$ concentration.

### 1.3.2. Kir channels are activated by $PI(4,5)P_2$.

Even before the molecular nature of Kir channels was revealed, they were observed to inactivate gradually when the cellular integrity was disrupted, for example, by membrane patch excision. Mg-ATP has been shown to reactivate the channel function following rundown, by process requiring ATP-hydrolysis. At that time it was suggested

that the reactivation occurs via channel phosphorylation by tightly linked protein kinases (see Ho et al., 1993). However, in 1996 Hilgemann and Ball showed that native $K_{ATP}$ channels in cardiac cells could be activated by $PI(4,5)P_2$ in the absence of Mg-ATP, and the activation by Mg-ATP was reversed by treatment with PI- and $PI(4,5)P_2$-specific PLC's. This study first showed that Kir channel rundown appears due to the gradual loss of $PI(4,5)P_2$ following patch excision (Hilgemann & Ball, 1996).

Since then, an emerging number of studies has appeared on various aspects of Kir channel regulation by $PI(4,5)P_2$ and other membrane phospholipids (see below). $PI(4,5)P_2$ has been found to activate almost all types of Kir channels increasing their open probability. The affinity towards $PI(4,5)P_2$, estimated by the time-course of channel activation by $PI(4,5)P_2$ (Fig.6 A) and inhibition by $PI(4,5)P_2$-antibody (Fig.6 B), is high for Kir1.1 and Kir2.1 channels, and lower for Kir3.1/3.4 and Kir3.2 channels (Huang et al., 1998).

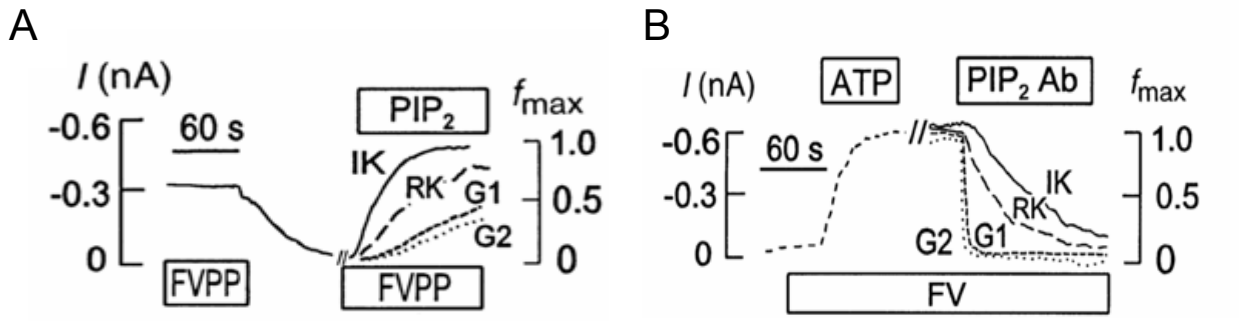


**Fig. 6.** $PI(4,5)P_2$ dependence of cloned inward rectifier $K^+$ channels Kir2.1 (IK), Kir1.1 (RK), Kir3.1/3.4 (G1) and Kir3.2 (G2). **A:** Rundown of inward rectifier channels and recovery by application of $PI(4,5)P_2$. The rundown was prevented by adding FV and PP in cytoplasmic solutions. **B:** Activation of inward rectifier $K^+$ channels by cytoplasmic ATP and inhibition by $PI(4,5)P_2$ antibodies. Channel activities were stable in FV solution after removal of ATP, and were completely inhibited by subsequent application of $PI(4,5)P_2$ antibody. Results are normalised (from Huang et al., 1998).

In several cases, activation by $PI(4,5)P_2$ interferes with the action of other agents specifically modulating Kir channel function. However, it is often difficult to distinguish whether these effects come from general increase of the channel $P_O$ or reflect the overlap of the interaction sites for $PI(4,5)P_2$ and regulatory factors.

$PI(4,5)P_2$ decreases the Kir1.1 channel pH sensitivity, possibly by causing conformational changes in the cytoplasmic domains (Leung et al., 2000). Phosphorylation

of Kir1.1 by PKA (see above) seems to enhance the channel interaction with $PI(4,5)P_2$ (Liou et al., 1999).

$PI(4,5)P_2$ synergises with activation of Kir3.x channels by $G_{\beta\gamma}$ subunits or intracellular $Na^+$ ions (Sui et al., 1998; Huang et al., 1998). Binding site for $PI(4,5)P_2$ on the Kir3.1/Kir3.4 channels might overlap physically with a binding site for arachidonic acid, that specifically inhibits these channels (Kim & Pleumsamran, 2000; Rogalski & Chavkin, 2001).

For Kir6.2/SUR channels, $PI(4,5)P_2$ strongly decreases the apparent affinity of channels for ATP (Baukrowitz et al., 1998; Shyng & Nichols, 1998; Fan & Makielski, 1999). It is still unclear if the competition between $PI(4,5)P_2$ and ATP is only functional or might also be physical, involving overlapping binding sites (see Shyng et al., 2000b; MacGregor et al., 2002). Mutational studies of the Kir6.2 protein have revealed that positively charged aminoacid residues important for the ATP- or $PI(4,5)P_2$-response of Kir6.2/SUR1 channels locate in the same regions of the Kir6.2 C-terminus (regions I-II in Fig.3), and some of them are probably involved in both responses without significantly affecting $P_O$ (Shyng et al., 2000b).

$PI(4,5)P_2$ also augments $P_O$ of heteromeric pH-sensitive Kir4.1/Kir5.1 channels, that are expressed in brainstem and presumably play an important role in the response to hypercapnia. The effect of $PI(4,5)P_2$ is exerted via the Kir5.1 subunit, as the homomeric Kir4.1 channels are not affected by $PI(4,5)P_2$ (Yang et al., 2000).

### 1.3.3. Specificities of Kir channels towards $PI(4,5)P_2$ and other phospholipids.

For some Kir channels, the activation by $PI(4,5)P_2$ has been compared with activation by other phospholipids (Table 2). Kir2.1 seems to have the highest specificity towards $PI(4,5)P_2$, as PI(4)P, $PI(3,5)P_2$ and $PI(3,4,5)P_3$ need to be at concentrations at least 10 times higher than $PI(4,5)P_2$ in order to activate the channel, and $PI(3,4)P_2$ can not activate it at all (see Table 2). It means that the Kir2.1 channel's activation by phosphoinositides requires the presence of phosphates both in the 4- and 5- positions on the inostiol ring, and is disturbed by phosphate in the 3-position. In contrast, Kir1.1 channels that also have a high affinity towards $PI(4,5)P_2$, are much less selective, as they can be activated by PI(4)P, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ with a potency comparable to $PI(4,5)P_2$.

| | DOG | PC | PE | PS | PI | PI(4)P | $PI(3,4)P_2$ | $PI(3,5)P_2$ | $PI(3,4,5)P_3$ |
|---|---|---|---|---|---|---|---|---|---|
| Kir1.1 | n.d | n.t. | n.t. | n.t. | n.t. | (1x) 20% | (1x) 45% | n.t. | (1x) 100% |
| Kir2.1 | n.t. | n.t. | n.t. | n.t. | - | (10x) 10% | - | (10x) 10% | (10x) 80% |
| Kir3.1/3.2 | n.t. | n.t. | n.t. | n.t. | n.t. | n.t. | (1x) 80% | (1x) 80% | n.t. |
| Kir3.1/3.4 | n.t. | n.t. | n.t. | n.t. | - | (10x) 20% | (1x) 70% | (1x) 70% | (1x) 120% |
| Kir6.2/SUR | - | - | - | (40x) 45% | (20x) 20% | (4x) 70% | n.t. | n.t. | n.t. |

**Table 2.** Activation of cloned mammalian Kir channels expressed in *Xenopus* oocytes by various phospholipids (Fan & Makielski, 1997; Rohács et al., 1999; Ho & Murrell-Lagnado, 1999a; Zeng et al., 2002). The activation of channels by phospholipids is expressed as percent of activation by $PI(4,5)P_2$. The values in parentheses indicate the amount of phospholipid relative to $PI(4,5)P_2$ necessary for the given percent of activation. "-" - no activation detected; "n.t." - not tested

Heterologously expressed Kir3.1/Kir3.2 and Kir3.1/Kir3.4 channels, that have lower $PI(4,5)P_2$-affinity compared to Kir1.1 and Kir2.1, are also activated by PIPs with phosphate in the 3-position of the inositol ring with a similar potency as $PI(4,5)P_2$. $G_{\beta\gamma}$-regulated Kir channels in native tissues are even less selective towards phospholipids, as the activated $K_{ACh}$ channels from rat atrial cells can be stimulated even by neutral PC in the same way as by PI (Kim & Bang, 1999). This possibly reflects the differences in the membrane phospholipid composition of frog oocytes and mammalian cells that would influence Kir channel modulation depending on what cells they are expressed in (Nasuhoglu et al., 2002).

Kir6.2/SUR channels have a lower affinity towards $PI(4,5)P_2$ than Kir2.1 and Kir1.1 channels (T. Baukrowitz, Jena, personal communication), and also seem to be not selective towards anionic phospholipids. They can be activated by PI, PI(4)P and non-inositol anionic phospholipid PS at high concentrations (Table 2). PC and PI do not affect the ATP-inhibition of Kir6.2/SUR1 channels. PI(4)P is able to recover the channels from ATP-inhibition, only with a lower potency than $PI(4,5)P_2$, whereas $PI(3,4,5)P_3$ has a potency similar to $PI(4,5)P_2$ in competing with ATP-inhibition (Shyng & Nichols, 1998; Baukrowitz et al., 1998). Moreover, an artificial anionic lipid DOGS-NTA can substitute for phosphoinositides in reducing the inhibition of Kir6.2/SUR2A channels by ATP or glibenclamide (Krauter et al., 2001).

### 1.3.4. How do Kir channels interact with anionic phospholipids?

Regions for interactions with membrane phospholipids have evolved in numerous proteins involved in cellular signalling, membrane transport or cytoskeleton organisation. Interactions with anionic phospholipids, like phosphoinositides, are mainly a result of electrostatic attraction between the positively charged face of the protein and the negatively charged membrane. In some cases the positively charged clusters are combined with hydrophobic structures enhancing incorporation into the lipid bilayer (see for review Janmey et al., 1999; Lemmon & Ferguson, 2000; Niggli, 2001). There are a very few examples of protein domains with high-affinity stereospecific phosphoinositide binding. These are the PH (pleckstrin homology) domains of PLCδ1 (specific for $PI(4,5)P_2$) and ARF exchange factor GRP1 (specific for $PI(3,4,5)P_3$). Interestingly, even in these cases the specificity appears to be significantly lower when the binding is assayed with phosphoinositides inserted into lipid bilayers, and not with the phosphoinositide headgroups, that shows an increase of electrostatic effects in the bilayer (Lemmon & Ferguson, 2000).

The aminoacid side chains on the protein surface that contact the lipids can be revealed by NMR, photoaffinity labelling or crystal structure studies of protein-phosphoinositide complexes. To date, no such information exists for the cytoplasmic regions of Kir channel proteins or other $PI(4,5)P_2$-sensitive ion channel and transporter subunits. They have been suggested to interact with anionic phospholipids via stretches of cationic residues (Fan & Makielski, 1997) or a PH-domain like structure (Cukras et al., 2002) in their cytoplasmic C-termini. The electrostatic nature of Kir channel's interaction with $PI(4,5)P_2$ is supported by the following facts: (i) Several Kir channels have been shown to be activated in excised patches not only by $PI(4,5)P_2$, but also by other anionic phospholipids (see Table 2). Anionic phospholipids were also able to compete *in vitro* with binding of the fluorescent ATP analogue, TNP-ATP, to the C-termini of Kir1.1, Kir6.1 and Kir6.2 proteins (MacGregor et al., 2002). (ii) The polyvalent cations like polylysine or neomycin are able to antagonise $PI(4,5)P_2$ activation of Kir channels (Hilgemann & Ball, 1996; Fan & Makielski, 1997; Huang et al., 1998) and also reverse the competition of $PI(4,5)P_2$ on TNP-ATP-binding *in vitro* (McGregor et al., 2002). (iii) $PI(4,5)P_2$-antagonism with ATP-inhibition of Kir6.2/SUR channels depends on the ionic strength of the solutions used in patch excision experiments (Krauter et al., 2001). (iv) The response of Kir

channels to $PI(4,5)P_2$ is reduced by mutations exchanging certain positively charged residues for neutral or negatively charged ones (see below).

The charge effects are required but not sufficient to permit interaction of phospholipids with Kir channels, as they are not activated by the hydrolysis products of $PI(4,5)P_2$ – $IP_3$ and DOG (Fan & Makielski, 1997; Shyng & Nichols, 1998), and these ligands are also unable to compete for the binding of Kir protein's C-termini with TNP-ATP *in vitro* (McGregor et al., 2002). Importance of interactions with the lipid tail of the phosphoinositide molecule has also been shown for some unrelated proteins, like cytoskeleton-binding protein profilin, that is able to bind only $PI(4,5)P_2$ and not its soluble headgroup $IP_3$ (Niggli, 2001). Activation of Kir2.1 and Kir3.1/Kir3.4 channels by $PI(4,5)P_2$ in excised patches depends on the length of the lipid side-chain in a way that $PI(4,5)P_2$ with longer acyl side-chains are more effective in Kir channel activation (Rohács et al., 1999). It means that Kir channels can be activated only by $PI(4,5)P_2$ effectively incorporated into the plasma membrane.

Cationic sites, that are not selective towards anionic phospholipids, are suggested to bind preferentially $PI(4,5)P_2$ in the plasma membrane of the native cells. On the average, $PI(4,5)P_2$ is a trivalent anion at pH7.0, and therefore it will bind to polyvalent cationic sites about 100 times more potently than monovalent phospholipids. $PI(4,5)P_2$ consitiutes about 1% of anionic phospholipids in most cells, whereas the abundance of $PI(3,4,5)P_3$ probably never exceeds a few percent of the level of $PI(4,5)P_2$. Thus, modest evolutionary improvements of nonspecific cationic binding sites might have achieved fairly specific binding sites for $PI(4,5)P_2$ in a physiological phospholipid milieu (see Hilgemann et al., 2001).

### 1.3.5. $PI(4,5)P_2$-dependent regulation of Kir channel activity *in vivo*.

Dependence of Kir channels on $PI(4,5)P_2$ *in vivo* has been proved by showing an effect of PLC-linked receptor activation on native and heterologously expressed Kir channels via depleting the membrane $PI(4,5)P_2$. Native $K_{ATP}$ channels and cloned Kir6.2/SUR1 channels were inhibited by activation of PLC-linked metabotropic purino-receptor $P_{2Y}$ or $M_1$ receptor (Baukrowitz et al., 1998; Xie et al., 1999; Oketani et al., 2002). Accordingly, coexpression of Kir6.2/SUR1 channels with PIP5K (PI-4-phosphate 5-kinase) decreased the ATP-sensitivity of the channels, presumably by increasing the amount of $PI(4,5)P_2$ in the plasma membrane (Shyng et al., 2000a). Heterologously

expressed Kir3.1/Kir3.4 heteromeric channels and native $K_{ACh}$ channels were inhibited by the stimulation of PLC-linked $M_1$ and $M_3$ receptors in a manner dependent on the channel's $PI(4,5)P_2$-affinity (Cho et al., 2001; Kobrinsky et al., 2000). Kir2.1 channels were used as biosensors registering PLC-mediated changes in $PI(4,5)P_2$ levels in *Drosophila* eye rhabdomeres during phototransduction (Hardie et al., 2001).

Several pathogenic factors modulate the phospoinositide composition in the plasma membrane and thus affect function of Kir channels or other phosphoinositide-dependent transmembrane proteins in experimental systems. These are the amyloid β protein, a neurotoxic factor in Alzheimer's disease (Yagyu et al., 2001), cell stress caused by photoirradiation (Fan & Neff, 2000), and L-palmitoylcarnitine (L-PC), a fatty acid metabolite that accumulates in the heart sarcolemma during ischaemia (Haruna et al., 2000). Oxidative stress - an important factor in ischaemic preconditioning - depresses the synthesis of $PI(4,5)P_2$ in heart sarcolemma (Mesaeli et al., 2000). These findings suggest that some diseases or environmental conditions could affect the activity of Kir family members by a decrease of the plasma membrane phosphoinositide (especially $PI(4,5)P_2$) content.

Some pharmacological substances affect Kir channels in a manner dependent on the channel's $PI(4,5)P_2$-affinity. In fact, heterologously expressed $G_{\beta\gamma}$-activated Kir channels, but not highly $PI(4,5)P_2$-sensitive Kir1.1 or Kir2.1 channels, were reported to be inhibited by membrane-permeable anaesthetics bupivacaine and lidocaine. The Kir3.1/Kir3.4 channel's inhibition rate by bupivacaine was decreased by introducing mutations increasing their $PI(4,5)P_2$-affinity. In this study it was assumed that anaesthetics act directly on the channel, regulating its binding to $PI(4,5)P_2$ (Zhou et al., 2001). However, these agents could exert their effect by decreasing $PI(4,5)P_2$ levels in the plasma membrane. This seems likely as lidocaine and chlorpromazine, also a membrane-permeable local anaesthetic, have been reported to increase the PLC activity with subsequent lowering of $PI(4,5)P_2$ levels at the cytoplasmic surface of the plasma membrane (Raucher & Sheetz, 2001). Several other commonly used drugs can modify the phosphoinositide levels in the plasma membrane of living cells (see for example Bucki et al., 2000; O'Donnell et al., 2000; Szewczyk & Pikula, 2000; Tharmapathy et al., 2000), and, therefore, as a consequence of their action, up- or downregulation of phosphoinositide-dependent membrane proteins, like Kir channels, is expected to occur.

### 1.3.6. Identification of Kir channel regions interacting with $PI(4,5)P_2$ by mutational analysis.

Mutational analysis of some Kir channel proteins has highlighted several aminoacid residues, primarily positively charged ones, important for Kir channel $PI(4,5)P_2$-response in excised membrane patches (Table 3). Most of the aminoacid residues important for the $PI(4,5)P_2$ response of one or several Kir channels are highly conserved in the Kir protein family. In some cases, mutations of the homologous residues are affecting $PI(4,5)P_2$ affinity of one channel, but do not affect the $PI(4,5)P_2$ affinity of another channel. These inconsistencies may arise from different methods used for detection of channel affinity towards $PI(4,5)P_2$ (application of $PI(4,5)P_2$ or $PI(4,5)P_2$-antibody to membrane patches). Mutations often influence the amount of current conducted by the channel: most of them completely inhibit or significantly reduce the Kir current (Table 3). The currently reported mutations affecting the Kir-channel $PI(4,5)P_2$ response are localised in the proximal C-terminus of Kir channel proteins and can be grouped in three regions (Fig.3, Table 3):

(i) Region I. The cluster of conserved positively charged aminoacid residues following the second transmembrane domain. This region has been suggested to play a predominant role in Kir channel-phosphoinositide interaction (Fan & Makielski, 1997). One conserved arginine residue within this region has been shown to be important for the Kir1.1, Kir6.2/SUR and Kir4.1/Kir5.1 channel response to $PI(4,5)P_2$.

(ii) Region II. Downstream region with concentrated conserved positively charged residues. This region contains another conserved arginine residue, that is important for $PI(4,5)P_2$ activation of three Kir channels (Kir1.1, Kir6.2/SUR and Kir2.1). One hydrophobic residue in this region has been identified as responsible for lower affinity of $G_{\beta\gamma}$-activated Kir channels towards $PI(4,5)P_2$, compared to the Kir2.1 channels (Zhang et al., 1999).

(iii) Region III. Region in the end of conserved proximal C-terminal part of Kir proteins. It contains two highly conserved arginine residues that are important for $PI(4,5)P_2$ response of the Kir6.2/SUR channels (Table 3; Shyng et al., 2000b).

| | Kir1.1 | Kir2.1 | Kir3.4 | Kir6.2 | Kir5.1 |
|---|---|---|---|---|---|
| Proximal N-terminus | *C49Y* | | | | |
| | *I51T* | | | | |
| M0 | | *D71V* | | | |
| | *V72E* | | | | |
| | *D74Y* | | | | |
| Proximal C-terminus I | **K181Q** !! | | | | |
| | R184Q | | | | |
| | K186Q | | | | |
| | K187Q | | | **R176A** !! | |
| | **R188Q** !! | | | **R177A** !!! | **R178Q** |
| | | *T192A* | | | |
| II | K190A | | | | |
| | K196A | | | K185Q | |
| | *A198T* | | | | |
| | K202A | | | | |
| | R203A | | | **R192A*** ! | |
| | K206A | K207Q | | **R195A*** !! | |
| | R212A | R213Q | | **R201A*** ! | |
| | *A214V* | | | | |
| | **R217Q** !! | **R218Q**!!! *R218W/Q* | | **R206A** !!! | |
| | **K218Q** !! | | | K207A | |
| | **S219A** *S219R* | | | | |
| | *L220F* | | | | |
| | | **L222I** !! | **I229L** | | |
| | | **R228Q** !! | | | |
| | | Q230K | **K237Q** | | |
| | | K233Q | | R221A | |
| | | | | **K222A** !!! | |
| | | R235Q | | | |
| | | *G300V* | | | |
| | | *E303K* | | | |
| III | *R311W/Q* | | | **R301A** !!! | |
| | **S313A** | *del. S314 Y315* | | | |
| | | | | | |
| | *V315G* | | | | |
| | | | | **R314A** ! | |
| | *F325C* | | | | |
| Distal C-terminus | *f.s. T332 K333.* | | | | |
| | | | | R325A | |
| | *f.s. P353* | | | | |
| | *M357T* | | | | |
| | | | | R365A | |
| | | | | R369A | |
| | | | | K377A | |
| | | | | K381A | |

**Table 3.** Kir mutations studied for their effect on channel activation by $PI(4,5)P_2$ (Baukrowitz et al., 1998; Shyng & Nichols, 1998; Huang et al., 1998; Liou et al., 1999; Zhang et al., 1999; Shyng et al., 2000b; Yang et al., 2000; Zeng et al., 2002) and known disease-causing mutations in the cytoplasmic domains of Kir proteins (designated in italics, see references in the text). Mutations that influence channel-$PI(4,5)P_2$ interactions are designated in bold. These mutations inhibit the channels' activation by $PI(4,5)P_2$, except mutations in Kir3.4, that have an opposite effect. "del." – deletion; "f.s." - frameshift. Aminoacid residues conserved in Kir protein family are highlighted in grey.

* - these mutations cause a modest decrease in channel-$PI(4,5)P_2$ response.

Some mutations are reported to cause: ! - nonsignificant current reduction; !! - significant current reduction; !!! - complete current inhibition

The practical significance of identifying the interaction sites for $PI(4,5)P_2$ and probably other phosphoinositides in Kir proteins arises from the physiological importance of Kir channels and their regulation. Mutations in genes encoding for Kir channel porteins produce three inherited diseases in man. Several mutations in Kir1.1-coding gene cause Bartter's syndrome type III - a renal tubular disorder characterised by salt-wasting, hypokalaemia (low plasma $K^+$), metabolic acidosis and marked loss of $Ca^{2+}$ in the urine (Simon et al., 1996; Jeck et al., 2001). Kir2.1-coding gene has been linked to Andersen's syndrome - a complex disorder characterised by periodic paralysis, cardiac arrhythmias and dysmorphic features. This disorder is the first to demonstrate a link between muscle and cardiac electrical phenotypes and developmental phenotypes (Plaster et al., 2001). Familial persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI) is a rare disorder of glucose homeostasis, leading to unregulated insulin secretion despite severe hypoglycaemia. Mutations associated with this disease have been identified both in Kir6.2 and SUR coding genes (Ashcroft, 2000).

Disease-causing mutations may occur throughout all regions of Kir protein-coding genes. These mutations often lead to premature termination of the Kir protein synthesis or to disruption of the transmembrane or pore domains' structure. In terms of channel regulation by intracellular factors, the mutations within the cytoplasmic domains are of special interest. Such mutations have been identified in genes coding for Kir1.x and Kir2.1 proteins. Some of these mutations are located close to the aminoacid residues important for $PI(4,5)P_2$-regulation of these channels (see Fig.3, Table 3), as for example in the proximal C-terminal regions I, II and III. In some cases the same (or homologous) residues are implicated in the $PI(4,5)P_2$-response and are affected by disease-causing mutations (Ashcroft, 2000; Jeck et al., 2001; Plaster et al., 2001; Ai et al., 2002).

Considering these parallels, the studies of Kir protein regions able to interact directly with $PI(4,5)P_2$ or other anionic phospholipids can improve our understanding of the reasons for functional impairment caused by some disease-causing mutations in Kir channel-coding genes. These data could be useful in therapy of these diseases, especially considering that gene therapy using Kir channel genes is probably a question of nearest future, as starting experiments on Kir2.1 channel expression in guinea pig hearts to overcome ventricular arrhythmia have already been performed recently (Ennis et al., 2002). This information can also help to predict possible consequences of several pathological conditions or side-effects of various commonly used drugs that modulate

phosphoinositide levels in the plasma membrane of the living cells (see above) and, therefore, can influence Kir channel function.

### 1.4. Objectives.

With a lot of electrophysiological evidence on Kir channel activation by phosphoinositides, and especially $PI(4,5)P_2$, there are still not enough biochemical data on Kir protein interactions with these lipids. In 1999, when this study was started, the only comparative *in vitro* biochemical studies reported were those by Huang and colleagues (Huang et al., 1998), where it was shown that the C-terminal cytoplasmic domains of Kir1.1, Kir2.1 and Kir3.1 proteins fused to glutathione-S-transferase (GST) bind lipid micellas formed from $PI(4,5)P_2$. In this study it was shown that the Kir1.1 and Kir2.1 C-terminal domains bind $PI(4,5)P_2$ with almost the same affinity, but the relevant domain from Kir3.1 protein has a significantly lower $PI(4,5)P_2$ affinity than the first two proteins. The mutation of conserved arginine residue in the Kir1.1 protein (R188Q) reduced the *in vitro* $PI(4,5)P_2$-affinity (Huang et al., 1998). It has also been shown that the Kir2.1 fragment approximately corresponding to the region II in Fig.3 is able to confer a higher $PI(4,5)P_2$ affinity to the Kir3.4/Kir2.1 chimeric channels, and several residues within this region are important for $PI(4,5)P_2$ response of Kir2.1 and Kir3.4 channels (see Table 3; Zhang et al., 1999). These data, however, left several questions open:

(i) do the cytoplasmic C-terminal regions of the Kir channel proteins contain one or several regions for $PI(4,5)P_2$ binding?

(ii) do the regions identified by mutational analysis as important for $PI(4,5)P_2$ regulation serve as modulators of one conserved $PI(4,5)P_2$-binding region or can directly bind $PI(4,5)P_2$?

(iii) why do some channels only require $PI(4,5)P_2$, whereas other channels need additional factors to stabilise the interaction of $PI(4,5)P_2$ with the channel (like $G_{\beta\gamma}$ subunits)?

To answer these questions we set up a standardised biochemical assay for studying Kir-$PI(4,5)P_2$ interaction that enabled us to compare directly Kir channel proteins from various subfamilies. If the protein-lipid interaction depends on the phospholipid insertion into the membrane, as it is in the case of Kir channel proteins (see above), a convenient semi-quantative biochemical method is to assay the isolated proteins or protein fragments with liposomes composed of the phospholipids of interest. Liposomes are vesicles in which

an aqueous volume is entirely enclosed by a membrane composed of lipid molecules. They may be composed of multiple concentric membrane lamellae or bound by a single bilayer membrane. *Small unilamellar vesicles* (SUVs) are defined as liposomes at the lowest limit of size possible for phospholipid vesicles (15-30 nm), and thus forming a relatively homogeneous population in terms of size (New, 1989). These features make SUVs suitable for detecting the lipid-protein binding by pulling down of complexes formed between labelled liposomes and proteins, as it has been done in our study. Using the liposome binding assay we conducted:

i) A systematic mapping of $PI(4,5)P_2$ interaction sites in the Kir2.1 protein by screening its cytoplasmic domains for regions interacting with $PI(4,5)P_2$;

ii) Comparison of the Kir2.1 protein cytoplasmic regions with homologous regions from other Kir proteins in order to find common and variable structural elements involved in $PI(4,5)P_2$-binding;

iii) Comparison of biochemical $PI(4,5)P_2$ binding with physiological data.

## 2. Materials and methods.

### 2.1. Materials

#### 2.1.1. Bacterial strains and cell lines

*E.coli* strains

XL1 Blue - for cloning recombinant DNA (Stratagene)

BL21, BL21 Codon Plus - for heterologous expression of recombinant GST-fusion proteins (Stratagene)

Cell lines

HEK293 - human, transformed, embryonic kindney fibroblasts (German Collection of Microorganisms and Cell Culture, DSMZ, Braunschweig, Germany)

CHO-K1 - chinese hamster ovary cells (German Collection of Microorganisms and Cell Culture, DSMZ, Braunschweig, Germany)

#### 2.1.2. Plasmids and vectors.

Commercially available vectors

pGEX-5x-2 - GST-gene fusion vector for expression of GST-fusion proteins in *E.coli* (Pharmacia Biotech)

pcDNA3 - for transient CMV-promoter-driven expression in mammalian cells (Invitrogen).

pEGFPC1 - EGFP-gene fusion vector for expression of proteins fused to the C-terminus of EGFP in mammalian cells (Clontech)

Plasmids used for generation of Kir protein fragments

| **Cloned cDNA** | **Plasmid** | **Source** |
|---|---|---|
| rat Kir1.1 | pSp64-Kir1.1 | provided by Dr. U.Brändle (Tübingen, Germany) |
| mouse Kir2.1 | pBSSK-Kir2.1 | provided by Prof. Y.Kubo (Tokyo, Japan) |
| mouse Kir3.1 | pBSSK-Kir3.1 | provided by Prof. Y.Kubo (Tokyo, Japan) |
| mouse Kir3.2 | pBSSK-Kir3.2 | provided by Prof. R.Murrell-Lagnado (Cambridge, UK) |
| mouse Kir6.2 | Sp6-Kir6.2 | provided by Prof. T.Baukrowitz (Jena, Germany) |
| | Sp6-Kir6.2 R50E | provided by Prof. T.Baukrowitz (Jena, Germany) |

Plasmids used for *in vitro* synthesis of Kir2.1 mRNA

| **Plasmid** | **Source** |
|---|---|
| pBSKS Kir2.1 | provided by Prof. Y.Kubo (Tokyo, Japan) |
| pBSKS Kir2.1 P186A | provided by Prof. Y.Kubo (Tokyo, Japan) |
| pBSKS Kir2.1 K188Q | provided by Prof. Y.Kubo (Tokyo, Japan) |
| pBSKS Kir2.1 R189Q | provided by Prof. Y.Kubo (Tokyo, Japan) |
| pBSKS Kir2.1 K188Q R189Q | this study |
| pBSKS Kir2.1 R343Q K346Q | this study |

Plasmids for expression and purification of GST-fused Kir protein fragments in *E.coli*

| **Plasmids containing Kir cDNA fragments *** | **Corresponding GST-fusion protein** | **Source** |
|---|---|---|
| **mouse Kir2.1** | | |
| pGEX-5X-2-Kir2.1 (175-428) | 2.1 C1-8 | produced by Dr. R.Schönherr (Jena) |
| pGEX-5X-2-Kir2.1 (175-428) K188Q | 2.1 C1-8 K188Q | this study |
| pGEX-5X-2-Kir2.1 (175-291) | 2.1 C1-4 | produced by Dr. R. Schönherr (Jena) |
| pGEX-5X-2-Kir2.1 (192-428) | 2.1 C2-8 | this study |
| pGEX-5X-2-Kir2.1 (292-428) | 2.1 C5-8 | this study |
| pGEX-5X-2-Kir2.1 (207-428) | 2.1 C3-8 | — " — |
| pGEX-5X-2-Kir2.1 (207-291) | 2.1 C3-4 | — " — |
| pGEX-5X-2-Kir2.1 (247-428) | 2.1 C4-8 | — " — |
| pGEX-5X-2-Kir2.1 (175-246) | 2.1 C1-3 | — " — |
| pGEX-5X-2-Kir2.1 (192-246) | 2.1 C2-3 | — " — |
| pGEX-5X-2-Kir2.1 (175-206) | 2.1 C1-2 | — " — |
| pGEX-5X-2-Kir2.1 (175-206) P186A | 2.1 C1-2 P186A | — " — |
| pGEX-5X-2-Kir2.1 (175-206) K188Q | 2.1 C1-2 K188Q | — " — |
| pGEX-5X-2-Kir2.1 (175-206) R189Q | 2.1 C1-2 R189Q | — " — |
| pGEX-5X-2-Kir2.1 (175-206) K188Q·R189Q | 2.1 C1-2 K188Q·R189Q | — " — |
| pGEX-5X-2-Kir2.1 (207-246) | 2.1 C3 | — " — |
| pGEX-5X-2-Kir2.1 (247-291) | 2.1 C4 | — " — |
| pGEX-5X-2-Kir2.1 (292-323) | 2.1 C5-6 | — " — |
| pGEX-5X-2-Kir2.1 (292-365) | 2.1 C5-7 | — " — |
| pGEX-5X-2-Kir2.1 (309-428) | 2.1 C6-8 | — " — |
| pGEX-5X-2-Kir2.1 (324-428) | 2.1 C7-8 | — " — |
| pGEX-5X-2-Kir2.1 (309-365) | 2.1 C6-7 | — " — |
| pGEX-5X-2-Kir2.1 (324-365) | 2.1 C7 | — " — |
| pGEX-5X-2-Kir2.1 (324-365) R343Q·K346Q | 2.1 C7 R343Q·K346Q | — " — |
| pGEX-5X-2-Kir2.1 (366-428) | 2.1 C8 | — " — |
| pGEX-5X-2-Kir2.1 (1-86) | 2.1 N1-5 | — " — |
| pGEX-5X-2-Kir2.1 (1-72) | 2.1 N1-4 | — " — |

| | | |
|---|---|---|
| **mouse Kir6.2** | | |
| pGEX-5X-2-Kir6.2 (163-390) | 6.2 C1-8 | this study |
| pGEX-5X-2-Kir6.2 (163-193) | 6.2 C1-2 | — " — |
| pGEX-5X-2-Kir6.2 (194-234) | 6.2 C3 | — " — |
| pGEX-5X-2-Kir6.2 (235-292) | 6.2 C4 | — " — |
| pGEX-5X-2-Kir6.2 (293-320) | 6.2 C5-6* | — " — |
| pGEX-5X-2-Kir6.2 (321-390) | 6.2 C7-8* | — " — |
| pGEX-5X-2-Kir6.2 (313-353) | 6.2 C7 | — " — |
| pGEX-5X-2-Kir6.2 (354-390) | 6.2 C8 | — " — |
| pGEX-5X-2-Kir6.2 (1-74) | 6.2 N1-5 | — " — |
| pGEX-5X-2-Kir6.2 (1-59) | 6.2 N1-4 | — " — |
| pGEX-5X-2-Kir6.2 (1-43) | 6.2 N1-3 | — " — |
| pGEX-5X-2-Kir6.2 (1-43) R27H·R31Q | 6.2 N1-3 R27H·R31Q | — " — |
| pGEX-5X-2-Kir6.2 (1-23) | 6.2 N1 | — " — |
| pGEX-5X-2-Kir6.2 (24-43) | 6.2 N2-3 | — " — |
| pGEX-5X-2-Kir6.2 (34-74) | 6.2 N3-5 | — " — |
| pGEX-5X-2-Kir6.2 (34-59) | 6.2 N3-4 | — " — |
| pGEX-5X-2-Kir6.2 (34-59) R50E | 6.2 N3-4 R50E | — " — |
| pGEX-5X-2-Kir6.2 (34-59) R54Q | 6.2 N3-4 R54Q | — " — |
| pGEX-5X-2-Kir6.2 (34-59) R54E | 6.2 N3-4 R54E | — " — |
| pGEX-5X-2-Kir6.2 (44-74) | 6.2 N4-5 (low solubility) | — " — |
| | | |
| **mouse Kir3.1** | | |
| pGEX-5X-2-Kir3.1 (325-501) | 3.1 C7-9 | — " — |
| pGEX-5X-2-Kir3.1 (325-367) | 3.1 C7 (low solubility) | — " — |
| pGEX-5X-2-Kir3.1 (368-429) | 3.1 C8 (low solubility) | — " — |
| pGEX-5X-2-Kir3.1 (430-501) | 3.1 C9 | — " — |
| | | |
| **mouse Kir3.2** | | |
| pGEX-5X-2-Kir3.2 (336-414) | 3.2 C7-8 | — " — |
| pGEX-5X-2-Kir3.2 (336-377) | 3.2 C7 (low solubility) | — " — |
| pGEX-5X-2-Kir3.2 (378-414) | 3.2 C8 | — " — |
| | | |
| **rat Kir1.1** | | |
| pGEX-5X-2-Kir1.1 (323-391) | 1.1 C7-8 | — " — |
| | | |

* Numbers in parentheses indicate the aminoacid positions flanking the resulting Kir protein fragments.

Plasmids for expression of the Kir2.1 protein in mammalian cells:

| **Plasmid** | **Source** |
|---|---|
| pEGFPC1-Kir2.1 | produced by Dr. R. Schönherr (Jena) |
| pEGFPC1-Kir2.1 K188Q·R189Q | this study |
| pEGFPC1-Kir2.1 R343Q·K346Q | this study |
| pcDNA3-Kir2.1 | this study |
| pcDNA3-Kir2.1 R343Q·K346Q | this study |

### 2.1.3. Other materials

Proteins

GST-PI3Kγ - provided by Prof. R. Klinger and C. Kirsch (Jena, Germany)

recombinant $G\beta_1\gamma_2$ - provided by Prof. R. Wetzker and Dr. A. Bondev (Jena, Germany)

Antibodies

Polyclonal rabbit anti-$G_\beta$ antibody M-14 (Santa Cruz Laboratories, Santa Cruz, CA, USA; provided by Dr. A.Bondev, Jena, Germany)

Polyclonal rabbit IgG, whole antibody, peroxidase-conjugated (from donkey) (Amersham Pharmacia Biotech)

Lipids

PI(4,5)$P_2$ from bovine brain (Sigma)

PI(4)P (Sigma)

PI from soybean (Sigma)

PC from egg yolk (Sigma)

PE from bovine brain (Sigma)

PS from bovine brain (Sigma)

Rh-PE (Avanti Polar Lipids, Alabaster, AL, USA)

Other Reagents and Chemicals

Expand High fidelity PCR Kit (Roche)

High Pure PCR Purification Kit (Roche)

DNA Clean & Concentrator Kit (Zymo Research)

Zymo Clean Gel extraction Kit (Zymo Research)

Qiaex Midi Plasmid purification Kit (Qiagen)

Thermosequenase mix (Amersham)

Restriction enzymes (New England Biolabs)

T4 DNA ligase & Ligase Buffer (Promega)

SequaGel XR sequencing solution (National Diagnostics)

SequaGel complete buffer reagent (National Diagnostics)

mMessage mMachine Kit (Ambion, Ausin, TX, USA)

IPTG (Roth)

Protease inhibitor tablets (Roche)

Lysozyme (Sigma)

DTT (Sigma)

TritonX100 (Fluka BioChemika)

Glutathion-Sepharose 4B (Amersham Pharmacia Biotech)

Immobilon-P Transfer Membrane (Millipore Corporation, Bedford, MA)

ECL+plus Western blotting detection system (Amersham Pharmacia Biotech)

BioMax MR-1 Film (Kodak Company, Rochester)

Dulbecco's Modified Eagle Medium (Life Technologies, Inc.)

Nutrient Mix F-12 (HAM) (Biochrom)

FCS (Life Technologies, Inc.)

Trypsin-EDTA (Life Technologies, Inc.)

Superfect Transfection Reagent (Qiagen)

### 2.1.4. Buffers and solutions

TY medium for growth of *E.coli*

| | |
|---|---|
| Bacto-Trypton | 10g |
| Yeast extract | 5g |
| NaCl | 5g |
| $H_2O$ | add 1l, autoclave |

for agar plates: 15g agar for 1l medium

Lipid binding buffer

| | |
|---|---|
| KCl | 100 mM |
| HEPES | 10 mM |
| pH 7.4 | |

Phosphate-buffered saline (PBS)

| | |
|---|---|
| $Na_2HPO_4 \cdot 7\ H_2O$ | 4.3mM |
| $KH_2PO_4$ | 1.4mM |
| NaCl | 137mM |
| KCl | 2.7mM |
| pH 7.4 | |

4x SDS-PAGE loading buffer

| | |
|---|---|
| TrisHCl pH 6.8 | 70mM |
| β-mercaptoethanol | 5% |
| glycerol | 40% |
| SDS | 3% |
| bromophenyl blue | 0.05% |

Protein storage buffer

| | |
|---|---|
| NaCl | 50mM |
| $MgCl_2$ | 5mM |
| HEPES | 50mM |
| glycerol | 50% |
| DTT | 1mM |
| pH 7.4 | |

Coomassie-stain

| | |
|---|---|
| Coomassie R250 (for gels) | 2 g/l |
| Acetic acid | 10% |
| Methanol | 50% |

| Destaining solution for Coomassie-stained gels | |
|---|---|
| acetic acid | 10% |
| ethanol | 10% |
| methanol | 5% |

| Semi-dry transfer buffer | |
|---|---|
| TrisBase | 48 mM |
| Glycine | 39 mM |
| SDS | 0.037 % |
| Methanol | 20 % |
| pH 9-10 | |

| NET-G buffer | |
|---|---|
| Na Cl | 150 mM |
| EDTA | 5 mM |
| Tris-HCl | 50 mM |
| Triton X100 | 0.05% |
| Gelatin | 0.04% |
| pH 7.5 | |

### 2.2. Methods.

#### 2.2.1. DNA manipulations.

For constructing plasmids expressing GST-fused fragments of channel proteins the relevant fragments of channel DNA were generated by PCR with oligonucleotide primers. All forward primers contained BamHI restriction sites in the 5' termini. The reverse primers contained in their 5' termini XhoI restriction sites (for cloning Kir1.1, Kir2.1, Kir3.1 and Kir3.2 fragments) or SalI restriction sites (for cloning Kir6.2 fragments). The obtained PCR fragments were purified, digested with the corresponding restriction enzymes and ligated to the pGEX-5X-2 expression vector digested at the same restriction sites. Point mutations were generated by overlap extension PCR. All constructs were verified by sequencing with fluorescently labeled primers on LI-COR 4000 sequencer (MWG-Biotech AG, Ebersberg, Germany). PCR primers were purchased from JenaBioScience GmbH (Jena, Germany), sequencing primers were purchased from MWG.

#### 2.2.2. Transformation of competent *E.coli* and plasmid preparation.

Competent *E.coli* XL1 cells were prepared and transformed using Z-Competent *E.coli* transformation kit & buffer set (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. For production of competent BL-21 (or BL-21 Codon Plus) cells a $CaCl_2$ method was used (Sambrook & Russel, 2001). For transformation, cells were incubated with 1 μg plasmid DNA for 30 min on ice, subjected to a heat chock at 42°C for 1 min and plated on a TY-agar plate containing the appropriate antibiotic. For selection of cells transformed with pBSSK-, pGEX- and pcDNA3-based vectors,

ampicillin (100 μg/ml) was used. Cells transformed with pEGFP-based vectors were selected on the media containing chloramphenicol (34 μg/ml). Plasmid DNA was purified from bacterial cultures using Qiagen plasmid-purification kits following the manufacturer's protocol.

### 2.2.3. Expression and purification of GST-fused proteins.

For testing the protein expression, clones of *E.coli* strain XL1-Blue transformed with pGEX5x2 or pGEX5x2 with cloned Kir fragments were grown in 2 ml YT medium with antibiotic for 3 hrs at 37°C, incuced by 1mM IPTG and grown for additional 2-3 hrs at 37°C. 1 ml of the culture was transfered to the 1.5 ml Eppendorf tube, cells were spinned down and resuspended in 45 μl of 2x Laemmli buffer. After heating at 95°C for 3 min, the samples were loaded on the 12% SDS-PAGE (Laemmli, 1970) and the expression of the desired proteins was checked by comparison with samples from uninduced cultures. A molecular weight standard that contains proteins of a defined size was loaded to allow estimation of the molecular weight of detected sample proteins. For the gel-electrophoresis, gel-chambers from Biometra were used and assembled according to the manufacturers instructions.

For large-scale protein preparation, GST-fusion proteins were expressed in *E.coli* strains BL-21 or BL-21Codon Plus. The cells were grown in 500 ml YT medium to $OD_{600}$ 0.6-0.7, induced with 0.1 mM IPTG, and grown for additional 4-5 hrs or overnight. Cells were harvested by centrifugation (6000g, 10 min at 4°C) and resuspended in PBS with protease inhibitors (1 tablet/50 ml PBS) and subjected to three times freeze-thaw cycle. Lysozyme was added up to 1-2 mg/ml and cells were incubated on ice for 15 min. DTT was added up to 5mM and cells were disrupted by sonication. After this, Triton-X100 was added to the sonicate up to 1-2% and cells were shaked on ice for 30 min. Alternatively, to avoid degradation of some fusion proteins the cells were lysed under mild conditions without freezing. Namely, they were resuspended in 25mM TrisHCl with protease inhibitors (1 tablet/50 ml buffer) and lysozyme (1-2 mg/ml) and incubated on ice for 15 min. EDTA was added up to 20mM, DTT was added up to 5mM and TritonX100 was added up to 1-2%. The lysate was shaked on ice for 30 min. Cell debris was spun down by centrifugation of the lysates obtained by both methods at 12000g for 10 min, and the supernatant was incubated with Glutathione Sepharose beads (500-600 μl of 50% slurry in PBS) on ice for 1hr. Beads were washed 3 times for 10 min with PBS, resuspended in

protein storage buffer and stored at -20°C. Protein concentration on the beads was estimated by the comparison with BSA diluted to known concentrations on Coomassie-stained 12% SDS-PAGE gel. For carrying out liposome binding assays protein concentration on the beads was adjusted to 0.5-0.75 mg/ml by diluting with fresh 50% Glutathion Sepharose. Depending on the solubility of the fusion protein, the total amount of immobilised protein ranged from 0.1 mg to 3 mg per 500 ml culture volume. All the proteins were checked before each assay on the Coomassie-stained 12% SDS-PAGE gel. For liposome binding assays the proteins were washed three times with lipid binding buffer.

### 2.2.4. Liposome binding assay with Glutathione-Sepharose-immobilised proteins.

Liposome binding assays were carried out in the Institute for Biochemistry II, University of Jena (Prof. R. Klinger). Mixed liposomes were prepared by I. Schwabe and C. Kirsch from PI(4,5)$P_2$, PC and Rh-PE. The latter was used for fluorescent labeling of liposomes. Phospholipids dissolved in chloroform:methanol:$H_2O$:1N HCl (20:9:1:10) were mixed to obtain the desired phospholipid composition of 2, 5, 10, 20 and 35 mol % PI(4,5)$P_2$ and 97, 94, 89, 79 and 64 mol% PC, respectively. Control PC/Rh-PE liposomes were taken to determine the background binding. For some experiments, liposomes containing PC and PI, PI(4)P, PE or PS at indicated ratios were prepared. Rh-PE ratio was always 1 mol%. Phospholipid mixtures were dried under $N_2$; lipid binding buffer was added to obtain a phospholipid concentration of 1 mg/ml and the lipid film was dissolved by homogenising and vortexing. The mixture was left at room temperature for 30 min, then homogenised and frozen at -80°C for 15 min, followed by sonication on ice (three times for 2 min with 1 min break). The resulting sonicate was centrifuged and the supernatant containing small unilamellar vesicles was used for binding assays with glutathione-Sepharose immobilised proteins.

For binding assay 0.1 ml of 50% gluthatione-4B-Sepharose with immobilised proteins in lipid binding buffer (20-40 μg/assay) were mixed with 0.1 ml of fluorescently labeled liposomes and incubated with agitation at 37°C for 1 hr. For assaying protein-liposome binding in the presense of βγ subunits of trimeric G-proteins, 0.1μM of recombinant $G_{\beta 1\gamma 2}$ was added per assay before starting the incubation. Following incubation, Sepharose was spun down, washed three times with 1 ml lipid binding buffer

and diluted in the buffer to the final volume of 0.12 ml. Two 0.05 ml specimen were taken from each sample and added to a 96-well plate. Binding of liposomes to immobilised proteins was quantified by fluorescence measurement (Fluoroscan II, Laborsystems GmbH, Frankfurt, Germany) using an excitation and emission wavelenth of 390 nm and 590 nm, respectively. The mean fluorescence value of two specimens was calculated for every sample. In addition, the fluorescence of liposomes prepared in every experiment was measured. The data obtained for the protein samples mixed with different liposomes and in different experiments were corrected by the ratio of the liposome's fluorescence. The resulting values were termed "fluorescence in arbitrary units" (a.u.), reflecting liposome binding of each protein sample.

### 2.2.5. Channel expression and electrophysiological measurements in *Xenopus* oocytes.

For synthesis of capped mRNA, the pBSSK-Kir2.1 plasmids with wild-type or mutant Kir2.1 cDNA were linearised with XhoI restriction endonuclease. mRNA was synthesised *in vitro* with T3 RNA polymerase according to manufacturer's instructions (mMessage mMachine kit, Ambion, Austin, TX, USA). Stage V oocytes were prepared from *Xenopus laevis* as described (Stühmer et al., 1992). Oocytes were injected with 10 ng mRNA per oocyte, if not indicated differently, and incubated at 18°C for 24 to 48 hrs. The bath solution for recording of the Kir2.1 channel currents contained (in mM): 90 KCl, 3 $MgCl_2$, 10 HEPES (pH 7.2 with KOH). In the experiment on coinjection of the wild-type and the mutant Kir2.1 mRNA the bath solution contained (in mM): 40KCl, 1.8 $CaCl_2$, 77 NaCl, 10 HEPES. Currents were measured at 20-23°C with a two-electrode voltage clamp amplifier (Turbo-TEC 10CD, NPI electronic, Tamm, Germany). Electrodes were filled with 2M KCl and had resistances of 0.6-0.8 MΩ. Experiment control including pulse generation and data recording was performed with the Pulse+PulseFit acquisition package (HEKA Elektronik, Lambrecht, Germany) running on an Apple Macintosh Quadra 650 computer. Currents in oocytes expressing Kir2.1 channels were measured by using voltage ramps from +50 mV to -100 mV (200 ms) from a holding potential of -10 mV. The current value at -100 mV was taken as the measured signal, while the current at +50mV was used for linear leakage correction. The current at +50 mV was assumed as the leakage current with a reversal potential of 0 mV. For data analysis, the programs PulseFit (HEKA Electronik) and Igor-Pro (Wave-Metrics, Lake Oswego, OR, USA) were used. Statistical

data are given as mean ± SEM (n = number of oocytes). Some statistical data for the measured currents are represented as box plots, where center line indicates a median, boxes indicate second and third quartile; whiskers and asterisks indicate extreme values and outliers, respectively. For statistical analyses Student's t test was used where appropriate. A value of $P<0.05$ was considered statistically significant.

### 2.2.6. Channel expression and electrophysiological measurements in cultured mammalian cells.

CHO-K1 cells were cultured in 250 ml culture-flasks in HAM'S F-12 supplemented with 10% fetal calf serum (FCS). HEK293 cells were cultured in 250 ml culture-flasks in Dulbecco's modified Eagle's medium-HAM'S F-12 supplemented with 10% FCS. Both cell lines were maintained at 37°C in a humidified atmosphere with 5% $CO_2$.

Subconfluent CHO-K1 and HEK293 cells were transfected by the SuperFect method (Qiagen, Hilden, Germany). The day before transfection cells were trypsinised and plated on glass coverslips. DNA of interest (2 μg/ dish), FCS-free medium (0.1 ml/ dish) and SuperFect reagent (5μl/dish) were mixed and incubated at room temperature for 5 min. Cells were washed with PBS. The DNA mixture (0.1 ml/dish) was resuspended in 0.6 ml media without FCS, vortexed and immediately added to the dish. Media supplemented with FCS was added (1.3 ml/dish) and cells were incubated at 37°C for 3-5 hrs. After incubation, the media was removed gently, cells were once washed with 2 ml PBS, and 2 ml of fresh media with FCS was added per dish.

The expressed Kir2.1 currents were measured in the whole-cell patch-clamp configuration 20-48 hrs after transfection. The recordings were performed using an EPC9 (HEKA Elektronik, Lambrecht, Germany) patch-clamp amplifier. Pulse protocol generation and data acquisition was controlled with the program Pulse (Heka Elektronik). Series resistance errors were compensated in the range of 70-80 %. Data were low-pass filtered at 1.33 kHz. Patch pipettes were fabricated from Kimax-51 glass (Kimble Glass, Vineland, NJ) and were of 1-3 MΩ resistance. All experiments were performed at 19-20°C. Bath solution contained (in mM): 135 NaCl, 5.4 KCl, 1.8.$CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.4. Pipette solution contained (in mM): 140 KCl, 2 $MgCl_2$, 1 EGTA, 5 HEPES, pH 7.3. Currents were measured in voltage ramps from -100 mV to +50 mV (200 ms) from a holding potential of 0 mV, or in ramps from –120 mV to +50 mV. The current value at

-100 mV was taken as the measured signal, while the current at +50mV was used for linear leakage correction. Data were analysed and presented as in the case of *Xenopus* oocyte expression data.

### 2.2.7. Laser confocal microscopy.

Fluorescent images were taken 15-63 hrs after transfection. Images were acquired in confocal mode using a laser scanning microscope (LSM510; Zeiss, Jena, Germany) equipped with a 63x water immersion objective (C-Apochromat, 63x/1.2W corr) and controlled by LSM510 software. Scans were averaged 4x, the pixel time was 1.76 μs. Images were digitized with 512x512 pixel spatial and 8-bit intensity resolution. All transmission images shown are differential interference contrast (DIC) images. Prior to imaging cells were washed 5x with buffer solution. EGFP was excited at 488 nm (Argon laser) and its fluorescence emission bandpass-filtered (505-550 nm).

### 2.2.8. Western blotting.

During the Western blot procedure, the proteins from an SDS-PAGE gel were electrophoretically transferred onto a PVDF membrane by a semi-dry method. The transfer chamber consisted of two flat electrode plates. On the anode plate, a sandwich-like stack consisting of four pieces of filter paper, the membrane, both cut to the size of the gel, the gel and finally four more filter papers was constructed. The filter paper was soaked in semi-dry transfer-buffer and the PVDF-membrane was pre wet in methanol for 10 seconds before it was also submerged in transfer-buffer for 15 minutes to allow for equilibration. The sandwich of filter paper, membrane, gel and filter paper was then connected to the cathode and the proteins were transferred at a set current of $2mA/cm^2$ for 1.5 hours. Afterwards, the membrane was removed from the blotting chamber and blocked in NET-G buffer for at least 1h. For detection of $G_\beta$ the membrane was incubated with rabbit anti-$G_\beta$ antibody solution (1:2000) in NET-G buffer for one hour at room temperature. After three times wash with NET-G buffer the membrane was incubated for 1h with a solution of secondary antibody (horseradish peroxidase coupled goat anti-rabbit Ig, 1:20000 in NET-G buffer). After the incubation, the membrane was washed again thoroughly for a minimum three times to remove unbound antibody. For detection of the $G_\beta$/antibody complex a ECL+plus detection reagent (Amersham Pharmacia Biotech) was applied to the membrane according to the manufacturers protocol. The membrane was thereafter exposed to the film for 5-10 sec.

# 3. Results

## 3.1. Identification of PI(4,5)$P_2$ binding regions in the C-terminal cytoplasmic domain of the Kir2.1 channel protein.

Kir2.1 channels have a simplest regulation pattern among the well-studied Kir channels – their activity in the membrane is dependent mainly on the presence of PI(4,5)$P_2$ and is not regulated by specific factors, like $G_{\beta\gamma}$ subunits, intracellular ATP or protons. These channels are also characterised by high affinity and specificity towards PI(4,5)$P_2$ (see "Introduction"). Therefore, the Kir2.1 channel protein is a good candidate for studying the distribution of prototypic PI(4,5)$P_2$ binding regions in Kir channels.

### 3.1.1. Mutations in the region I of the proximal cytoplasmic C-terminus reduce the expression of functional Kir2.1 channels in *Xenopus* oocytes.

Like other Kir family proteins, the Kir2.1 protein contains a region of positively charged aminoacid residues (region I in Fig.7) in the cytoplasmic C-terminus next to the predicted second transmembrane domain, M2. Aminoacid residues in the homologous regions from other Kir channel proteins have been found to be important for channels' activation by PI(4,5)$P_2$ (see "Introduction", Table 3).

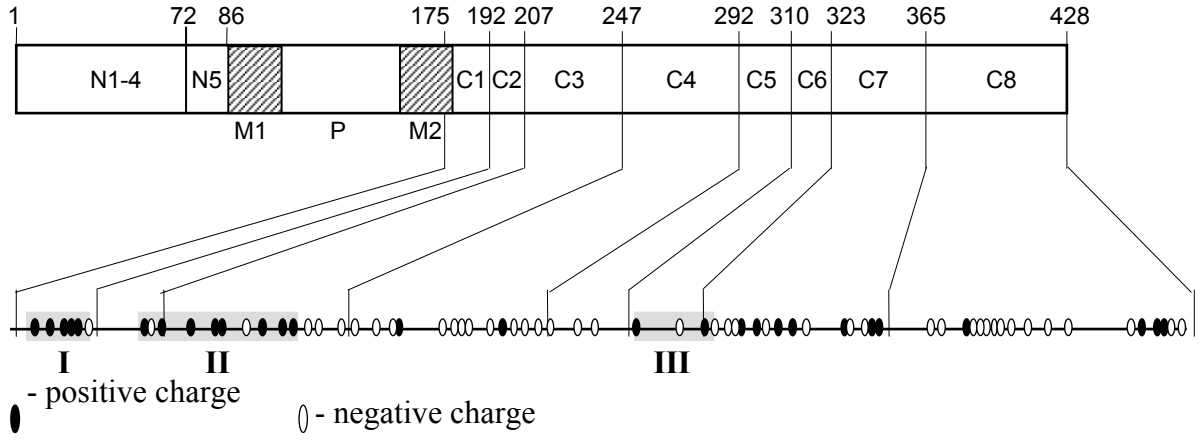


**Fig. 7.** Schematic presentation of the Kir2.1 protein. Numbers indicate the amino acid positions. M1, M2 - transmembrane domains; P - pore domain; N1-N5, C1-C8 - sections of Kir2.1 cytoplasmic N- and C-termini used in this study. **Bottom:** distribution of positively (black) and negatively (white ovals) charged aminoacid residues. Regions I-III containing aminoacid residues important for Kir-PI(4,5)$P_2$ interaction (defined in "Introduction") are boxed in grey (after Soom et al., 2001).

Mutation of the arginine residue 188 within this region in Kir1.1 channel protein (R188Q) decreased the functional expression of this channel in *Xenopus* oocytes and accelerated the time-course of Kir1.1 channel inhibition by $PI(4,5)P_2$ antibodies. This mutation also reduced the binding of the purified C-terminus of Kir1.1 protein to $PI(4,5)P_2$-containing liposomes (Huang et al., 1998). Based on these data we supposed that the homologous residues in Kir2.1 protein are important for the expression of functional Kir2.1 channels and $PI(4,5)P_2$ binding.

Interactions of Kir channels with $PI(4,5)P_2$ are supposed to be mainly of electrostatic nature, involving interactions of positively charged residues in the protein with the negatively charged phosphate groups on the lipid (see "Introduction"). Therefore, we neutralised two positively charged residues within region I, introducing point-mutations K188Q, R189Q (corresponding to Kir1.1 R188Q), and a double mutation K188Q·R189Q. As a control, we introduced mutation P186A not changing the charge, but increasing slightly the hydrophobicity of this region.

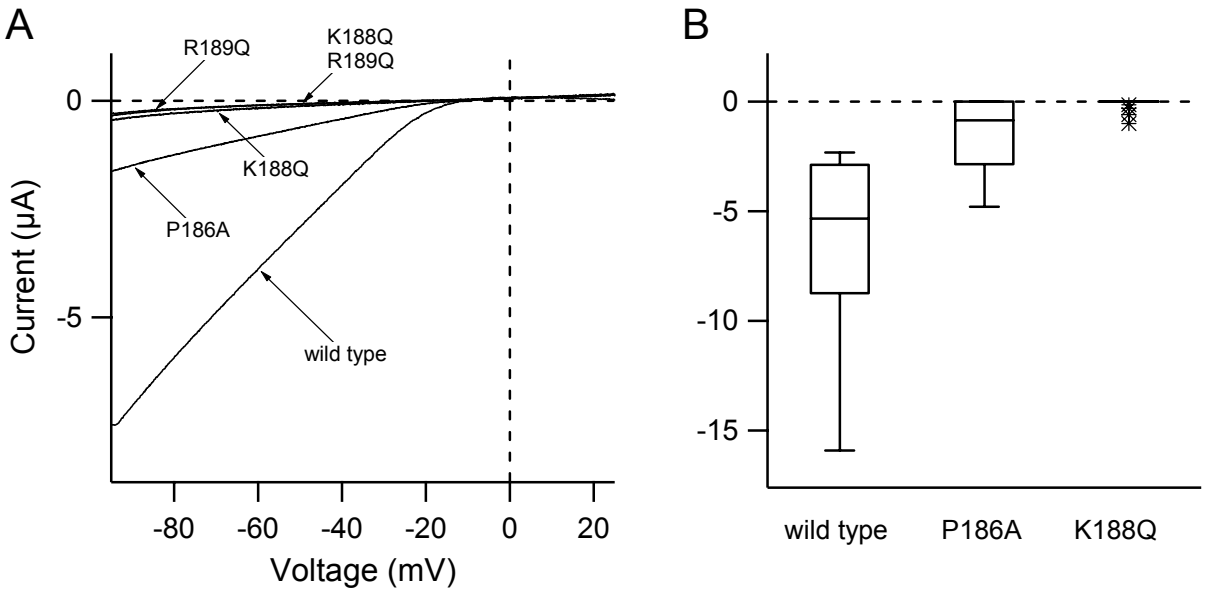


**Fig. 8.** Effects of point mutations in Kir2.1 C-terminal region I on the expression of Kir2.1 channels.

**A:** Current traces in response to voltage ramps (-100 to +50 mV in 2 s) from *Xenopus* oocytes expressing wild-type Kir2.1 channels and the mutants P186A, K188Q, R189Q and K188Q·R189Q (10 ng mRNA / oocyte, 24 hrs after injection).

**B:** Box plots representing the statistics for the currents elicited by the wild-type Kir2.1 and the mutants P186A and K188Q at -100 mV (n = 17-22) (from Soom et al., 2001).

The effect of the mutations on Kir2.1 channel functional expression was tested in *Xenopus* oocytes. Expression of Kir2.1 channels was assayed by recording whole-oocyte currents using two-electrode voltage-clamp techniques. Oocytes were clamped at holding membrane potential of -10 mV, the voltage was then changed in ramps from -100 mV to +50 mV during 2 s, and the corresponding current change was recorded.

As shown in Fig.8, oocytes injected with the wild-type Kir2.1 elicited characteristic inwardly rectifying currents, whereas oocytes expressing mutant channels had either smaller or no currents. At -100 mV oocytes expressing the wild-type channels conducted an average current of -6.2 ± 1.0 μA (mean ± SEM; n=17). Mutant P186A yielded -1.4 ± 0.4 μA (n=20) and mutant K188Q only some residual current (-0.09 ± 0.05 μA; n=22). Mutants R189Q and K188Q·R189Q did not show detectable Kir-specific currents at all. Thus, the studied mutations in the PKKR region reduced the expression of functional Kir2.1 channels in the following order: K188Q·R189Q, R189Q>K188Q>P186A>wild-type.

### 3.1.2. Effect of mutations in the proximal C-terminal region I on Kir2.1-PI(4,5)$P_2$ binding *in vitro*.

The reduction or complete loss of Kir2.1 current in the mutants studied could be caused by a destabilising effect of the mutations on the channel-PI(4,5)$P_2$ interaction. Interactions with PI(4,5)$P_2$ can be studied by different direct and indirect methods. In our experiments we assayed heterologously expressed proteins containing fragments of the Kir2.1 C-terminal cytoplasmic domain *in vitro* with mixed liposomes (small unilamellar vesicles) composed of PC and PI(4,5)$P_2$ in various ratios (see "Introduction" and "Materials and methods"). The fragments of the Kir2.1 protein were expressed as fusions with glutathione-S-transferase (GST) to allow their one-step affinity purification (described in “Materials and methods“) and easier handling of short protein fragments (from 100 to 30 a.a. residues). As a control, we tested the binding of GST alone and GST-fused PI3Kγ, which is known to use PI(4,5)$P_2$ as one of its substrates and binds PI(4,5)$P_2$ *in vitro* (for a review, see Vanhaesebroeck et al., 2001). To assay the binding we used liposomes containing increasing concentrations of PI(4,5)$P_2$ (Fig.9). GST-PI3Kγ showed a stronger binding to all types of liposomes, that, however, increased with the addition of PI(4,5)$P_2$. The GST-PI3Kγ binding varied in independent experiments, especially at high PI(4,5)$P_2$ concentrations, possibly reflecting the differences between the liposomes and

protein batches. At 35 mol% $PI(4,5)P_2$ the fluorescence value for GST was 0.34 ± 0.17 and for GST-PI3Kγ 3.9 ± 1.2 arbitrary fluorescence units (mean ± SD). Therefore, we chose as a threshold value for $PI(4,5)P_2$-binding detection 1 arbitrary fluorescence unit, which is about the GST value plus three standard deviations. In the following experiments on determining Kir2.1 $PI(4,5)P_2$ binding regions we considered all fluorescence values below 1 arbitrary unit as absence of $PI(4,5)P_2$ binding.

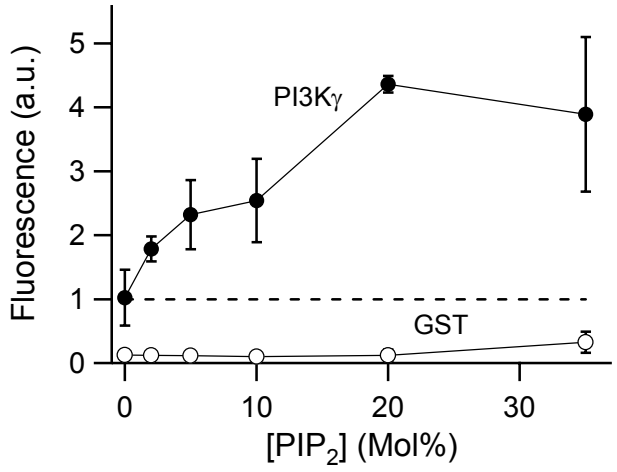


**Fig. 9.** Averaged binding data of GST alone and GST-PI3Kγ (PI3Kγ) to liposomes as a function of the $PI(4,5)P_2$ concentration. Error bars indicate ± SD (n = 3-13). The dashed line marks the threshold for $PI(4,5)P_2$ binding (from Soom et al., 2001).

We divided the Kir2.1 C-terminus into 8 consecutive sections C1-C8 (Fig. 7), and named the GST-fusions used in our study after the sections of Kir2.1 they contained. As mentioned in the "Introduction", the $PI(4,5)P_2$ concentration in the plasma membrane of the living cells is thought to be locally concentrated. Therefore, it is difficult to determine the "right" $PI(4,5)P_2$ concentration in the liposomes that would enable to detect the Kir protein regions binding or not binding $PI(4,5)P_2$ in the native channel. For this reason, we assayed the GST-fused fragments of Kir2.1 protein with liposomes containing 35 mol% $PI(4,5)P_2$ in order to find all the regions potentially able for phosphoinositide binding and exclude the ones that are not able for this binding even at high $PI(4,5)P_2$ concentration. Liposomes with lower $PI(4,5)P_2$ concentrations were used to define the differences in $PI(4,5)P_2$-affinities between the various $PI(4,5)P_2$-binding fragments; or the wild-type and mutated fragments. In comparison with GST and GST-PI3Kγ, we then tested the whole C-

terminus of Kir2.1 (sections C1 through C8 in Fig.7; C1-8) in the wild-type form and containing the single-site mutation K188Q in region I (Fig.10 A).

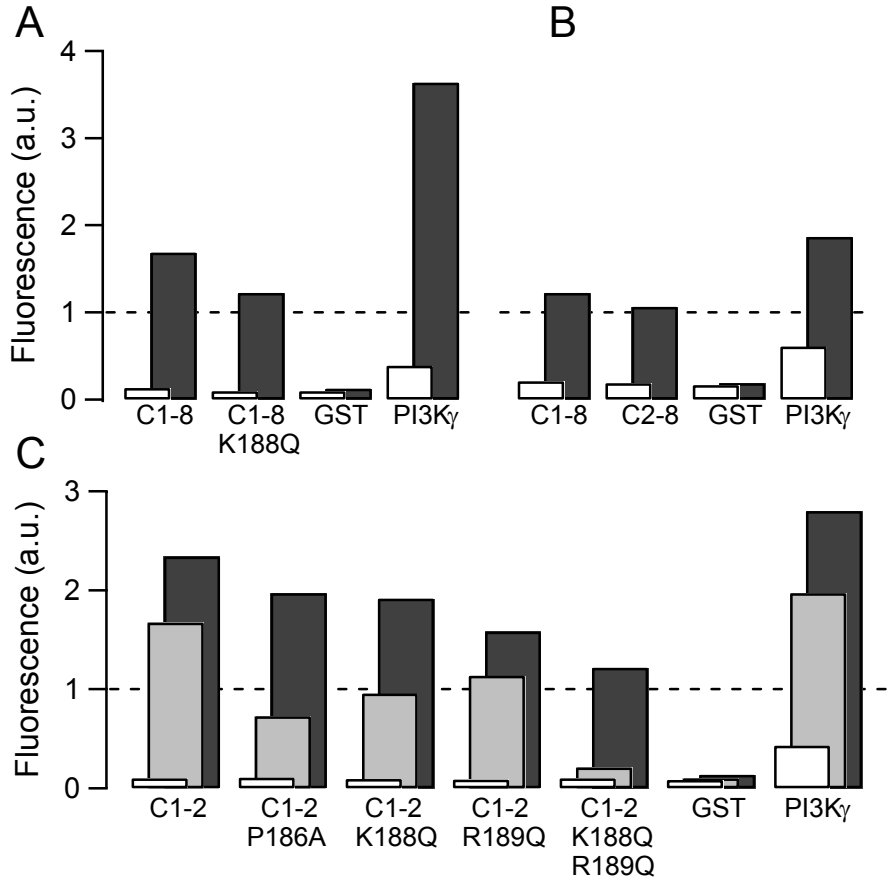


**Fig. 10.** Evaluation of region I in the Kir2.1 protein for $PI(4,5)P_2$ binding.
**A-B:** Independent $PI(4,5)P_2$ binding determinations to the indicated constructs. Data are shown for liposomes containing 35 mol% $PI(4,5)P_2$ (dark grey) and for liposomes without $PI(4,5)P_2$ (white).
**C:** Influence of mutations P186A, K188Q, R189Q and K188Q·R189Q on C1-2 fragment binding to liposomes containing no $PI(4,5)P_2$ (white), 10 mol% $PI(4,5)P_2$ (light grey) and 35 mol% $PI(4,5)P_2$ (dark grey). The mean data of two independent experiments are presented (after Soom et al., 2001).

In an independent experiment the $PI(4,5)P_2$ binding to C1-8 was compared with a fragment lacking region I (C2-8) (Fig.10 B). In both cases we did not find qualitative changes in $PI(4,5)P_2$ binding compared to the wild-type C1-8 fragment. In other words, neither the point mutation in region I, nor its complete absence abolished *in vitro* binding of the Kir2.1 C-terminus to the liposomes containing 35 mol% $PI(4,5)P_2$. This strongly suggests that other $PI(4,5)P_2$ binding regions must exist in the Kir2.1 C-terminus, compensating for the inactivation of region I and making it impossible to detect differences in $PI(4,5)P_2$ binding in the background of the full-length C-terminus.

Therefore, we tried to reduce the size of the tested fragments, in order to exclude additional PI(4,5)$P_2$ binding sites. As shown in Fig.10 C (leftmost bar) a GST-fused short C-terminal segment containing only 32 a.a. residues (C1-2) was still able to bind PI(4,5)$P_2$-containing liposomes. When assayed with liposomes containing 35 mol% PI(4,5)$P_2$, the mutated fragments showed a decrease in binding compared to the wild-type fragment, with the strongest effect for R189Q and K188Q·R189Q mutations (Fig.10 C). Nevertheless, as all the proteins bound the liposomes above the threshold, we could not distinguish them in the qualitative PI(4,5)$P_2$ binding assay. For this reason we assayed the same proteins with the liposomes containing 10 mol% PI(4,5)$P_2$ (Fig.10 C). Under these conditions the wild-type fragment was still binding PI(4,5)$P_2$ above the threshold. At the same time the mutant K188Q·R189Q reduced binding almost to the level of GST control. The effect of the single mutants P186A, K188Q and R189Q was weaker: they reduced the PI(4,5)$P_2$ binding to about 44%, 57%, and 68% of the wild-type, respectively.

These experiments showed that the mutations P186A, K188Q, R189Q, and K188Q·R189Q decrease PI(4,5)$P_2$ affinity of the Kir2.1 region C1-2 in the following order: K188Q·R189Q > P186A > K188Q > R189Q > wild-type. The reduction of PI(4,5)$P_2$ binding could be a basis for the physiological effect of these mutations on Kir2.1 expression, although the level of inhibition of Kir2.1 currents and PI(4,5)$P_2$ binding does not strictly correlate. Such correlation is observed only for the mutant K188Q·R189Q, as it leads to strong reduction both of Kir2.1 functional expression and *in vitro* PI(4,5)$P_2$ binding. Mutants K188Q and R189Q also abolish Kir2.1 expression, but do not inhibit so strongly the PI(4,5)$P_2$ binding. The mutant P186A still allows for formation of functional channels, but has a stronger effect on PI(4,5)$P_2$ binding than the K188Q and R189Q mutants. This indicates the existence of factors that could influence the biochemical characteristics of the PI(4,5)$P_2$-binding region C1-2 *in vivo*.

### 3.1.3. Screening the Kir2.1 protein cytoplasmic C-terminal domain for minimal PI(4,5)$P_2$ binding regions.

The above data suggested that other PI(4,5)$P_2$-binding regions could exist in the C-terminal part of Kir2.1 protein, in addition to region I. In fact, the Kir2.1 region between aminoacid residues 206-245 (corresponding to the section C3 and the region II in Fig.7) meanwhile was shown to confer the high PI(4,5)$P_2$ affinity to Kir3.4 channels (Zhang et al., 1999). Several residues within this region were identified as important for interaction

of Kir2.1 and other Kir channels with PI(4,5)$P_2$ *in vivo* (see "Introduction"). Therefore, for a further screen of the Kir2.1 C-terminus for minimal PI(4,5)$P_2$-binding sites we constructed GST fusions of overlapping Kir2.1 fragments and tested them for PI(4,5)$P_2$ binding *in vitro*.

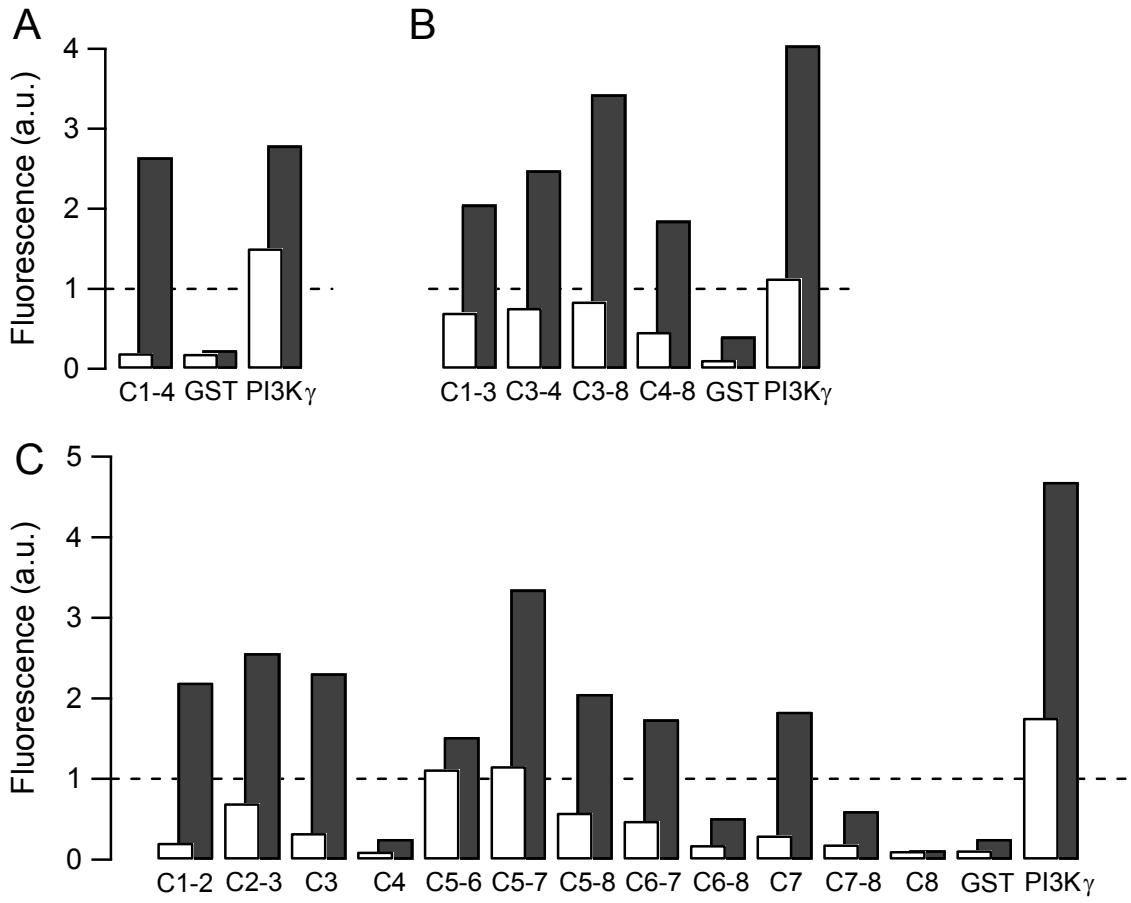


**Fig. 11.** Identification of PI(4,5)$P_2$ binding regions in the C-terminus of Kir2.1 protein. **A-C:** Each panel shows the data from one PI(4,5)$P_2$ binding experiment. The labeling of the protein constructs and liposomes is as in Fig.10 (after Soom et al., 2001).

Fig.11 shows the data from 3 independent experiments, in which GST-fused overlapping C-terminal fragments were tested for binding to liposomes containing 35 mol% PI(4,5)$P_2$ binding. GST-PI3Kγ usually showed a higher binding to the control liposomes without PI(4,5)$P_2$ than the Kir2.1 GST fusions, reflecting its lower specificity towards phospholipids. Two Kir2.1 fragments, C5-6 and C5-7, also bound unspecifically to the control liposomes (Fig.11 C). Possibly, in these short fragments the hydrophobic segment C5 influenced the lipid binding more than in the longer C5-containing constructs. Therefore, we judged upon PI(4,5)$P_2$ affinity of these proteins by the difference between binding to the control and experimental (PI(4,5)$P_2$-containing) liposomes. For the fragment

C5-6 this difference was 0.4 a.u. and for C5-7 2.2 a.u., so we considered them as not binding and binding PI(4,5)$P_2$, respectively. For comparison, the equivalent values for GST and C5-8 were, 0.2 and 1.5 a.u., respectively.

Several fragments bound PI(4,5)$P_2$ above the threshold. We found that fragments containing the putative PI(4,5)$P_2$ binding segment C3, like C1-4 (Fig.11 A), C1-3, C3-4, C3-8 (Fig.11 B) bound the liposomes with 35 mol% PI(4,5)$P_2$. At the same time fragment C4-8 lacking the segment C3 was still binding PI(4,5)$P_2$-containing liposomes (Fig.11 B). Judging from distribution of charged amino acid residues in the Kir2.1 C-terminus (Fig.7) we suspected that there could be one more PI(4,5)$P_2$-binding site in segment C7.

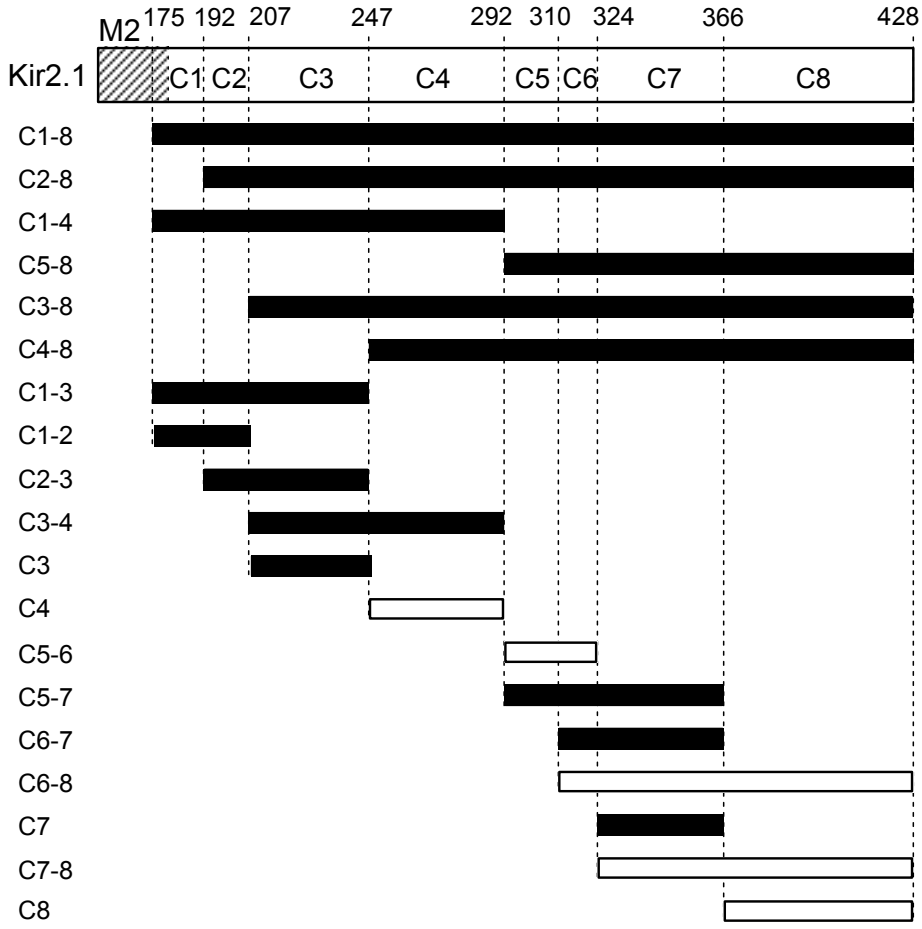


**Fig. 12.** Summary of the data from experiments on *in vitro* PI(4,5)$P_2$ binding of GST-fused C-terminal Kir2.1 fragments. The Kir2.1 C-terminus is schematically represented, position of the M2 domain is indicated. Black bars indicate fragments that bind to liposomes containing 35 mol% PI(4,5)$P_2$ above the threshold; white bars indicate fragments binding PI(4,5)$P_2$ below the threshold (after Soom et al., 2001).

The summary of PI(4,5)$P_2$-binding data from several experiments performed under equivalent conditions is given in Fig.12. The shortest PI(4,5)$P_2$-binding regions other than C1-2 were C3 (res. 206-247) and C7 (res. 324-365). In the study of Zhang et al. (1999) regions 246-270 and 292-310 were not tested for their importance for PI(4,5)$P_2$ response, as the Kir3.4/Kir2.1 chimeras containing these regions produced non-functional channels. According to our data the fragments containing these regions (C4, C5-6) do not bind PI(4,5)$P_2$, but the latter one may influence PI(4,5)$P_2$ binding by the region C7. Indeed, some fragments containing this last PI(4,5)$P_2$-binding region (C6-8 and C7-8) did not show PI(4,5)$P_2$ binding, whereas other fragments (C5-8, C5-7, and C6-7) did. Thus, C8 has a negative effect on PI(4,5)$P_2$ binding by fragments C7 and C6-7. This can be explained by high density of negatively charged residues in this region (Fig.7), which prevents electrostatic binding between the positive charges in the region C7 and PI(4,5)$P_2$. The region C5 antagonises this effect possibly due to its already mentioned hydrophobicity that enhances the protein–lipid interactions when present in the constructs.

### 3.1.4. Comparison of the Kir2.1 C-terminal PI(4,5)$P_2$-binding regions.

Having identified three regions in the Kir2.1 protein that were independently binding PI(4,5)$P_2$, we further compared their affinity to PI(4,5)$P_2$ by assaying binding to liposomes containing different PI(4,5)$P_2$ concentrations (Fig.13 A). Under these experimental conditions the fragments C1-2 and C3 showed a similar PI(4,5)$P_2$ binding pattern. They bound PI(4,5)$P_2$ already at 5 mol% and reached their maximal PI(4,5)$P_2$ affinity at 20 mol%. Thus the segment C3 is an independent binding site that binds PI(4,5)$P_2$/PC liposomes with almost the same affinity as the segment C1-2 (res. 175-206). PI(4,5)$P_2$ binding by the distal fragment C7 was weaker. At 5 and 20 mol% PI(4,5)$P_2$, the binding was below the threshold of 1 a.u. Only at 35 mol% PI(4,5)$P_2$ it almost reached the PI(4,5)$P_2$-binding strength of the first two fragments. The weaker PI(4,5)$P_2$ affinity may reflect a higher number of negatively charged residues in this region (Fig.7). These data characterise fragments 175-206 and 207-246 as "strong" and fragment 324-365 as "weak" *in vitro* PI(4,5)$P_2$-binding sites.

The experimental PC-based liposomes used so far in our study provide a useful tool to distinguish the proteins potentially able to bind PI(4,5)$P_2$. However, this liposome composition is far from the natural phospholipid surrounding of the channel proteins in the plasma membrane. The channel cytoplasmic domains should face the inner side of the

plasma membrane rich in anionic phospholipids and also containing PI(4,5)$P_2$ precursors PI and PI(4)P (for example, reviewed by Langner & Kubica, 1999; McIntosh, 1999). To test the identified PI(4,5)$P_2$ binding regions in conditions approximated to the phospholipid milieu of the plasma membrane inner surface, we compared the binding of these fragments to liposomes containing phospholipids abundant at the cytoplasmic side of the plasma membrane - PE and PS. Either PI, PI(4)P or PI(4,5)$P_2$ were added to these liposomes at low (5 mol%) or high (25 mol%) concentrations (Fig.13 B).

The GST-fused fragments C1-2 and C3, and the positive control PI3Kγ (but not GST) bound to the control liposomes without phosphatidylinositol or phosphoinositides, most probably due to the presence of anionic phospholipid PS. Interestingly, the C1-2 and C3 fragments, that showed a similar binding affinities to PC or PC/PI(4,5)$P_2$ liposomes, differed in their binding to PE/PS/PC-based liposomes. Binding to control liposomes was stronger for the C3 fragment than for the C1-2 fragment. Addition of PI or phosphoinositides increased the binding of C1-2 and C3 fragments to the liposomes correspondingly to the increase of negative charge on the added lipids. Binding at high inositol phospholipid concentration (25 mol%) was stronger than at low concentration (5 mol%), especially for the C1-2 fragment. The phosphoinositide affinity of this fragment seemed to be enhanced by PS, as for example, the concentration-dependent increase in its PI(4,5)$P_2$ binding was much steeper in the PS/PE/PC-based liposomes than in PC-based liposomes (compare with Fig.13 A). For the C3 fragment and PI3Kγ this concentration-dependent increase in binding was less pronounced. As these proteins showed also a strong binding to the control liposomes, this could indicate the saturation of binding due to the strong electrostatic interactions with anionic phospholipids.

The fragment C7 showed weaker binding properties than the fragments C1-2 and C3. It bound weakly to control PE/PS/PC liposomes, compared to GST. Binding was noticeable only for liposomes containing 25 mol% PI(4)P or PI(4,5)$P_2$. In an additional experiment (Fig.13 C) we checked the binding of the fragment C7 to PE, PS or PI at low (10 or 15 mol%) and high (70 or 90 mol%) concentrations. The control proteins GST and GST-fused fragment C8 showed only a weak binding to all the liposomes tested. The fragment C7 also did not markedly bind liposomes containing neutral PE or anionic PI, but bound liposomes with 70 mol% PS - the concentration generating a negative charge approximately equal to 25 mol% of PI(4,5)$P_2$. Although PI has the same net charge as PS,

the steric differences between their headgroups may make it easier for the protein to bind the uncompensated negatively charged phosphate on PS than on PI.

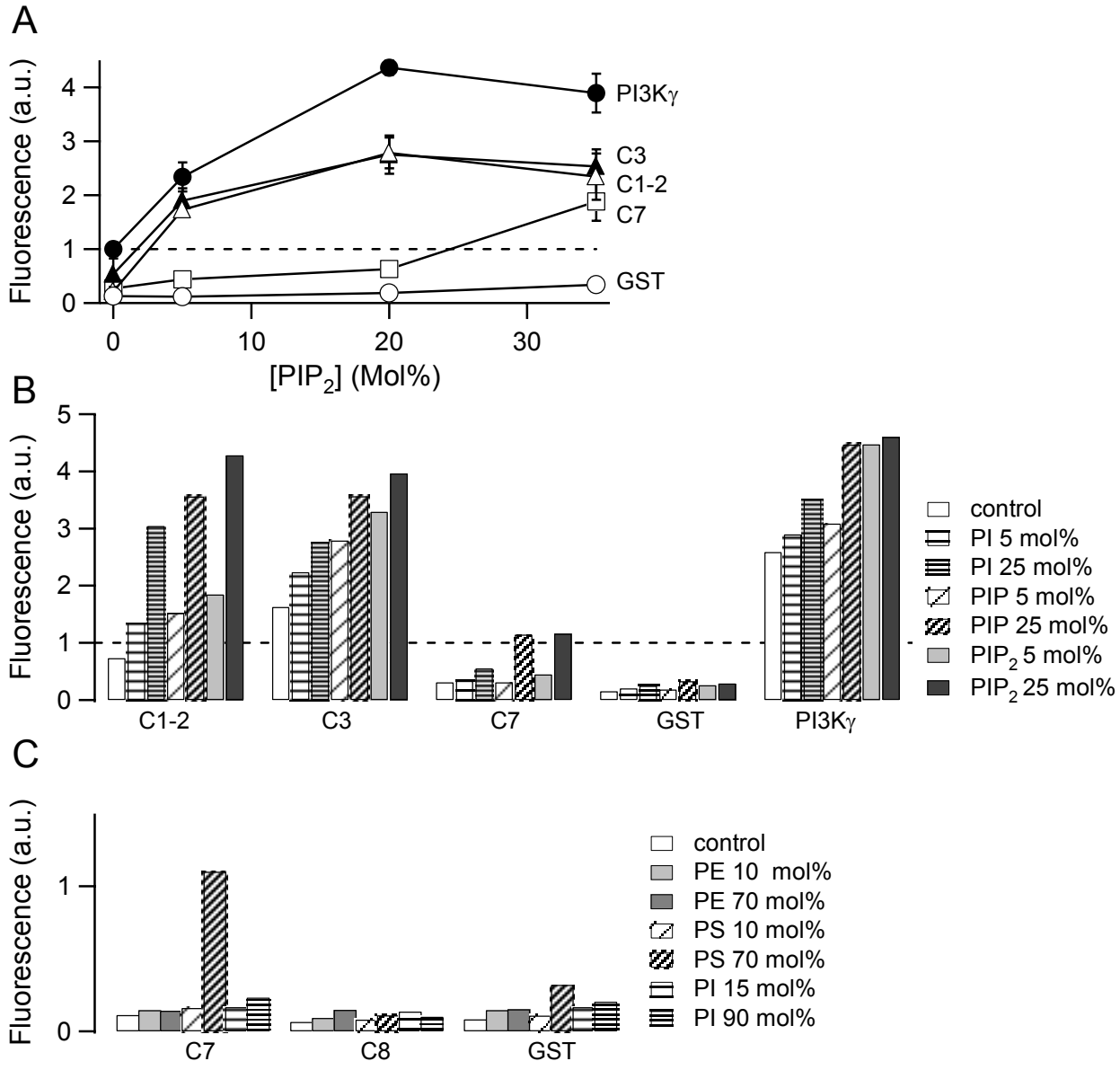


**Fig. 13.** Comparison of three PI(4,5)$P_2$ binding sites in the Kir2.1 C-terminus.
**A:** Binding of GST fusion proteins was assayed as a function of PI(4,5)$P_2$ concentration (mean ± SEM; n=3-13) in PC/PI(4,5)$P_2$ liposomes (from Soom et al., 2001).
**B, C:** Binding of GST-fused Kir2.1 fragments to various phospholipids.
**B:** Binding to liposomes composed of (in mol%) 49 PE : 20 PS : 30 PC : 1 Rh-PE (control) and the same liposomes with PI, PI(4)P or PI(4,5)$P_2$ partially substituting for PC in indicated concentrations. Data are the mean of two independent experiments. **C:** Results of single experiment on the C7 fragment binding to liposomes composed of 99 mol% PC : 1 mol% Rh-PE (control) or with PE, PS or PI partially substituting for PC at indicated concentrations.

Thus, the phospholipid binding of all the three Kir2.1 fragments studied includes a significant electrostatic component, as they are able to bind the non-inositol anionic phospholipid PS, and their binding to inositol phospholipids depends on the charge on the inositol headgroup. The fragment C1-2 binds PS-containing liposomes with about two times lower affinity than the fragment C3, thus being more specific towards PI and its derivatives PI(4)P and PI(4,5)$P_2$. The region C7 is less potent in electrostatic binding compared to the first two regions. It does not bind PI but binds PI(4)P and PI(4,5)$P_2$ at high concentrations. In its binding to PI(4)P and PI(4,5)$P_2$ the fragment C7 is similar to the fragment C1-2, as a steep increase in binding is caused by an increase in phosphoinositide concentration. These differences in biochemical affinities towards various phospholipids in the Kir2.1 C-terminal PI(4,5)$P_2$ binding regions could play a role in the specificity of Kir2.1 channel regulation by phosphoinositides *in vivo*.

### 3.1.5. Influence of mutations in the Kir2.1 distal C-terminal PI(4,5)$P_2$ binding region on channel expression in *Xenopus* oocytes and mammalian cells.

To test the possible functional relevance of the PI(4,5)$P_2$-binding region in Kir2.1 distal C-terminus, we neutralised two closely located positively charged residues R343 and K346 within this region, introducing a double mutation R343Q·K346Q. As shown in Fig.14 A, this mutation reduces *in vitro* PI(4,5)$P_2$ binding of the fragment C7. When this mutant was expressed in *Xenopus* oocytes for 24 hrs (46 ng/oocyte), it gave almost no current in comparison with the same amount of injected wild-type Kir2.1 (Fig.14 B, left panel; -0.4 ± 0.31 μA and -15 ± 1.82 μA, respectively).

To assess the ability of mutant Kir2.1 subunits to form functional heteromultimeric channels with wild-type subunits, we compared currents induced by injection of wild-type Kir2.1 mRNA to those elicited by coinjected mutant and wild-type Kir2.1 mRNAs. Coexpression of wild-type and R343Q·K346Q Kir2.1 induced inwardly rectifying potassium currents (Fig.14 B, left panel). Current amplitudes at -100 mV measured 24 hrs after injection were -8.3 ± 1.5 μA for oocytes injected with wild-type Kir2.1 (23 ng/oocyte) and -5.1 ± 0.9 μA for coinjected wild-type and R343Q·K346Q mutant (23 ng/oocyte each).

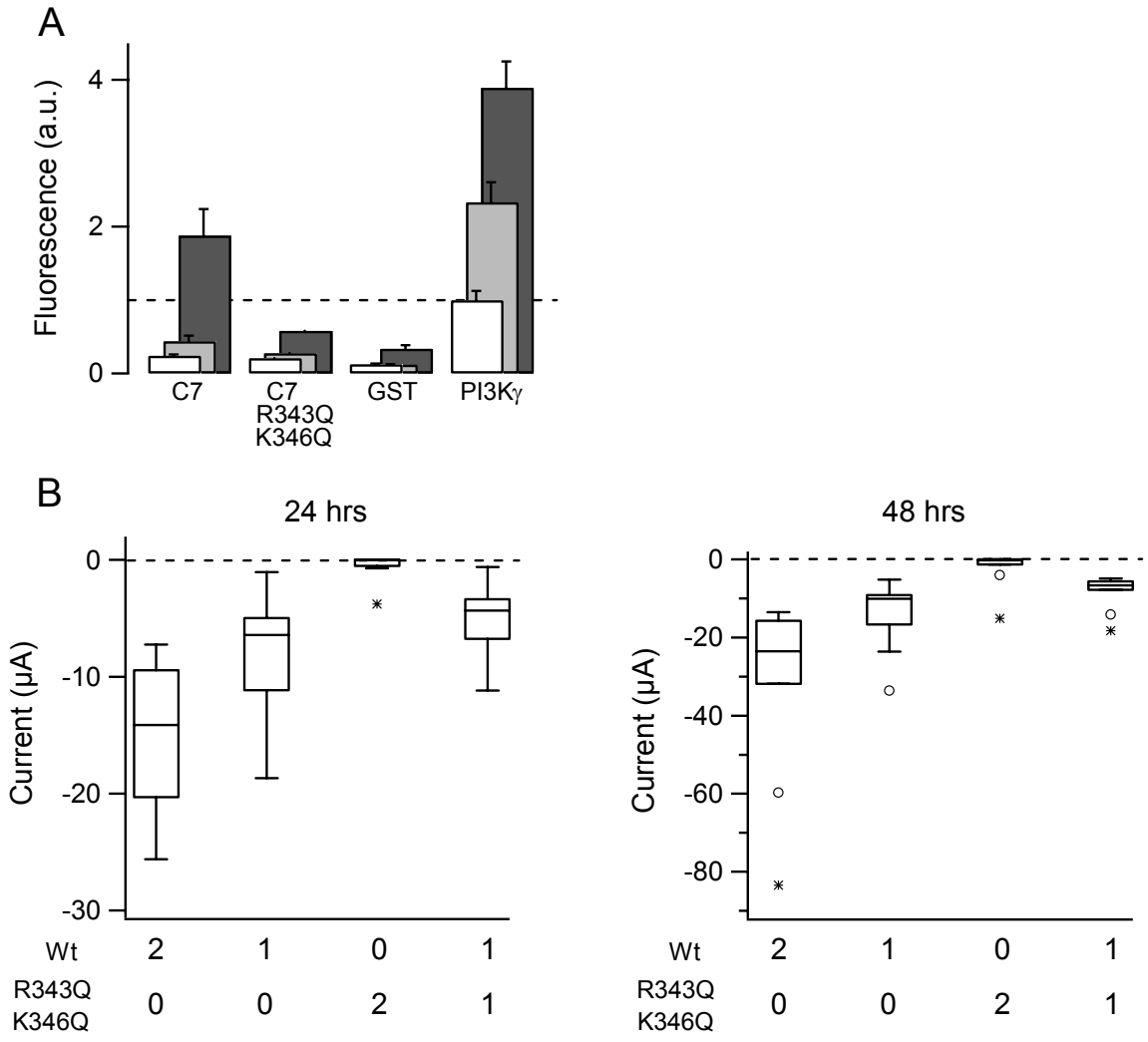


**Fig. 14.** Influence of mutation R343Q·K346Q on PI(4,5)$P_2$ binding and Kir2.1 channel expression in *Xenopus* oocytes.

**A:** Mutation R343Q·K346Q reduces PI(4,5)$P_2$ binding of the Kir2.1 C7 fragment. Binding was assayed with liposomes containing no PI(4,5)$P_2$ (white), 5 mol% PI(4,5)$P_2$ (light grey) and 35 mol% PI(4,5)$P_2$ (dark grey); data are represented as mean ± SEM for at least two independent experiments (from Soom et al., 2001).

**B:** Box plots representing the statistics for the currents measured at -100 mV in oocytes expressing the wild-type (Wt), the mutant (R343Q·K346Q) or coinjected wild-type and mutant mRNAs (n=11-12). Numbers under the boxes indicate the relative amount of corresponding mRNA: 0 - no mRNA injected, 1 - 23 ng/oocyte, 2 - 46 ng/oocyte. Currents were measured in the same oocytes 24 hrs (left panel) and 48 hrs (right panel) after mRNA injection.

In 48 hrs the average currents elicited by all the channel combinations increased proportionally (Fig.14 B, right panel). Oocytes injected with 46 ng/oocyte wild-type Kir2.1 and R343Q·K346Q conducted, respectively, -29.76 ± 6.64 μA and -1.81 ± 1.25 μA. Oocytes injected with 23 ng/oocyte wild-type Kir2.1 conducted -13.24 ± 2.54 μA and

coinjected with 23 ng/oocyte each of wild-type Kir2.1 and R343Q·K346Q mutant produced -7.9 ± 1.18 μA. Coinjected wild-type and mutant mRNA (23 ng/oocyte each) conducted about 60% of the current produced by 23 ng/oocyte wild-type mRNA ($P<0.05$). This indicates that R343Q·K346Q subunits coassemble with wild-type Kir2.1 subunits and cause a weak negative dominant suppression of the channel function when expressed in *Xenopus* oocytes.

It should be noted that although the average current elicited by R343Q·K346Q mutant in this experiment was low, two oocytes from 12 showed a substantial inward current after 48 hrs (-4.05 and -15.1 μA). The expression of the R343Q·K346Q mutant varied in oocyte preparations, like in one of the oocyte batches the average current produced by the R343Q·K346Q mutant in 48 hrs was -7.1 ± 0.77 μA versus -33.18 ± 4.96 μA of the wild-type current (n=7). This expression pattern is different from, for example, K188Q·R189Q mutant, which produced no current neither 24, nor 48 hours after injection in all the oocyte batches tested.

To compare the influence of R343Q·K346Q mutation on the Kir2.1 channel expression in *Xenopus* oocytes with expression in mammalian cells, we transiently overexpressed the R343Q·K346Q mutant channels in two mammalian cell lines, CHO-K1 and HEK293. The channel-coding sequences were subcloned into the expression vector containing the sequence for the enhanced green fluorescent protein (pEGFP-C1) to produce channel proteins with the EGFP fused to their N-termini. This enables an easier selection of transfected cells and monitoring subcellular distribution of the channel proteins. As a control, we used the EGFP-fused wild-type Kir2.1 and the mutant K188Q·R189Q that did not express in *Xenopus* oocytes and decreased the $PI(4,5)P_2$ binding of the Kir2.1 protein region C1-2.

The expression pattern of the channel proteins was similar in the two cell lines used. The control cells transfected with the pEGFP-C1 plasmid showed a homogenous signal throughout the cytoplasm, including the nucleus (Fig.15 A). Most of the cells transfected either with wild-type or the mutant channel constructs exhibited a distinct fluorescent signal in the plasma membrane and also a spot-like signal in the cytoplasm (Fig.15 B-D). Some cells only showed the cytoplasmic signal, but there were no pronounced differences in the signal distribution between the cells transfected with the wild-type or the mutant constructs.

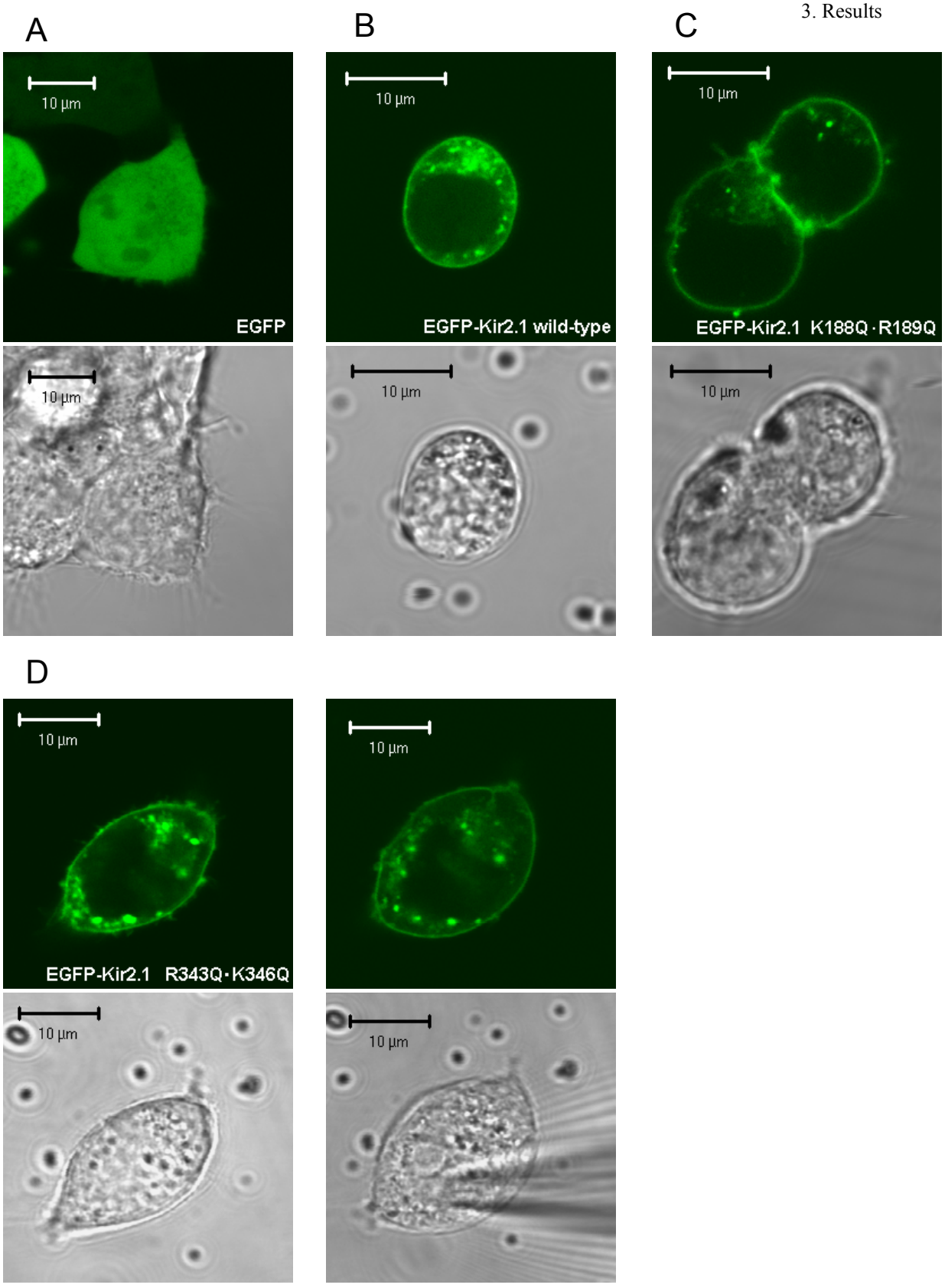


**Fig. 15.** Subcellular distribution of EGFP-Kir2.1. Representative images were taken from HEK293 cells transfected either with pEGFP-C1 control (**A**), pEGFP-C1-Kir2.1 wild-type (**B**) or the indicated mutants (**C**-**D**), 15-20 hrs after transfection. The corresponding transmission images are shown under the confocal fluorescence images of each cell. In (**D**) the same cell was imaged before (left panel) and during (right panel) the whole-cell patch-clamp experiment.

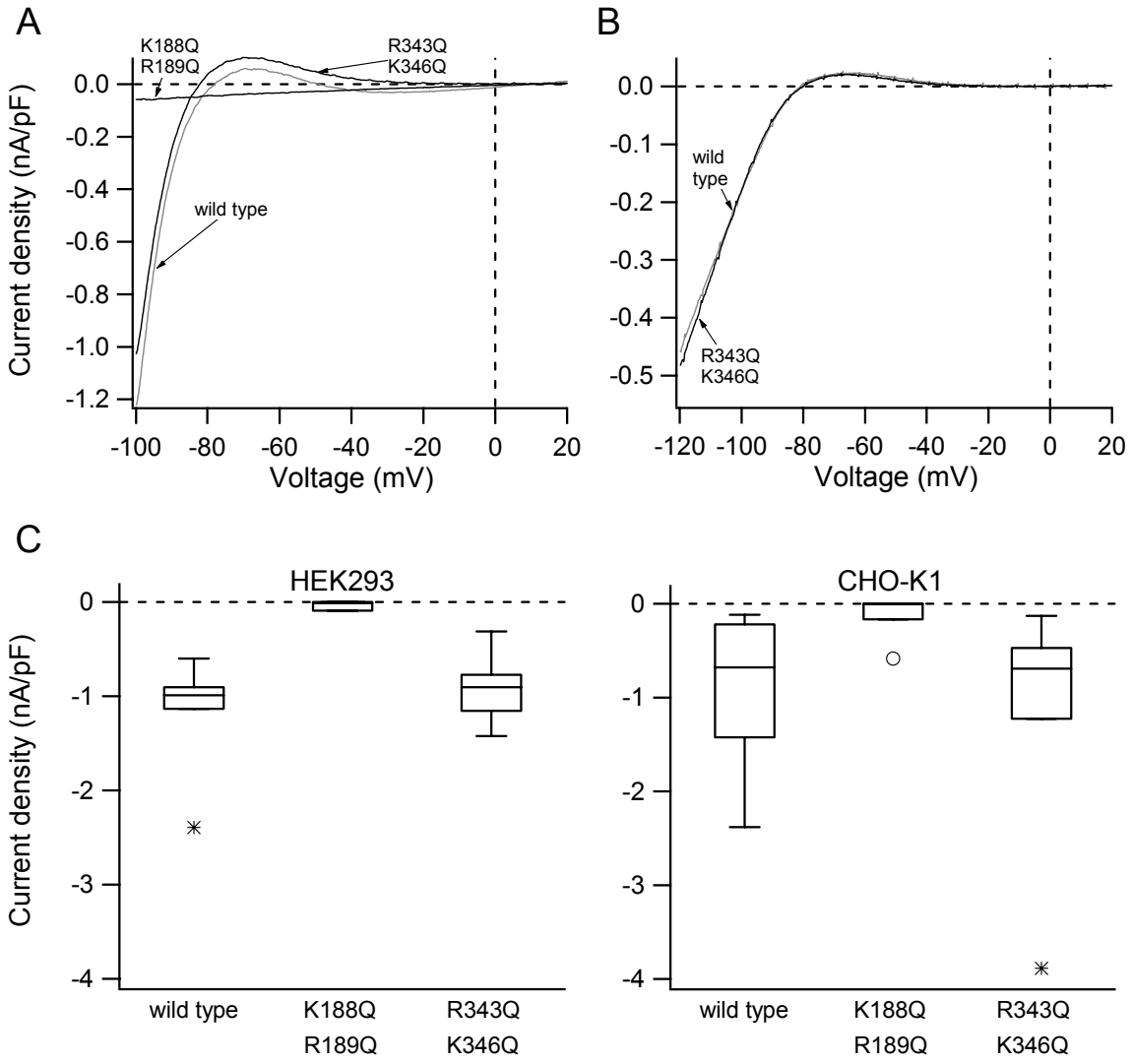


**Fig. 16.** Expression of EGFP-fused Kir2.1 channels in HEK293 and CHO-K1 mammalian cell lines.

**A:** Traces showing the whole-cell current densities in response to voltage ramps (from -100 to +50 mV in 300 ms) recorded from the HEK293 cells expressing the EGFP-fused wild-type Kir2.1 channels or the mutants K188Q·R189Q or R343Q·K346Q.

**B:** Whole-cell current density traces in response to voltage ramps (from -120 to +50 mV in 300 ms) recorded from the HEK293 cells coexpressing the EGFP and Kir2.1 wild-type or mutant R343Q·K346Q channels.

**C:** Box plots representing the statistics for the whole-cell current densities measured at -100 mV in HEK293 (left panel) or CHO-K1 (right panel) cells expressing the EGFP-fused wild-type Kir2.1 and the mutants K188Q·R189Q and R343Q·K346Q (n=5-6).

In the cells exhibiting plasma membrane fluorescence signal, the Kir2.1 currents were measured 24-48 hrs after transfection by the whole-cell patch-clamp method. The control mutant K188Q·R189Q only showed some residual current, consistent with its oocyte expression data. However, the R343Q·K346Q mutant expressed in both cell lines

showed, unlike in *Xenopus* oocytes, wild-type-like currents (Fig.16 A shows as an example the representative traces recorded from HEK293 cells). The same results were obtained when the cells were cotransfected with separate vectors containing sequences coding for untagged Kir2.1 and EGFP (pCDNA3-Kir2.1 and pEGFP-C1), indicating that fusing to EGFP did not affect the properties of the expressed channels (Fig.16 B).

In HEK293 cells the average current densities at -100 mV were -1.2 ± 0.31 nA/pF for the wild-type, -0.04 ± 0.03 nA/pF for the K188Q·R189Q, mutant and -0.91 ± 0.16 for the R343Q·K346Q mutant (n=5-6; Fig.16 C, left panel). In CHO-K1 cells the current densities were -0.92 ± 0.37 nA/pF for the wild-type Kir2.1, -0.15 ± 0.11 nA/pF for the K188Q·R189Q mutant and -1.18 ± 0.56 nA/pF for the R343Q·K346Q mutant (n=5-6; Fig.16 C, right panel).

Thus, residues R343 and K346 within the Kir2.1 protein segment C7 are important for $PI(4,5)P_2$ binding of this fragment *in vitro* and for expression of functional Kir2.1 channels in *Xenopus* oocytes. Neutralisation of these residues does not impair function of the Kir2.1 channels in the tested mammalian cell lines.

### 3.1.6. Comparison of the distal C-termini of Kir2.1 and Kir1.1 proteins.

As already mentioned, Kir2.1 channels have a higher affinity towards $PI(4,5)P_2$, compared to regulated Kir channels (Kir3.x or Kir6.x/SUR). Kir1.1 is another Kir channel with a similarily high affinity towards $PI(4,5)P_2$ (Huang et al., 1998). The distal C-terminus of Kir1.1 protein, approximately corresponding to the fragment C7-8 of Kir2.1 (Fig.17 A), is truncated in one of the Bartter's syndrome mutations (Δ331) and has been shown to be important for Kir1.1 channel opening. The Kir1.1 region homologous to Kir2.1 aminoacid residues 332-352 within the fragment C7 was found to be absolutely required for the Kir1.1 channel function in *Xenopus* oocyte expression system (Flagg et al., 1999). Another Bartter's syndrome mutation truncates the Kir1.1 protein from aminoacid residue 354 (corresponding to res. 355 in Kir2.1), but does not influence expression of functional Kir1.1 channels in *Xenopus* oocytes or mammalian cells (Jeck et al., 2001).

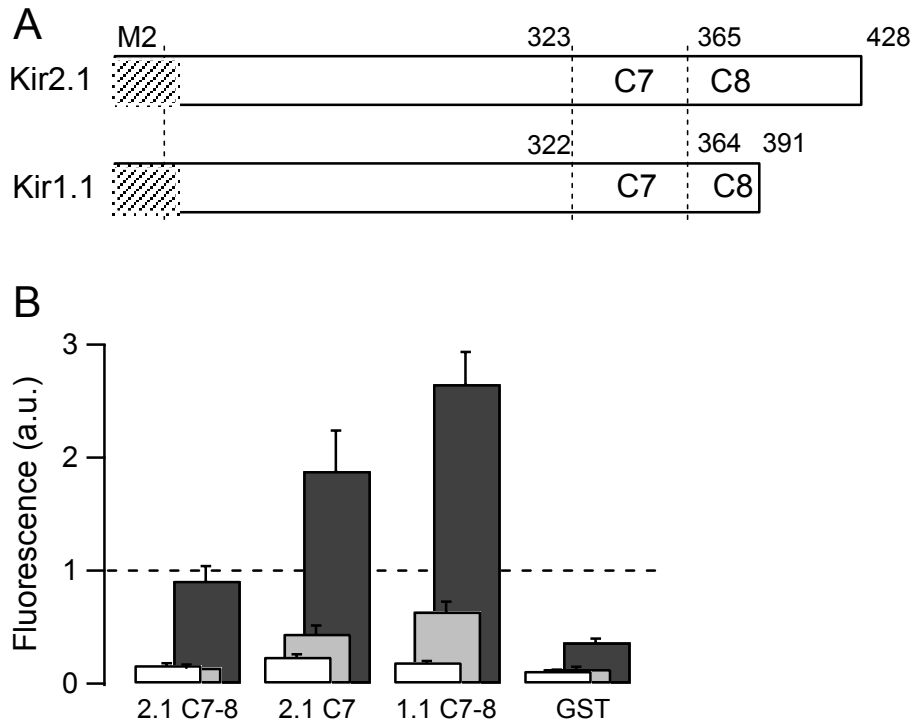


**Fig. 17.** Distal C-terminal portion of Kir1.1 protein binds to PI(4,5)$P_2$-containing liposomes.
**A:** Schematic presentation of cytoplasmic C-termini of Kir2.1 and Kir1.1 proteins. Numbers indicate the aminoacid positions. The part of M2 domain is indicated.
**B:** Binding of the Kir2.1 C7-8 (2.1 C7-8), Kir2.1 C7 (2.1 C7) and Kir1.1 C7-8 (1.1 C7-8) regions to liposomes without PI(4,5)$P_2$ (white) and with 5 mol% (light grey) or 35 mol% (dark grey) PI(4,5)$P_2$ (n=3).

The Kir1.1 distal C-terminus contains a region with concentrated positively charged aminoacid residues, like the homologous part of the Kir2.1 protein. However, in Kir1.1 this stretch of positive charges involves both regions corresponding to C7 and C8 of Kir2.1 protein, except the last 19 aminoacid residues of the Kir1.1 protein that contain the ER-export signal (Ma et al., 2001). Therefore, instead testing PI(4,5)$P_2$ binding of the separated Kir1.1 fragments C7 and C8, we tested the full Kir1.1 distal C-terminal fragment C7-8 in comparison with the Kir2.1 fragments C7-8 and C7. The results (Fig.17 B) showed that this region bound to PI(4,5)$P_2$/PC liposomes over the GST control both at low and high PI(4,5)$P_2$ concentrations. The binding of Kir1.1 C7-8 was stronger than the binding of the Kir2.1 region C7, but it increased in a similar way with an increase in PI(4,5)$P_2$ concentration from 5 mol% to 35 mol% (Fig.17 B). This indicates the Kir1.1 protein distal C-terminal portion as potentially able for interactions with PI(4,5)$P_2$, similarily to the region C7 of the Kir2.1 protein.

### 3.2. Identification of PI(4,5)$P_2$-binding regions in the cytoplasmic C-terminus of the Kir6.2 channel protein.

According to our data, the Kir2.1 channel protein has regions able to interact *in vitro* with PI(4,5)$P_2$ both in the proximal and distal C-terminus, and the Kir1.1 protein distal C-terminus also can bind PI(4,5)$P_2$. As mentioned above, the proximal cytoplasmic C-termini are conserved among Kir family proteins, whereas distal C-termini are variable. However, both proximal and distal parts of the C-terminus may be involved in regulation of some Kir channels by specific ligands. For example, in the Kir6.2 protein the aminoacid residues responsible for the Kir6.2/SUR channel ATP-inhibition have been identified both in the proximal and distal parts of the C-terminus. Kir6.2/SUR channels have a more complex regulation than Kir2.1 channels, as their PI(4,5)$P_2$-activation is antagonised by ATP-inhibition, and they have also lower affinity and specificity towards PI(4,5)$P_2$ (see "Introduction"). We attempted to find the regions able for physical interaction with PI(4,5)$P_2$ within the Kir6.2 protein with an approach analogous to our identification of PI(4,5)$P_2$-binding regions in the Kir2.1 protein.

#### 3.2.1. Screening the Kir6.2 cytoplasmic C-terminus for minimal PI(4,5)$P_2$-binding regions.

The C-terminus of Kir6.2 was divided into sections approximately corresponding to the fragments studied in the Kir2.1 protein (see Fig.18 A). The results of three individual experiments on PI(4,5)$P_2$ binding of these regions are shown in Fig.18 B-D, and the summarised data for the Kir6.2 C-terminal PI(4,5)$P_2$-binding regions are given in Fig.18 A. Two consecutive regions in the proximal C-terminus of the Kir6.2 protein (C1-2 and C3) bound PI(4,5)$P_2$ like the homologous fragments from the Kir2.1 C-terminus. A region homologous to C7 of Kir2.1 bound the liposomes containing 35 mol% PI(4,5)$P_2$ at the threshold border, and thus we did not consider it as substantially binding PI(4,5)$P_2$. Remarkably, the extreme Kir6.2 C-terminal fragment (C8) bound PI(4,5)$P_2$-containing liposomes.

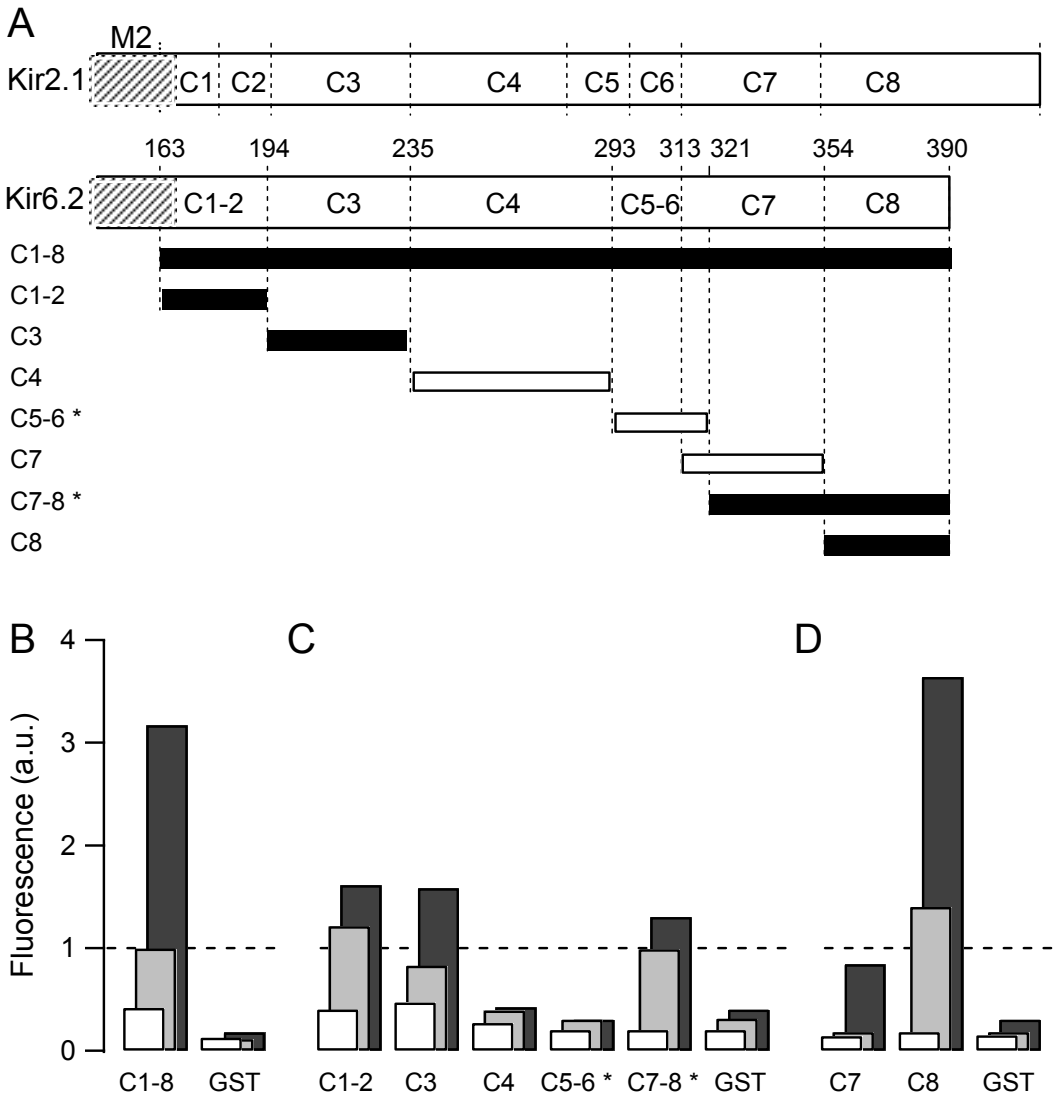


**Fig. 18.** Indentification of PI(4,5)$P_2$ binding domains in the Kir6.2 C-terminus.
**A:** Summary of the data from *in vitro* PI(4,5)$P_2$ binding of GST-fused Kir6.2 C-terminal fragments. Black bars indicate fragments binding to the liposomes with 35 mol% PI(4,5)$P_2$ above the threshold, white bars - fragments binding below the threshold. Kir2.1 and Kir6.2 cytoplasmic C-termini are schematically represented with indication of the sections of both proteins used in our study. Hatched area indicates a part of the M2 region.
* the border between these fragments is shifted towards the fragment C7 by 8 a.a.res..
**B-D:** Each panel shows the data from one PI(4,5)$P_2$ binding experiment with the GST fusions of the indicated Kir6.2 fragments. Data are shown for liposomes containing 35 mol % PI(4,5)$P_2$ (dark grey), 5 mol% PI(4,5)$P_2$ (light grey) and without PI(4,5)$P_2$ (white).

### 3.2.2. Comparison of the Kir6.2 C-terminal PI(4,5)$P_2$ binding regions.

The comparison of three PI(4,5)$P_2$ binding regions from Kir6.2 C-terminus (C1-2, C3, and C8) is shown in Fig.19 A. The fragment C8 had strongest PI(4,5)$P_2$-binding affinity, about 1.5 times higher than the affinity of the fragments C1-2 and C3, both at low (5 mol%) and high (35 mol%) PI(4,5)$P_2$ concentrations. Fig.19 B illustrates the differences in PI(4,5)$P_2$-binding of the corresponding distal C-terminal fragments from the Kir2.1 and Kir6.2 proteins. The Kir6.2 region C7 bound PI(4,5)$P_2$ weaker than the C7 region from the Kir2.1 protein, while the Kir6.2 fragment C8 was, in contrast to the Kir2.1 C8 region, binding strongly to PI(4,5)$P_2$-containing liposomes.

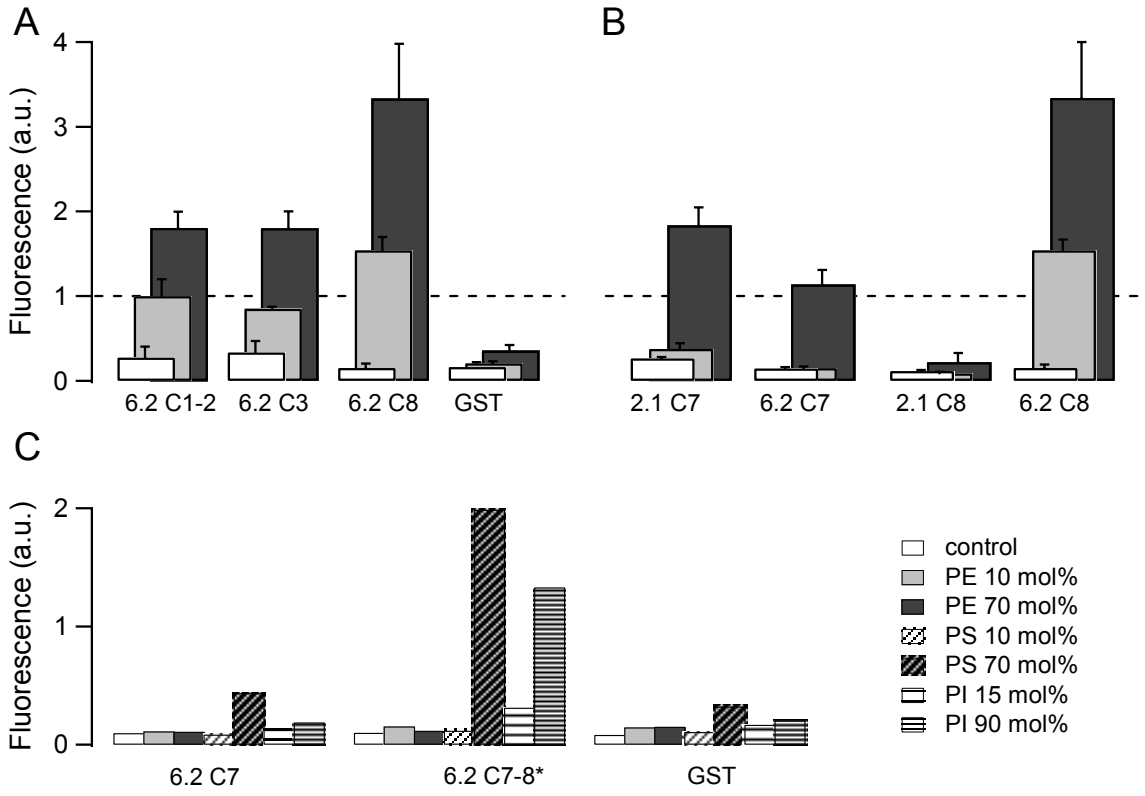


**Fig. 19.** The PI(4,5)$P_2$ binding regions in Kir6.2 C-terminus.
**A:** Comparison of GST-fused Kir6.2 PI(4,5)$P_2$ binding regions.
**B:** Comparison of PI(4,5)$P_2$ binding of relevant distal C-terminal fragments from Kir2.1 and Kir6.2. In **A, B** the labeling of the protein constructs and the liposomes is as in Fig.18. Data are represented as mean ± SEM (n=3-4).
**C:** Binding of the Kir6.2 distal C-terminal fragments to liposomes composed of 99 mol% PC : 1 mol% Rh-PE (control) and liposomes with PE, PS or PI partially substituting for PC at indicated concentrations.

To assay the binding specificity of the region C8 to different phospholipids, we tested the binding of the GST-fused fragments C7 and C7-8* (this fragment lacks 8 amino acid residues from the beginning of the C7 region, see Fig.18 A) to liposomes containing neutral phospholipid PE or anionic phospholipids PS or PI (Fig.19 C, the similar experiment as in Fig.13 C). Both fragments did not bind liposomes containing neutral PE. The fragment C7-8* bound liposomes containing 10 mol% PS or 15 mol% PI (a charge approximately equal to 3 or 5 mol% $PI(4,5)P_2$, respectively) at the level of the GST control, but bound strongly to liposomes with 70 mol% PS or 90 mol% PI (generating a charge approximately equal to 25 mol% or 30 mol% $PI(4,5)P_2$, respectively). Binding to the liposomes containing 70 mol% PS appeared to be stronger, possibly reflecting the above suggested better accessibility of its negative charge. As the fragment C7 did not bind any of the liposomes, the PI or PS binding of the fragment C7-8* is obviously acquired by the region C8.

Thus, the distribution of $PI(4,5)P_2$-binding regions in the Kir2.1 and Kir6.2 cytoplasmic C-termini is similar in the regions proximal to the second membrane-spanning domain, but differs in the distal parts. The isolated Kir6.2 extreme C-terminus is a strong $PI(4,5)P_2$-binding protein fragment, showing specificity towards $PI(4,5)P_2$ at low phospholipid concentrations, but potentially able for interactions with other anionic phospholipids, like PI or even a non-inositol phospholipid PS.

### 3.3. The cytoplasmic N-termini of Kir2.1 and Kir6.2 proteins bind $PI(4,5)P_2$.

Most of the recent studies on Kir-$PI(4,5)P_2$ interplay have assumed that Kir channels interact with $PI(4,5)P_2$ via their cytoplasmic C-termini. We found the Kir2.1 GST fused N-terminal cytoplasmic domain containing aminoacid residues 1-86 (segment N1-5 in Fig.20A) to bind liposomes containing 35 mol% $PI(4,5)P_2$ at the border of threshold, although stronger than the GST-control (Fig.20 B). Comparison with the corresponding Kir6.2 N-terminal fragment N1-5 (res. 1-74, see Fig.20 A) showed that the Kir6.2 fragment bound the liposomes with the same $PI(4,5)P_2$ concentration markedly stronger (Fig.20 B). However, both GST fused Kir2.1 and Kir6.2 N1-5 fragments produced proteins with low solubility, difficult to purify and to handle in biochemical assays, most possibly due to the presence of the hydrophobic region M0 (Fig.20 A, see "Introduction").

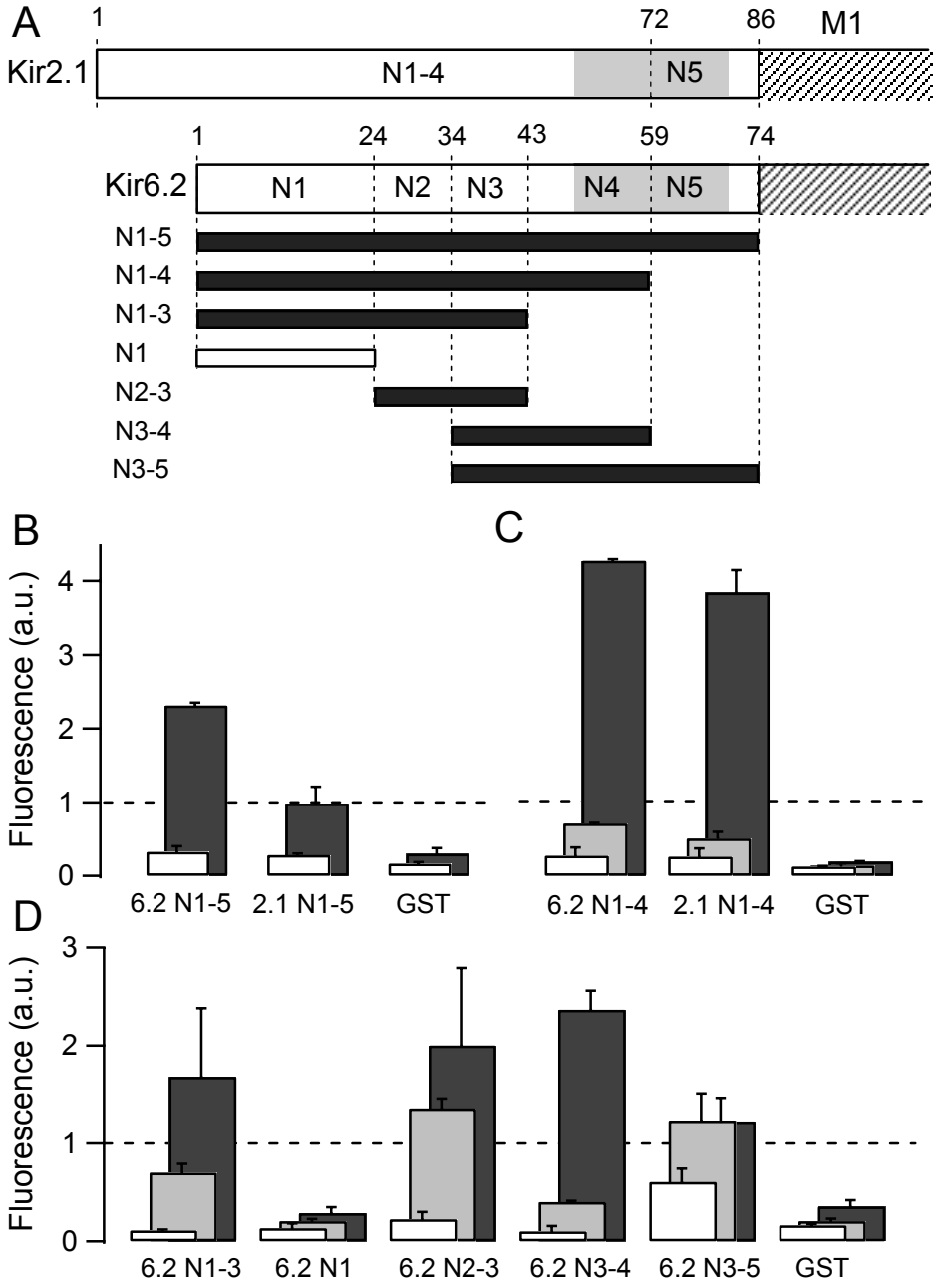


**Fig. 20.** Screening of the Kir6.2 N-terminus for PI(4,5)$P_2$ binding regions.
**A:** Schematic representation of Kir2.1 and Kir6.2 cytoplasmic N-termini with indication of the sections of both proteins used in our study. The M0 region is shaded in grey; a part of the M1 region is hatched. Summary of the data on *in vitro* PI(4,5)$P_2$ binding of Kir6.2 N-terminal fragments is represented with indication of fragments as in Fig.16 A.
**B, C:** Comparison of PI(4,5)$P_2$-binding of the corresponding GST fused N-terminal fragments from Kir6.2 and Kir2.1 proteins.
**D:** PI(4,5)$P_2$-binding of the GST fused Kir6.2 N-terminal fragments.
In **B-D** liposomes are indincated as in Fig.18. Data are represented as mean ± SEM (n=3).

To obtain proteins with higher solubility, we constructed GST fusions of the N-terminal fragments that leave out the part of M0 region proximal to the M1 domain

(fragments N1-4 in Fig.20 A), and tested their $PI(4,5)P_2$ binding (Fig.20 C). Both constructs showed a significantly higher $PI(4,5)P_2$ binding than the previous ones, with Kir2.1 N1-4 fragment binding only slightly weaker than the relevant Kir6.2 fragment both at low and high $PI(4,5)P_2$ concentrations. Thus, the presence of the hydrophobic region N5 in the N-terminal constructs reduces *in vitro* $PI(4,5)P_2$ binding of the resulting proteins. As the Kir6.2 N-terminal domain showed a stronger binding to the liposomes, and some unpublished observations suggested the involvement of the Kir6.2 cytoplasmic N-terminal residues into channel regulation by $PI(4,5)P_2$ (T. Baukrowitz, FSU Jena), we concentrated on it to identify minimal $PI(4,5)P_2$ binding regions.

The results of $PI(4,5)P_2$-binding experiments of GST fused overlapping fragments of Kir6.2 N-terminus are shown in Fig.20 D and the data are summarised in Fig.20 A. The only fragment unable to bind liposomes with $PI(4,5)P_2$ was the first third of the N-terminus (region N1), whereas the fragments containing the rest of the N-terminal domain bound $PI(4,5)P_2$ above the GST control already at 5 mol% $PI(4,5)P_2$. Consistent with the inhibitory role of the fragment N5, $PI(4,5)P_2$ binding of the fragment N3-5 did not become stronger with increasing $PI(4,5)P_2$ concentration in the liposomes from 5 mol% to 35 mol%. At the same time, at low $PI(4,5)P_2$ concentration (5 mol%) the fragment N3-5 showed a higher $PI(4,5)P_2$-specific liposome binding than the fragment N3-4, indicating that under some conditions the fragment N5 could increase $PI(4,5)P_2$-binding.

Although the region N4 contains the part of hydrophobic M0 domain (Fig.20 A), it seems to enhance $PI(4,5)P_2$-binding in the absence of the region N5. Average liposome binding of the fragments N1-4 (Fig.20 C) and N3-4 (Fig.20 D) increased steeper with increase in $PI(4,5)P_2$ concentration than binding of fragments N1-3 and N2-3. The isolated GST fused fragment N4-5 could not be studied, as it had a very low solubility and did not give reproducible results in the liposome binding assay.

The regions N2 to N5 are too short (less than 20 aminoacid residues) to be studied as individual GST-fusions. For that reason we attempted to further examine the $PI(4,5)P_2$ binding of the Kir6.2 N-terminus by site-directed mutagenesis. Several residues located in the Kir6.2 region N3-4 were shown to play a role in regulation of Kir6.2/SUR channels by ATP (Tucker et al., 1998) or by $PI(4,5)P_2$ (T. Baukrowitz, personal communication). We studied two single point-mutations within this region that are known to produce different effects on the Kir6.2 $PI(4,5)P_2$ affinity *in vivo*.

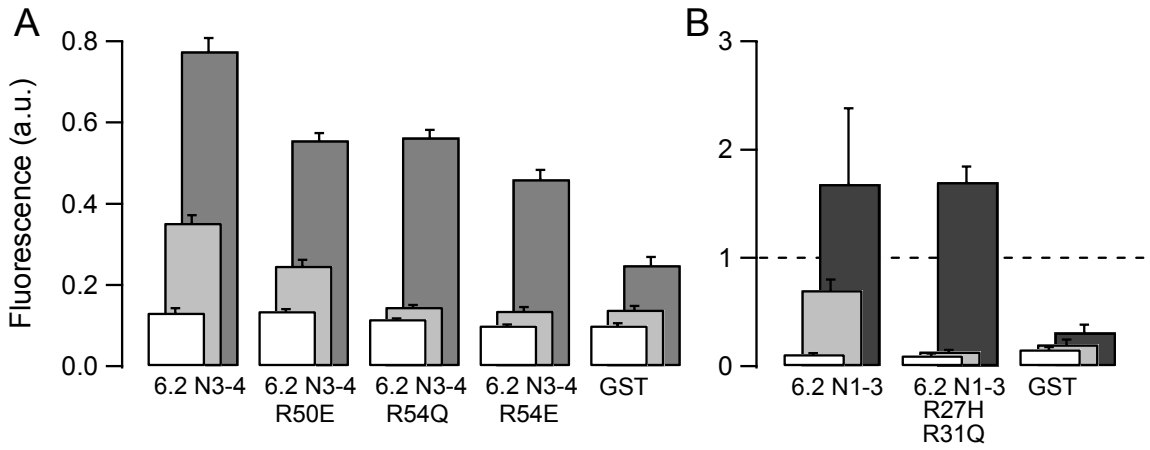


**Fig. 21.** Influence of point mutations in Kir6.2 N-terminal fragments on PI(4,5)$P_2$ binding.
**A:** Comparison of binding of Kir6.2 N3-4 fragment (6.2 N3-4) and the corresponding fragments with mutations R50E (6.2 N3-4 R50E), R54Q (6.2 N3-4 R50Q) and R54E (6.2. N3-4 R54E) to liposomes without PI(4,5)$P_2$ (white) and containing 10 mol% (light grey) or 20 mol% (grey) PI(4,5)$P_2$. Data are presented as mean ± SEM of three parallel experiments.
**B:** Comparison of Kir6.2 N1-3 fragment (N1-3) and the relevant R27H·R31Q mutant (N1-3 R27H·R31Q) binding to liposomes without PI(4,5)$P_2$ (white) and containing 5 mol% (light grey) or 35 mol% (dark grey) PI(4,5)$P_2$ (mean ± SEM, n=3).

A conserved arginine residue R54 was mutated to glutamine (R54Q) or glutamate (R54E). These mutations have been shown to weaken Kir6.2-PI(4,5)$P_2$ interaction in inside-out membrane patches when expressed in *Xenopus* oocytes, with R54E producing a stronger effect than R54Q (T. Baukrowitz, personal communication). The mutation of another arginine residue to glutamate (R50E) does not change the channel's PI(4,5)$P_2$ affinity *in vivo*, antagonising only with the ATP-inhibition of the channel. When this mutation was introduced into the GST-fused fragment N3-4, the binding to the liposomes containing 20 mol% or 10 mol% PI(4,5)$P_2$ was decreased to about 2/3 of the wild-type protein, although it remained higher than the GST-control (Fig.21 A). Mutations of arginine residue in position 54 also decreased PI(4,5)$P_2$ binding, but to a different degree. At 20 mol% PI(4,5)$P_2$ mutant R54Q had the same binding as mutant R50E, whereas mutant R54E bound slightly weaker. At 10 mol% PI(4,5)$P_2$ binding of both mutants did not exceed the level of the GST control (Fig.21 A). Thus, under some conditions (10 mol% PI(4,5)$P_2$) the mutations in position 54 produced a stronger effect than the mutation in position 50, eliminating the PI(4,5)$P_2$-binding of the studied Kir6.2 N-terminal fragment.

The Kir6.2 fragment N2-3 showed, along with the fragment N3-4, a high level of PI(4,5)$P_2$-binding. Alignment of the Kir6.2 and Kir2.1 proteins shows that the Kir6.2 fragment N2-3 contains only two non-conserved positively charged aminoacid residues (arginines R27 and R31). To check if these charges could be responsible for higher *in vitro* PI(4,5)$P_2$ sensitivity of the Kir6.2 N-terminal domain, we mutated these residues within the N1-3 fragment to histidine and glutamine residues as found in the corresponding positions in the Kir2.1 protein. The resulting N1-3 R27H·R31Q mutant protein was assayed with PI(4,5)$P_2$-containing liposomes in comparison with the wild-type N1-3 fragment. As Fig.21 B shows, the binding of the mutant protein to the liposomes containing 35 mol% PI(4,5)$P_2$ was at the wild-type level, but did not exceed the binding of the GST-control at 5 mol% PI(4,5)$P_2$. When the Kir6.2 R27H·R31Q mutant was expressed in *Xenopus* oocytes together with the SUR2A subunit, it produced robust currents similar to or higher than the wild-type Kir6.2 currents that did not differ from the wild-type in PI(4,5)$P_2$ sensitivity in inside-out membrane patches (T. Baukrowitz, personal communication). It appears as if the effect of this mutation on reducing *in vitro* PI(4,5)$P_2$ sensitivity is not significant for a physiological PI(4,5)$P_2$ response.

### 3.4. PI(4,5)$P_2$ binding by distal C-termini of $G_{\beta\gamma}$-regulated Kir channel proteins.

Having identified PI(4,5)$P_2$ binding regions in the distal C-termini of Kir2.1, Kir1.1 and Kir6.2 proteins, we questioned whether the distribution of distal PI(4,5)$P_2$ binding regions could be dependent on the presence of other regulatory regions in Kir channels. The Kir3.1 and Kir3.2 proteins form the tetraheteromeric $G_{\beta\gamma}$-regulated Kir channels in brain. It has been shown that the C-termini of Kir3.1 and Kir3.2 proteins interact physically with $G_{\beta\gamma}$, although experiments on pointing out the exact site of this interaction in Kir3.1 protein have led to somewhat controversal results. Several studies have identified the Kir3.1 fragments overlapping in the region corresponding to the segment C7 of the Kir proteins examined in this study as important for $G_{\beta\gamma}$-binding *in vitro* or *in vivo*. Additionally, the unique distal C-terminal region of the Kir3.1 protein has been supposed to confer a higher affinity towards $G_{\beta\gamma}$. However, some studies have suggested that this unique distal C-terminus is not required for the $G_{\beta\gamma}$-activation of the native cardiac Kir3.1/Kir3.4 heteromultimers, but instead might act as an inhibitor of these channels and

determine the intracellular retention of the Kir3.1 protein expressed without a heteromerisation partner (see "Introduction").

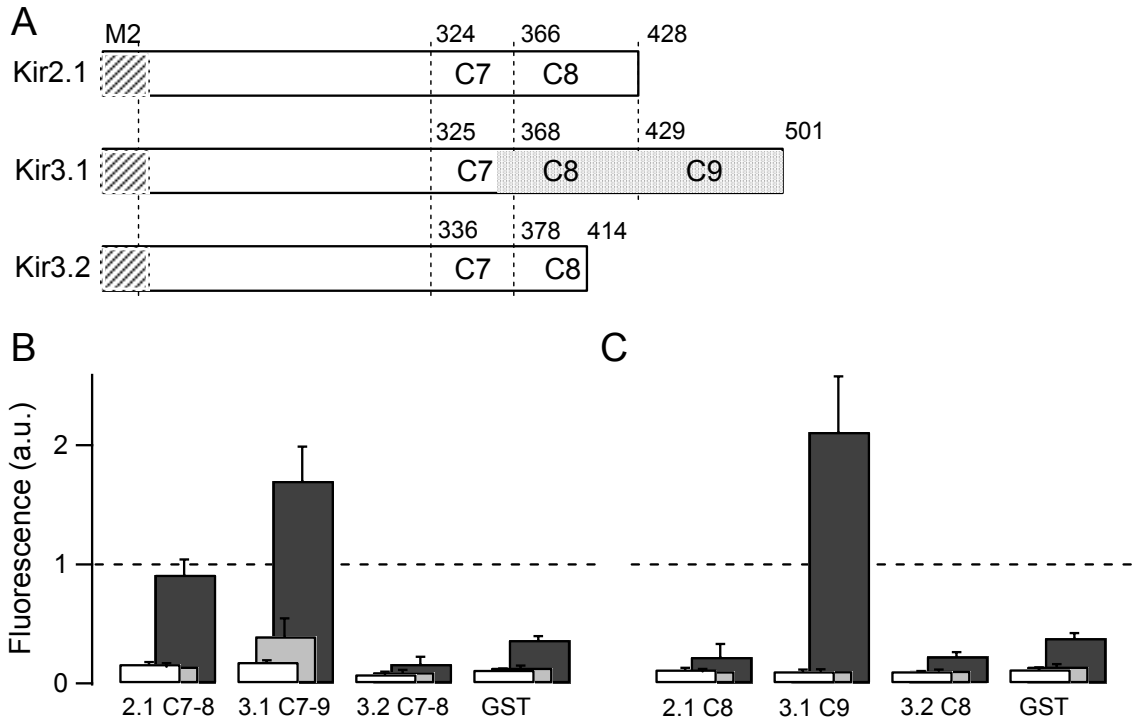


**Fig. 22.** Comparison of PI(4,5)$P_2$ binding of distal C-terminal fragments of Kir2.1, Kir3.1 and Kir3.2 channel proteins.
**A:** Schematic representation of cytoplasmic C-termini of the Kir2.1, Kir3.1 and Kir3.2 proteins. Numbers indicate the aminoacid positions. A part of the M2 domain is hatched; the unique C-terminal part of Kir3.1 protein is shaded in grey.
**B:** Liposome binding of the Kir2.1 C7-8 region (2.1 C7-8) and the corresponding regions from Kir3.1 (3.1 C7-9) and Kir3.2 (3.2 C7-8) proteins.
**C:** Liposome binding of the extreme C-terminal fragments from Kir2.1 (2.1 C8), Kir3.1 (3.1 C9) and Kir3.2 (3.2 C8) proteins.
In **B,C** proteins were assayed to the liposomes without PI(4,5)$P_2$ (white) and with 5 mol% (light grey) or 35 mol% (dark grey) PI(4,5)$P_2$ (n=3).

Binding to $G_{\beta\gamma}$ subunits has been proposed to stabilise the open state of heteromeric Kir3.1/3.4 or homomeric Kir3.2 channels and, thus, increase their affinity towards PI(4,5)$P_2$ (Huang et al., 1998). We constructed the GST fusions of distal C-terminal fragments of Kir3.1 and Kir3.2 proteins starting from the region corresponding to the Kir2.1 C7 and up to the end of the protein (Fig.22 A) and tested the resulting fragments for *in vitro* PI(4,5)$P_2$ binding (Fig.22 B). The relevant region from the Kir3.1 protein (C7-9)

bound PI(4,5)$P_2$-containing liposomes with a higher affinity than the Kir2.1 C7-8 region, whereas the Kir3.2 protein fragment C7-8 did not show any binding over the GST control level.

The GST-fusions of the fragments C7 from Kir3.1 and Kir3.2, and also the fragment C8 from Kir3.1 had low solubility, and thus it was not possible to test them for PI(4,5)$P_2$ binding with reproducible results. The only individual fragments we could compare were the extreme C-terminal portions of the Kir2.1, Kir3.1 and Kir3.2 proteins (fragments 2.1 C8, 3.1 C9 and 3.2 C8, respectively). The results presented in Fig.22 C show that, while the Kir2.1 and Kir3.2 C8 fragments did not bind PI(4,5)$P_2$ containing liposomes, the extreme part of the unique distal C-terminus of Kir3.1 (fragment C9) bound liposomes containing 35 mol% PI(4,5)$P_2$. As this fragment did not bind liposomes with 5 mol% PI(4,5)$P_2$, we can suggest that other regions in the Kir3.1 distal C-terminus (fragments C7 and C8) are also involved in the observed *in vitro* PI(4,5)$P_2$-binding of the Kir3.1 distal C-terminal region C7-C9.

Considering the existing evidence for the increase of the Kir3.x channels' PI(4,5)$P_2$ affinity upon activation by $G_{\beta\gamma}$, we wanted to check if binding of the distal C-terminal portion of the Kir3.1 protein to $G_{\beta\gamma}$ subunits could influence its interaction with PI(4,5)$P_2$. Kir2.1 channels are not activated by $G_{\beta\gamma}$ and the GST fused cytoplasmic C-terminal domain of this protein has been reported not to bind recombinant $G_{\beta\gamma}$ subunits in a biochemical assay (Huang et al., 1995; Zhang et al., 1999). We tested whether the addition of $G_{\beta\gamma}$ could increase the affinity of GST-fused Kir3.1 distal C-terminus (C7-9; res. 325-501) towards PI(4,5)$P_2$-containing liposomes in comparison with the relevant GST-fused Kir2.1 region (C7-8, res. 324-428). These proteins were assayed with liposomes containing 35 mol% PI(4,5)$P_2$, as at this concentration they both bound to liposomes above the GST level (see Fig.22 B). PI3Kγ has been shown to interact directly not only with PI(4,5)$P_2$ but also with $G_{\beta\gamma}$ subunits of heterotrimeric G-proteins, presumably via two distinct contact sites (Leopoldt et al., 1998), and therefore in these experiments we used GST-PI3Kγ as a positive control. The mean data of two independent experiments are shown in Fig.23.

GST (negative control) did not show substantial binding to PI(4,5)$P_2$-containing liposomes independently on the presence of $G_{\beta\gamma}$. GST-PI3Kγ interacted strongly with liposomes containing 35 mol% PI(4,5)$P_2$ and $G_{\beta\gamma}$ did not markedly alter the binding strength. However, when GST-PI3Kγ was assayed with liposomes containing only 5 mol% PI(4,5)$P_2$, binding in the presence of $G_{\beta\gamma}$ was about 1.5 times stronger, reaching now the

level of binding to liposomes with 35 mol% $PI(4,5)P_2$ (Fig.23, rightmost bar). At 35 mol% $PI(4,5)P_2$ binding of Kir3.1 fragment C7-9 to the liposomes was approximately 1.6 times stronger than of the Kir2.1 fragment C7-8, but still about 2 times weaker than binding of the PI3Kγ control at the same $PI(4,5)P_2$ concentration. Binding of both the Kir2.1 and Kir3.1 fragments increased in the presence of $G_{\beta\gamma}$ subunits, but this increase was more pronounced in the latter case. The Kir3.1 fragment C7-9 now bound to liposomes about 2.1 times stronger compared to the Kir2.1 fragment C7-8, and reached the level of PI3Kγ binding (Fig.23).

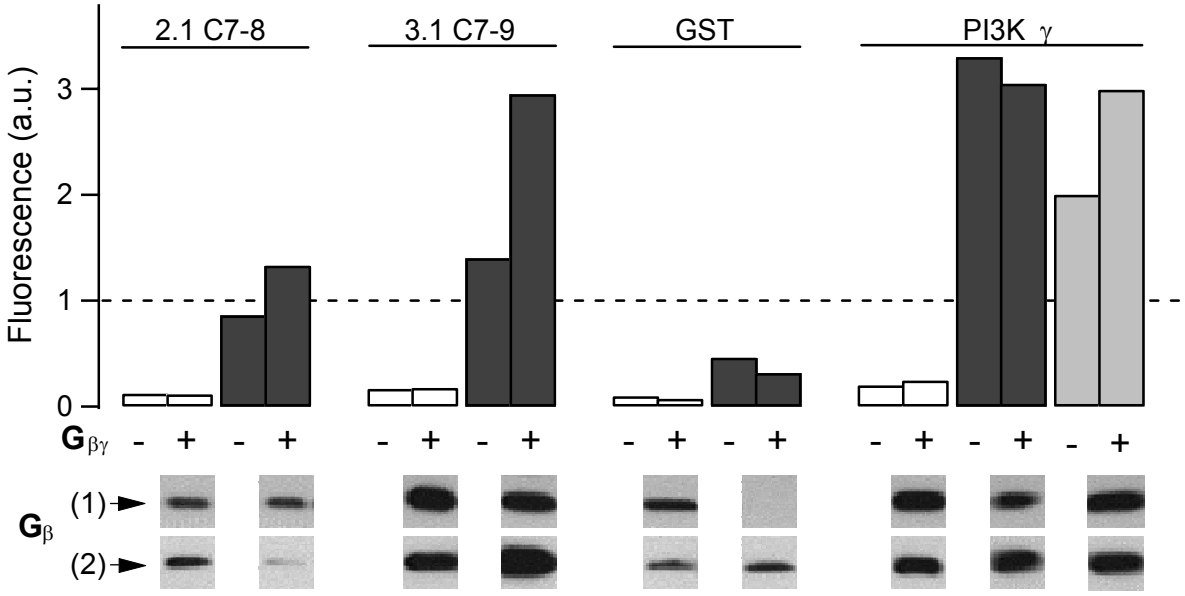


**Fig. 23.** Binding of the distal C-termini from Kir2.1 (2.1 C7-8) and Kir3.1 (3.1 C7-9) to liposomes without (white) and with 35 mol% (dark grey) $PI(4,5)P_2$. The positive control PI3Kγ was additionally tested for binding with lipsomes containing 5 mol% $PI(4,5)P_2$ (light grey). Binding was assayed in the absence (-) and presence (+) of 0.1μM recombinant $G_{\beta1\gamma2}$ subunits ($G_{\beta\gamma}$). Mean data from two independent experiments are shown. The reactions from both liposome binding experiments indicated as (1) and (2) were probed on one Western blot. The corresponding sections of the Western blots with $G_\beta$ immunoreactivity (indicated by an arrow) are shown below the bars.

After estimating the amount of fluorescence bound to the Sepharose-immobilised proteins, the samples from the reactions where $G_{\beta\gamma}$ was added were probed with an antibody against $G_\beta$ to check if the binding of $G_{\beta\gamma}$ to the tested proteins did occur. The resulting Western blots of the samples from both experiments showed some variations of the $G_\beta$ signal between the experiments, liposomes and proteins. In most cases the background signal of a similar strength was present in samples with GST and GST fused Kir2.1 C7-8 (Fig.23). Possibly, liposomes influenced the properties of the assayed

proteins, increasing the background $G_{\beta\gamma}$ binding level. Nevertheless, this background signal was always weaker than the signal found in GST fused Kir3.1 C7-9 and PI3Kγ samples both in the presence or absence of $PI(4,5)P_2$ in the liposomes. It should be noted that the $G_\beta$ signal in GST-PI3Kγ samples was not stronger than the signal in GST-Kir3.1 C7-9 samples, although PI3Kγ has two sites for $G_{\beta\gamma}$ interaction (see above). An explanation for this could be that as the weight/volume protein concentrations were adjusted in the assays, the final molar concentration of GST-PI3Kγ appeared to be 3 times lower than of GST-Kir3.1 C7-9. However, these results show that the positive control PI3Kγ and the distal C-terminal fragment (C7-9) of the Kir3.1 protein bound $G_{\beta\gamma}$ in the presence of liposomes. The greater increase in $PI(4,5)P_2$-binding of the Kir3.1 C7-9 versus Kir2.1 C7-8 fragment in the presence of $G_{\beta\gamma}$ points to an interplay of $G_{\beta\gamma}$- and $PI(4,5)P_2$-interactions. In the case of PI3Kγ control, the basal affinity towards liposomes with 35 mol% $PI(4,5)P_2$ was probably already too strong to detect the further increase in the presence of $G_{\beta\gamma}$. It could also be possible that $G_{\beta\gamma}$ bound the $PI(4,5)P_2$-containing liposomes, but given a significant molar excess of the GST-fused proteins over $G_{\beta\gamma}$ (more than 30-fold) in our assay, it seems unlikely that an increase in liposome signal bound to the proteins could be due to additional pull-down of liposomes by the $G_{\beta\gamma}$ subunits. Taken together, these experiments show that the Kir3.1 distal C-terminal region is able to bind simultaneously $PI(4,5)P_2$ and recombinant $G_{\beta\gamma}$ subunits. These two ligands seem not to compete with each other, but instead, $G_{\beta\gamma}$ enhances $PI(4,5)P_2$-binding of the indicated region.

# 4. Discussion.

## 4.1. Studying protein - PI(4,5)$P_2$ interactions *in vitro*.

The method used in this study for determining interactions between fragments of Kir channel proteins and various phospholipids was the *in vitro* binding assay with mixed liposomes. One of the questions that arise while discussing the obtained results is how reliable this method is for identifying protein regions able for interactions with, for example, PI(4,5)$P_2$. Binding to PI(4,5)$P_2$-containing liposomes could be simply a function of the overall protein charge, and not a result of specific interactions. Since the exact PI(4,5)$P_2$ concentration that Kir channels meet in the plasma membrane is unknown, we have used, whenever possible, both liposomes with high (35 mol%) and low (5 or 10 mol%) PI(4,5)$P_2$ concentration to estimate binding to the GST-fusion proteins. During our study, GST, GST-PI3Kγ and 36 GST-fusions with N- and C-terminal fragments of Kir channel proteins were assayed with liposomes containing PI(4,5)$P_2$ at both concentrations. The studied proteins varied in their molecular weights. Therefore, to compare these proteins by their charges, a value termed as "protein charge density" was calculated for each protein as follows:

protein charge density = theoretical charge at pH 7.4 / theoretical molecular weight

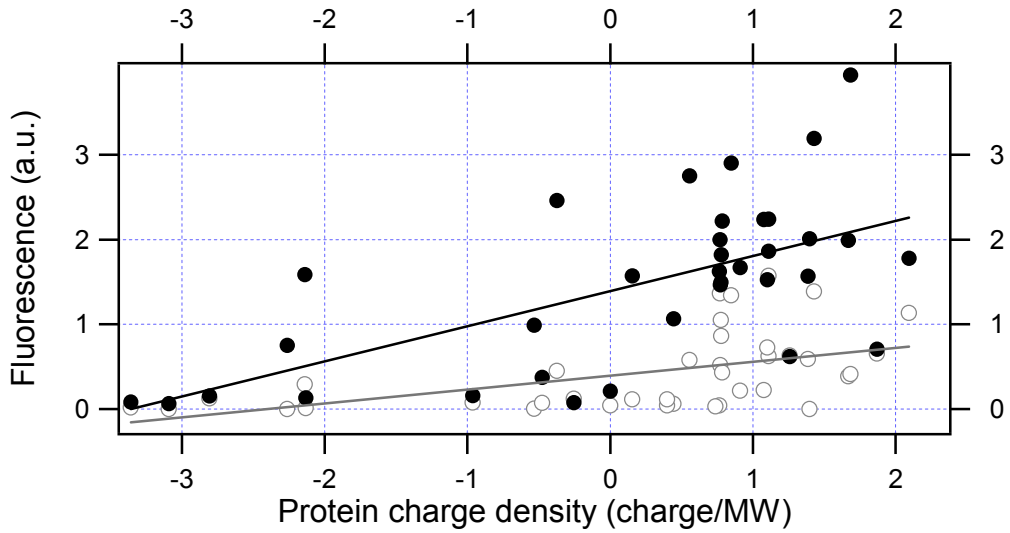


**Fig. 24.** A scatter diagram showing the correlation between charge density of the studied proteins and the amount of fluorescence signal from bound liposomes containing 5 or 10 mol% PI(4,5)$P_2$ (open grey circles) or 35 mol% PI(4,5)$P_2$ (filled black circles). The charge density of GST was substracted from the charge density calculated for each GST-fusion protein; binding to control liposomes without PI(4,5)$P_2$ was substracted from binding to PI(4,5)$P_2$-containing liposomes.

For example, the GST-fused protein fragments Kir2.1 (175-428) and Kir3.2 (378-414) have the same charge density (-2.13), but the first fragment, being about 7 times longer than the second one, contains regions with clustered positive charges, and thus shows a stronger PI(4,5)$P_2$ binding than the second one. Possibly for the same reason, the negatively charged GST-fused Kir2.1 (175-428) fragment shows PI(4,5)$P_2$-binding similar to the positively charged GST-fused Kir6.2 (1-43) fragment (charge density +1.39). As we have already seen for Kir6.2 N-terminal fragments, hydrophobic regions may reduce the protein binding to liposomes with high PI(4,5)$P_2$ concentration. Thus, the PI(4,5)$P_2$ binding observed in the liposome assay is not determined solely by the overall charge of the protein, but depends also on charge distribution within the protein molecule and the presence of hydrophobic regions. This combination of electrostatic and hydrophobic effects has been shown to determine interaction of several natural and model peptides with experimental membranes (reviewed by Cafiso, 1999). To what extent are the data obtained *in vitro* reflecting the *in vivo* situation? The examples where the biochemical data do not fall together with the physiological evidences will be discussed below and mostly concern the mutational analysis of the channel proteins. Although isolated protein fragments fused to GST may adopt conformations changing their lipid-binding properties compared to intact protein, the *in vitro* liposome assay is useful for identifying protein regions potentially able for interaction with different phospholipids.

### 4.2. Role of cytoplasmic domains of Kir channel proteins in Kir channel function and PI(4,5)$P_2$-response.

#### 4.2.1. Region I in the cytoplasmic C-terminus proximal to the second transmembrane domain.

The role of the positively charged cytoplasmic C-terminal region proximal to the second transmembrane domain (region I in Fig.3, see "Introduction") in the channel's response to phosphoinositides has been demonstrated for several Kir channels. Mutations in this region led to an increased inhibition of the Kir1.1 channel by PI(4,5)$P_2$ antibodies (Huang et al., 1998; Zeng et al., 2002) and decreased the Kir6.2/SUR1 channel sensitivity to PI(4,5)$P_2$ or phoshpoinositides (Fan & Makielski, 1997; Baukrowitz et al., 1998; Shyng & Nichols, 1998; Shyng et al., 2000b). As already mentioned, the direct effect on PI(4,5)$P_2$ binding was shown for the mutation of the highly conserved arginine residue at position 188 of Kir1.1 channel protein to glutamine (R188Q) (Huang et al., 1998). Mutation of the

homologous residue in Kir6.2 protein to alanine (R177A) resulted in closed channels when mutated Kir6.2 subunits were coexpressed with SUR1 subunits in mammalian cells (Shyng & Nichols, 1998). These channels did not conduct any $K^+$ current, but could be rescued by coexpression with active Kir6.2 subunits, producing channels with altered $PI(4,5)P_2$ sensitivity (Shyng et al., 2000b). In our study, mutation of the homologous residue in Kir2.1 channel protein to glutamine (R189Q) resulted in channels conducting no current, and also decreased the binding of the proximal C-terminal fragment to $PI(4,5)P_2$-containting vesicles (fragment C1-2; aminoacid residues 175-206). Mutation of the neighbouring lysine residue (K188Q) in Kir2.1 led to the channels conducting only some residual current when expressed in *Xenopus* oocytes and also decreased the *in vitro* $PI(4,5)P_2$-binding of the relevant C-terminal fragment. The arginine residue in the homologous position in Kir6.2 protein has been identified as important for Kir6.2/SUR1 channel expression and $PI(4,5)P_2$-response in several studies. Mutation of this residue to alanine (R176A) reduces the intrinsic activity and slows the $PI(4,5)P_2$-response of the channels expressed in *Xenopus* oocytes (Baukrowitz et al., 1998) or in mammalian cells (Shyng & Nichols, 1998; Shyng et al., 2000b). In the latter study an increase of the current of the mutant channel was observed with $PI(4,5)P_2$-addition. A more severe effect on the Kir6.2/SUR1 channel expression was observed with changing this arginine residue to glutamic acid (R176E) that resulted in closed channels when expressed in mammalian cells (Fan & Makielski, 1997).

The effect of the double mutation of these adjacent positively charged residues to alanines (R176A·R177A) in Kir6.2 was a significant decrease in the Kir6.2/SUR1 current in mammalian cells and slowing of the channel's response to polyphosphoinositides (Fan & Makielski, 1997). According to our results, when homologous positively charged residues were mutated together in Kir2.1 channels (K188Q·R189Q), the resulting mutant channels did not produce any specific Kir current in *Xenopus* oocytes and mammalian cells. The subcellular distribution of the mutant channels in mammalian cells was not affected. This mutation produced a much greater decrease in *in vitro* $PI(4,5)P_2$ binding of the proximal C-terminal Kir2.1 protein fragment than single mutations. This could indicate the cooperative role of K188 and R189 residues in $PI(4,5)P_2$ binding of the Kir2.1 proximal cytoplasmic C-terminus. Alternatively, the effect on channel functional expression produced already by single mutations could mean that even a weak reduction in $PI(4,5)P_2$ binding of this region observed *in vitro* is physiologically significant. Although it

is difficult to compare the results of mutations in different Kir channels (Kir2.1, Kir1.1, and Kir6.2) obtained in different expression systems (*Xenopus* oocytes and mammalian cells) and with different mutagenesis, our data support the role of the discussed residues in Kir channel open state stabilisation and/or $PI(4,5)P_2$-interaction.

Our biochemical data show that the proximal C-terminal fragments of Kir2.1 and Kir6.2 channel proteins (fragments C1-2 in Fig.13 A) that include region I of the cytoplasmic C-terminus, bind substantially to liposomes containing $PI(4,5)P_2$ both at low and high concentrations. At least for the Kir2.1 fragment this binding includes a significant electrostatic component, as the strength of its interaction with PI, PI(4)P and $PI(4,5)P_2$ depends on the number of negative charges on the phosphoinositol headgroup. These data strengthen the earlier hypothesis that electrostatic mechanism plays an important role in the interaction of the positive charge cluster adjacent to the Kir protein second transmembrane domains with $PI(4,5)P_2$ and possibly other plasma membrane phosphoinositides. However, the effect of the P186A mutation, that does not change the charge of the Kir2.1 protein, but leads to decrease in Kir2.1 channel functional expression and *in vitro* $PI(4,5)P_2$-affinity, is pointing to the role of non-electrostatic interactions or conformational constraints in $PI(4,5)P_2$ binding.

### 4.2.2. Other regions in the proximal cytoplasmic C-terminus.

The first 70 aminoacid residues of the cytoplasmic C-termini of Kir2.1 and Kir6.2 proteins contain two consecutive fragments binding independently $PI(4,5)P_2$-containing liposomes. These are fragments C1-2 (containing region I of the cytoplasmic C-terminus) and C3 (corresponding to region II of the cytoplasmic C-terminus). These consecutive fragments had almost the same affinity in binding liposomes composed of PC and $PI(4,5)P_2$. The downstream region from the Kir2.1 protein (C3, aminoacid residues 207-246) was less selective towards phosphoinositides, showing also strong binding to liposomes containing non-inositol phospholipid PS. Such a high potential for electrostatic interactions could explain the identification of this region as responsible for high $PI(4,5)P_2$ affinity of Kir2.1 channels (Zhang et al., 1999). These data support the physiological observations showing that mutations of several aminoacid residues (mainly positively charged) in region II of the cytoplasmic C-terminus reduce $PI(4,5)P_2$ affinity of Kir2.1 and Kir6.2/SUR1 channels (Zhang et al., 1999; Shyng et al., 2000b). These mutations have

various effects on channel functional expression, from insignificant reduction to complete inhibition of expressed currents (see "Introduction", Table 3).

While screening the cytoplasmic domains of Kir2.1 and Kir6.2 proteins for *in vitro* $PI(4,5)P_2$ binding we found that the fragments including region III of the cytoplasmic C-termini from both proteins did not bind $PI(4,5)P_2$-containing liposomes (the Kir2.1 fragment C5-6 and the Kir6.2 fragment C5-6*). This region, however, contains two highly conserved arginine residues which, when mutated to alanines in Kir6.2 channel protein, inhibited or reduced the Kir6.2/SUR1 currents expressed in mammalian cells (mutations R301A and R314A, see "Introduction", Table 3). These mutations, as well as substitutions of the same residues with cysteines, impaired the $PI(4,5)P_2$ response of Kir6.2/SUR1 channels (Shyng et al., 2000b; John et al., 2001). Mutations of the conserved tryptophan and phenylalanine residues within this region abolish Kir6.2/SUR1 channel activity and interfere with membrane association of Kir6.2 C-terminal domain (Cukras et al., 2002). Our data suggest that either Kir6.2 residues R301 and R314 are not directly involved in $PI(4,5)P_2$-binding, or that it is not possible to detect *in vitro* $PI(4,5)P_2$-binding mediated by these residues within an isolated protein fragment. The residue homologous to R301 in the Kir1.1 protein (R311) is mutated to glutamine or tryptophan in some cases of antenatal Bartter's syndrome (see "Introduction", Table 3). This residue has been proposed to form an intrasubunit triad with N-terminal lysine 80 and arginine 41 residues, providing a gating structure sensitive to the changes in intracellular pH (reviewed by Ruppersberg, 2000). Considering the absolute conservation of this residue in the Kir protein family it is possible that in other Kir channels it also interacts with distant residues in the cytoplasmic domains, resulting in a site for binding channel-regulating molecules, like $PI(4,5)P_2$. Consistent with this idea, region 307-315 has been recently suggested to be a part of a PH-domain-like structure in the C-terminus of Kir6.2 protein (Cukras et al., 2002).

### 4.2.3. $PI(4,5)P_2$-binding regions in the distal C-termini of Kir proteins.

Our experiments on *in vitro* liposome binding revealed the presence of regions potentially able to bind $PI(4,5)P_2$ and other anionic phospholipids in the variable distal C-termini of Kir2.1, Kir1.1, Kir3.1 and Kir6.2 channel proteins. These regions differed in their localisation and $PI(4,5)P_2$-binding strength. For the sake of simplicity, we will discuss separately the data obtained for the more conserved part of the distal C-terminus,

corresponding to the segment C7 in the studied proteins, and the data obtained for the extreme C-terminal fragments, corresponding to the segments C8(-C9).

In the Kir2.1 protein, the region in the distal C-terminus between aminoacid residues 324-365 was able to bind PI(4,5)$P_2$ and PI(4)P-containing liposomes, although weaker than the fragments from the proximal C-terminus. In the study of Zhang and colleagues (Zhang et al., 1999) Kir2.1 segments 316-341 and 342-366, which split the region 324-365 in two, did not confer high PI(4,5)$P_2$ affinity to Kir3.4/Kir2.1 chimeras. This could mean that only the intact region 324-365 is able to bind PI(4,5)$P_2$ or that this region is really a low-affinity PI(4,5)$P_2$-binding segment. This part of the distal C-terminus does not contain highly conserved positively charged residues, like regions I, II and III from the proximal C-terminus. Instead, it has several conserved aromatic and aliphatic residues. A highly conserved tyrosine residue has been shown to be phosphorylated in the Kir1.1 channel with a subsequent increase in channel endocytosis (Y337, see Sterling et al., 2002). Another absolutely conserved residue is the phenylalanine found in position 344 in Kir2.1 protein. This residue is located in the motif $^{343}$RFHK$^{346}$ conserved in the Kir2.x subfamily proteins but unconserved in the Kir family. In our study, mutation of positively charged R343 and K346 within this motif to glutamines inhibited *in vitro* PI(4,5)$P_2$-binding of this region. This mutation also reduced or completely abolished functional channel expression in *Xenopus* oocytes, but did not change Kir2.1 expression and subcellular localisation in mammalian cells. The mutation R343Q·K346Q might specifically impair the Kir2.1 channel trafficking to the plasma membrane when expressed in *Xenopus* oocytes, and not change the functional channel properties. Alternatively, the mutation can still alter the PI(4,5)$P_2$ sensitivity of the channels, but this effect might be dependent on the actual PI(4,5)$P_2$ content in the plasma membrane. PI(4,5)$P_2$ has been reported to be much less abundant in *Xenopus* oocytes than in HEK293 and other mammalian cells (less than 1% and 5% of the cellular PS amount, respectively). A logical explanation could be that in oocytes surface membranes represent a very small fraction of total cell membrane, whereas PI(4,5)$P_2$ is mainly a surface membrane phospholipid (Nasuhoglu et al., 2002). Variability of the mutant expression in oocytes could be connected as well with fluctuations in the phospholipid composition in the plasma membrane of oocytes from different batches. Also, the altered PI(4,5)$P_2$ sensitivity should not necessarily lead to a decreased functional channel expression at least in mammalian cells, as for example some Kir6.2 mutations altering PI(4,5)$P_2$ sensitivity of Kir6.2/SUR1

channels lead only to a nonsignificant current reduction (Shyng et al., 2000b; see "Introduction", Table 3).

The R343Q·K346Q mutation did not produce as drastic effect on channel function like the control K188Q·R189Q mutation, but was weakly dominant over the wild-type subunits expressed in *Xenopus* oocytes. This suggests that if the region where the R343Q·K346Q mutation is localised plays a role in *in vivo* $PI(4,5)P_2$ binding of the Kir2.1 channel, it should serve for a different purpose than the $PI(4,5)P_2$ binding by the proximal C-terminal positively charged cluster.

The corresponding regions from several other Kir channel proteins are involved in the regulation of these channels. The distal C-terminus of the Kir1.1 protein (323-391) also bound PC/$PI(4,5)P_2$ liposomes with a strength comparable with the binding of the Kir2.1 segment 324-365. This region has been already shown to play an important role in Kir1.1 channel function. Deletion of the residues 332-351 from the Kir1.1 protein (corresponding to aminoacid residues 334-352 in Kir2.1) led to non-functional Kir1.1 channels expressed in *Xenopus* oocytes (Flagg et al., 1999). A shorter truncation – from the residue H354, also found in one of the Bartter's syndrome cases, did not impair the expression of functional Kir1.1 channels in *Xenopus* oocytes and mammalian cells (Jeck et al., 2001). The residues 332-351 are not involved in the Kir1.1 channel trafficking to the plasma membrane, as the Kir1.1 protein truncated at aminoacid residue 331 was expressed at the surface of *Xenopus* oocytes. This truncation caused an intermediate dominant-negative effect when coexpressed with the wild-type Kir1.1. However, when a single truncated mutant was incorporated into a tetrameric concatamer, it conferred a complete dominant negative effect. It was concluded that the distal C-terminus of the Kir1.1 protein is controlling the efficiency of oligomerisation of channel subunits (Flagg et al., 1999). This region also contains sites important for Kir1.1 pH sensitivity (H342 and H354) and an already mentioned endocytosis motif.

The corresponding distal C-terminal fragment from Kir6.2 protein (aminoacid residues 313-353) bound $PI(4,5)P_2$-liposomes at a lower rate than the Kir2.1 region. Mutation of the Kir6.2 lysine residue (K332N) in position homologous to Kir2.1 R343 was shown to be without an effect on ATP sensitivity of Kir6.2ΔC36 channels expressed in *Xenopus* oocytes (Tucker et al., 1998). Mutation of the same residue (K332A) did not significantly alter the expression of functional Kir6.2/SUR1 channels in mammalian cells (Shyng et al., 2000b). Thus, in the Kir6.2 protein, the positively charged aminoacid residue

in this position is at least not involved in the channel's functional expression and ATP-response. At the same time, residue N335 in the Kir6.2 protein, corresponding to Kir2.1 K346, is located in the $^{334}$GNTI$^{337}$-motif important for ATP inhibition of the Kir6.2 channels. Mutation N335Q produces a 2-fold decrease in ATP sensitivity of Kir6.2/SUR1 channels expressed in *Xenopus* oocytes, and replacement of the whole motif with the corresponding region from Kir2.1 results in channels with 40-fold decreased ATP response (Drain et al., 1998). It is not known if this replacement is accompanied by increased PI(4,5)$P_2$ sensitivity. In an attempt to increase the Kir6.2 PI(4,5)$P_2$ affinity, we constructed a Kir6.2/Kir2.1 chimera containing the region 324-365 from Kir2.1 replacing the corresponding region 313-353 in Kir6.2 but this chimera produced non-functional channels when coexpressed with SUR2A subunits in *Xenopus* oocytes (T. Baukrowitz, personal communication). Thus, the whole region involving aminoacid residues 313-353 is important for Kir6.2/SUR channel function.

The Kir2.1 protein region 316-341, which overlaps the distal PI(4,5)$P_2$-binding region 324-365, has been shown to cause formation of the Kir3.4 channels insensitive to $G_{\beta\gamma}$-mediated agonist-dependent activation. The leucine residue located in the corresponding Kir3.4 and Kir3.1 protein regions was shown to be involved in this activation (He et al., 1999). In our study the distal C-terminus of the Kir3.2 protein did not bind PI(4,5)$P_2$, whereas the corresponding region from the Kir3.1 protein did. Considering the homology shared by the C-termini of the Kir3.2 and Kir3.4 proteins, we could suggest that these proteins possess only one site for $G_{\beta\gamma}$ binding in their C-termini, whereas in the Kir3.1 protein, the $G_{\beta\gamma}$ binding site overlaps with distal PI(4,5)$P_2$-binding region. The $G_{\beta\gamma}$ dependent activity and lower PI(4,5)$P_2$-affinity of Kir3.x channels could be explained in a way that the Kir3.4 and Kir3.2 proteins have a region for $G_{\beta\gamma}$ binding in place of an additional PI(4,5)$P_2$ binding site.

As the binding to PI(4,5)$P_2$ and other anionic phospholipids depends greatly on the positioning of charged aminoacid residues in the protein, we compared the distribution of charged residues in the regions corresponding to the segment C7 from various Kir family proteins (Fig.25). This comparison shows that Kir proteins forming channels with high PI(4,5)$P_2$ affinity (Kir1.1 and Kir2.1) or conferring PI(4,5)$P_2$ sensitivity to other channels (Kir5.1) contain closely positioned positively charged residues in this region (Fig.25, boxed). In other proteins, forming Kir channels reported to be less sensitive (Kir3.x and Kir6.2) or insensitive (Kir4.1) towards PI(4,5)$P_2$, there are no neighbouring cationic

residues and/or there are more negatively charged residues in these regions. Thus, the location of positively charged residues correlates with the PI(4,5)$P_2$-affinities determined for some Kir channels and also with the data on PI(4,5)$P_2$ binding obtained in our study. There are no published data on the PI(4,5)$P_2$-regulation of the Kir7.1 channel, but by analogy (Fig.25) we can expect it to be more sensitive towards PI(4,5)$P_2$ than Kir3.x or the Kir4.1 channels.

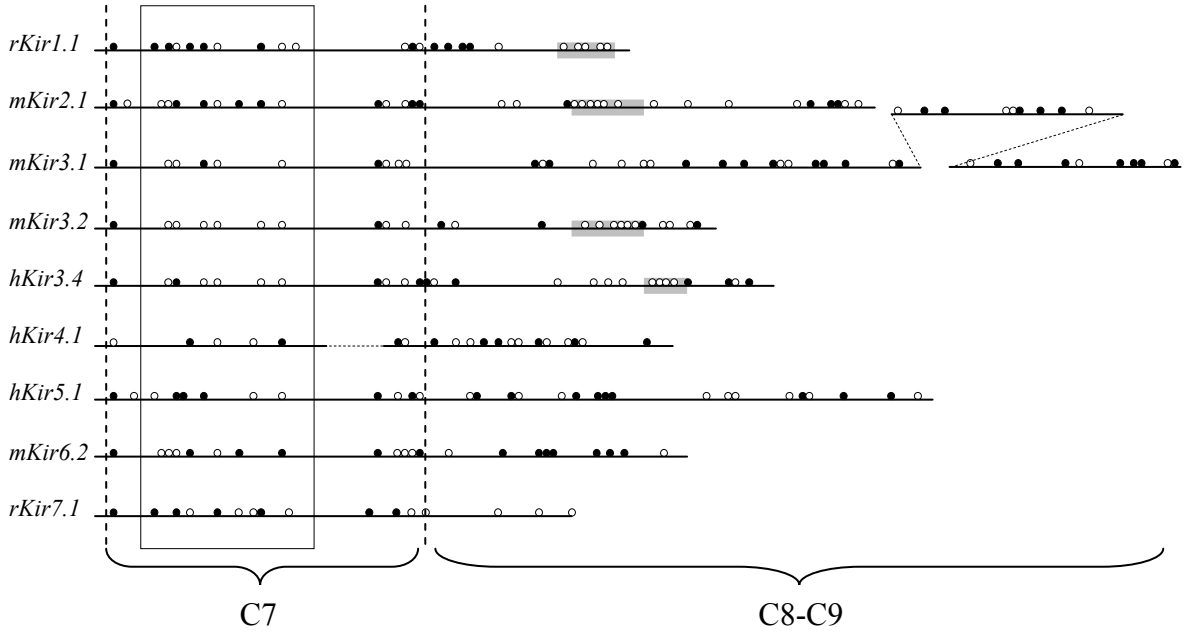


**Fig. 25.** Distribution of positively (black) and negatively (white) charged aminoacid residues in the distal C-termini of the Kir channel proteins from various subfamilies. Regions corresponding to the segments C7 and C8-C9 used in our study are indicated. The part of the C7 segment showing the highest variability in the number of positively and negatively charged residues is boxed. Clusters of negatively charged residues in the extreme C-termini are boxed in grey.

Summarising the above observations we speculate that the Kir proteins' region encompassing the more conserved part of the distal C-terminus has diverged providing structures determining channel-specific regulation. In some of them, like Kir2.1 and Kir1.1, it might add to the channel's PI(4,5)$P_2$-sensitivity, whereas in others, like Kir6.2 or Kir3.x it contains regions involved in channel regulation by ATP or $G_{\beta\gamma}$ subunits, respectively.

### 4.2.4. Extreme C-terminal regions of Kir proteins

The variable extreme C-termini of several Kir channel proteins contain sequences which determine their trafficking to the plasma membrane. Sequences promoting effective export from the endoplasmic reticulum (ER) or post-ER-trafficking have been found in either distal C-termini or both N- and C-termini of Kir1.1, Kir2.1, Kir3.2 and Kir3.4 proteins (Kennedy et al., 1999; Ma et al., 2001; Stockklausner et al., 2001; Ma et al., 2002). Kir2.x family proteins also have a motif for PDZ-domain binding in the extreme C-terminus, that promotes their specific targeting to discrete plasma membrane sites in brain neurones. The serine residue in this region is phosphorylated by PKA that causes the channels to dissociate from PDZ-domain-containing proteins (Cohen et al., 1996; Nehring et al., 2000; Leonoudakis et al., 2001). Kir proteins that are not effectively expressed at the cell surface without a heteromerisation partner or associated subunits contain in their extreme C-termini signals for ER retention/retrieval (Kir6.x), for association with intermediate filaments or microtubules and probably ER-localisation (Kir3.1) or for lysosomal targeting (Kir3.3) (Kennedy et al., 1999; Zerangue et al., 1999; Ma et al., 2002). The surface localisation of Kir3.1 is effectively promoted by association with Kir3.2 and Kir3.4, whereas the lysosomal targeting signal of Kir3.3 seems to dominate over the forward trafficking signals in the heteromerisation partners (Ma et al., 2002).

In Fig.25 we can see that Kir proteins effectively transported to the cell surface contain clusters of acidic residues in their extreme C-termini (Kir1.1, Kir2.1, Kir3.2, Kir3.4). These clusters are most probably necessary for efficient post-Golgi trafficking of these proteins, presumably for the targeting of channels from the endosomes to the cell surface (Ma et al., 2002). At the same time, proteins that do not produce functional homomers because of stacking in the ER (Kir6.2 and Kir3.1) or are inefficiently transported to the plasma membrane (Kir4.1; Stockklausner et al., 2001) do not possess such anionic clusters, but instead contain repeated positively charged residues. The Kir5.1 protein does not produce functional homomeric channels and contains clustered positively charged residues, thus probably also being stuck in the ER. Consistent with high concentration of anionic residues, the extreme C-terminal regions from the Kir2.1 and Kir3.2 proteins tested in our study did not bind the liposomes containing $PI(4,5)P_2$. However, the extreme C-termini from the Kir6.2 and Kir3.1 channel proteins showed $PI(4,5)P_2$-binding. We can suggest that biochemical properties of the extreme C-terminal portions of the Kir6.2 and Kir3.1 channel proteins enable their intracellular retention by

binding to anionic phospholipids in the intracellular membranes or to other anionic molecules at the stage of channel assembly from heteromeric subunits and trafficking to the plasma membrane. Nevertheless, analysis of some published observations shows that these regions might also be involved in $PI(4,5)P_2$ response of the channels already localised at the plasma membrane.

The fragment containing 36 extreme C-termial aminoacid residues of the Kir6.2 protein (fragment C8) showed a high level of binding to $PI(4,5)P_2$-containing liposomes, and also bound PS- and PI-containing liposomes. Positively charged aminoacid residues in this region were found to be not important for Kir6.2/SUR1 channel expression and $PI(4,5)P_2$ response in mammalian cells when mutated individually to alanines (Shyng et al., 2000b). However, some data on Kir6.2-$PI(4,5)P_2$ interactions point to the possibility of involvement of the extreme C-terminus in the $PI(4,5)P_2$ response of Kir6.2/SUR channels:

(i) Deletion of 36 aminoacid residues from the C-terminus of Kir6.2 prolongs the time of $PI(4,5)P_2$ application necessary for decreasing the ATP inhibition of Kir6.2/SUR1 channels expressed in *Xenopus* oocytes (Baukrowitz et al., 1998). In other words, this deletion of the extreme C-terminal portion of the Kir6.2 protein decreases the $PI(4,5)P_2$ sensitivity of Kir6.2/SUR1 channels. Deletion of 26 aminoacid residues from the C-terminus of Kir6.2 greatly decreases the open probability of the Kir6.2Δ26 channels (Drain et al., 1998).

(ii) $PI(4,5)P_2$ reduces the inhibition of Kir6.2/SUR2A channels by sunfonylureas (glibenclamide) by a mechanism distinct from $PI(4,5)P_2$ action on the ATP inhibition (Krauter et al., 2001). $PI(4,5)P_2$ might act by uncoupling Kir6.2 from SUR subunits via binding to the Kir6.2 extreme C-terminus, which is known to interact with the SUR protein at least during channel trafficking to the plasma membrane (Zerangue et al., 1999).

The Kir6.2 extreme C-terminal region contains a serine residue, that is a subject of PKA phosphorylation increasing the Kir6.2/SUR channel open probability (Béguin et al., 1999). The stimulatory effect of PKA-phosphorylation (although at different sites) on channel-$PI(4,5)P_2$ interactions has been shown for Kir1.1 channels (see "Introduction"), and could also take place in the case of Kir6.2/SUR channels.

Studying *in vitro* $PI(4,5)P_2$ interactions of the distal C-terminal regions from $G_{\beta\gamma}$-activated Kir channel proteins we found that the distal C-terminal portion of the Kir3.1 protein binds $PI(4,5)P_2$-containing vesicles. Although an aspartate residue in the Kir3.2 protein (D225) located upstream of the regions tested by us has been proposed to interact

with $Na^+$ ions and to promote the functional interaction of the Kir3.1/Kir3.2 channels with $PI(4,5)P_2$ (Ho & Murrell-Lagnado, 1999b), it does not exclude the involvement of the downstream region of the Kir3.1 protein in $PI(4,5)P_2$ binding. This speculation is supported by the following observations showing that at least in the *Xenopus* oocyte expression system the Kir3.1 subunits or even only the cytoplasmic domains might confer a higher $PI(4,5)P_2$ affinity to $G_{\beta\gamma}$-regulated Kir channels:

(i) When the heteromeric Kir3.1/3.4 and homomeric Kir3.2 channels expressed in *Xenopus* oocytes were compared for their inhibition with $PI(4,5)P_2$-antibody upon activation with $G_{\beta\gamma}$, the Kir3.1/3.4 channels were inhibited more slowly. Without $G_{\beta\gamma}$ activation both channels were inhibited by the $PI(4,5)P_2$-antibody at similar rates (Huang et al., 1998). This could mean that the Kir3.1 protein adds a stronger $PI(4,5)P_2$ affinity to the heteromeric channel complex, although this is physiologically noticeable only after activation of the channel by $G_{\beta\gamma}$ subunits.

(ii) The Kir3.1/Kir3.2 chimera containing the transmembrane regions from Kir3.2 and cytoplasmic regions from Kir3.1, when expressed as a homomer in *Xenopus* oocytes, had a much greater open probability than the Kir3.2 homomeric channels. In excised patches the chimeric currents ran down rapidly as the Kir3.1/Kir3.2 heteromeric currents, but upon application of MgATP a greater increase in chimeric currents was observed in comparison with Kir3.1/Kir3.2 currents (Ho & Murrell-Lagnado, 1999a). As MgATP is known to exert its effect on Kir channel activation by an increase in plasma membrane $PI(4,5)P_2$ (Hilgemann & Ball, 1996), and $PI(4,5)P_2$ is increasing the open probability of Kir channels, the above data could point to a higher $PI(4,5)P_2$ affinity of the cytoplasmic regions of the Kir3.1 protein than of the Kir3.2 protein. As the main difference of Kir3.1 from other Kir3.x subfamily proteins lies within its unique distal C-terminus, one can suggest that this region causes the effects connected with the presence of Kir3.1 cytoplasmic domains.

In addition, according to our data, the isolated Kir3.1 distal C-terminal fragment can bind *in vitro* both $PI(4,5)P_2$ and recombinant $G_{\beta\gamma}$ subunits, with an increase in affinity towards $PI(4,5)P_2$. It means that for the $G_{\beta\gamma}$-regulated Kir channels containing Kir3.1 subunits the suggested stabilising effect of $G_{\beta\gamma}$ on $PI(4,5)P_2$ interactions could involve the distal C-terminus of the Kir3.1 protein. We have also observed an increase of PI3Kγ-$PI(4,5)P_2$ interaction in the presence of $G_{\beta\gamma}$ subunits. The fact that these functionally and

structurally distant proteins respond similarily to $G_{\beta\gamma}$ binding could mean that this is a general feature of proteins binding both $PI(4,5)P_2$ and $G_{\beta\gamma}$ subunits.

### 4.2.5. Involvement of the N-terminal cytoplasmic domain in Kir channel-$PI(4,5)P_2$ interactions.

The GST-fused Kir2.1 N-terminus (aa 1-86) has been reported previously not to bind significantly $PI(4,5)P_2$ micellas (Huang et al., 1998). The Kir2.1 N-terminus was shown to be not responsible for high $PI(4,5)P_2$ affinity, because when parts of it (aminoacid fragments 1-34 and 35-56) were introduced into the Kir3.4 protein, they did not change the kinetics of Kir3.4 current block by $PI(4,5)P_2$-antibodies (Zhang et al., 1999). In our study, the GST-fused N-terminus of the Kir2.1 protein (1-86) bound liposomes containing 35 mol% $PI(4,5)P_2$ at the threshold level, but stronger than the GST-control. A shorter fragment, leaving out the part of the hydrophobic M0 domain (aminoacid residues 1-72) and thus producing a fusion protein with an increased solubility, bound the liposomes with the same $PI(4,5)P_2$ concentration above the threshold. Thus, we cannot exclude that the Kir2.1 N-terminal domain is also involved in $PI(4,5)P_2$ interactions of this channel. This suggestion is supported by a very recent report of mutations within the Kir2.1 cytoplasmic N-terminus decreasing the Kir2.1 $PI(4,5)P_2$ affinity (Lopes et al., 2002).

The N-terminal domain of the Kir6.2 protein (aminoacid residues 1-74) showed a stronger binding to $PI(4,5)P_2$-containing liposomes compared to the relevant Kir2.1 domain. We found that Kir6.2 N-terminal regions able for $PI(4,5)P_2$ binding start with the aminoacid residue 24. The N-terminal arginine residue within the M0 domain, conserved in Kir2.x, Kir3.x and Kir6.x proteins, is involved in the $PI(4,5)P_2$ response of Kir6.2/SUR2A channels (T. Baukrowitz, unpublished) and Kir2.1 channels (Lopes et al., 2002). This residue preceeds the position where an aromatic aminoacid residue (phenylalanine or tryptophan) is found in all Kir family proteins. In our experiments, exchange of this residue to glutamine or glutamate (mutations R54Q or R54E), decreased the $PI(4,5)P_2$ binding of the Kir6.2 N-terminal aminoacid residues 34-59. The control mutation R50E, that is not influencing the channel $PI(4,5)P_2$ response, but is involved in channel ATP sensitivity, produced a weaker effect on the fragment binding. The fact that two mutations with the same effect on the overall charge of the Kir6.2 fragment 34-59 (R50E and R54E) have different strengths in reducing $PI(4,5)P_2$ affinity of this protein

fragment supports the importance of position of charged aminoacid residues in addition to the overall electrostatic mechanism of $PI(4,5)P_2$ binding. Our data suggest that the arginine residue in position 54 within the M0 domain of the Kir6.2 protein and probably the homologous residues in other Kir channel proteins might interact directly with $PI(4,5)P_2$ in the plasma membrane.

The M0 domains from various Kir proteins contain several conserved charged aminoacid residues, analogously to the cytoplasmic region proximal to the second transmembrane domain. Such regions are generally required for stabilising transmembrane helices and providing their correct orientation in the plasma membrane via electrostatic interaction with anionic phospholipids (see van Klompenburg et al., 1997). In addition, they contain several conserved aliphatic and aromatic residues, that could promote penetration of M0 into the plasma membrane. Thus, it would be logical to expect that the region proximal to the first transmembrane domain is involved in binding of $PI(4,5)P_2$ and probably other anionic phospholipids for maintaining the proper structure of Kir channels.

Outside the highly conserved M0 region, the Kir protein N-termini contain channel-specific regions, since often the exchange of the N-termini between Kir proteins from different subfamilies results in formation of non-functional channels (see Hough et al., 2000; He et al., 2002). For example, exchange of the Kir2.1 N-terminus with the corresponding domain from Kir6.2 resulted in channels expressed at the surface of *Xenopus* oocytes, but conducting no current (Hough et al., 2000). Aminoacid residues in the proximal N-termini have been identified as important for specific regulation of several Kir channels (see "Introduction", Fig.3). We have constructed the Kir6.2 mutation R27H·R31Q, exchanging two arginine residues in the proximal N-terminus for the residues found in the corresponding positions in the Kir2.1 protein. This mutation reduced the $PI(4,5)P_2$ interaction of the Kir6.2 N-terminal fragment 1-43, but did not change the $PI(4,5)P_2$ sensitivity of Kir6.2/SUR2 channels expressed in *Xenopus* oocytes. These residues are obviously not involved in the $PI(4,5)P_2$ response of the channel, in spite of their effect on *in vitro* binding, or the introduced mutations were not strong enough to produce the effect *in vivo*. The Kir6.2 N-terminal fragment involving the mutated residues has been shown to interact with the C-terminal fragment, that includes the Kir protein region III, already discussed above (Jones et al., 2001). Thus, we have two interacting regions, with contrasting features: one (N-terminal) binds $PI(4,5)P_2$ *in vitro*, but seems not to influence the *in vivo* $PI(4,5)P_2$ affinity, whereas another (C-terminal) does not influence

$PI(4,5)P_2$ binding *in vitro*, but is certainly important for $PI(4,5)P_2$ response *in vivo*. Possibly, the C-terminal region determines the structure of its interaction partner region in the N-terminus, promoting $PI(4,5)P_2$ binding of the assembled channel complex.

### 4.3. The cytoplasmic $PI(4,5)P_2$-binding regions of Kir channel proteins and Kir channel gating model.

We have shown for Kir2.1 and Kir6.2 proteins that regions able to bind $PI(4,5)P_2$ are present in their N- and C-terminal domains. Several specific interactions of various Kir channels involve both of their cytoplasmic domains, suggesting that these domains form a complex in the channel's three-dimensional structure. In the Kir6.2 protein, N- and C-terminal aminoacid residues have been shown to be important for the ATP inhibition of Kir6.2Δ36 channels (Tucker et al., 1998). Aminoacid residues both in the beginning of the Kir6.2 protein and in its C-terminus are involved in functional coupling to SUR1 subunits (Reimann et al., 1999; John et al., 2001). Several studies have shown that N-terminal domains of $G_{\beta\gamma}$-activated Kir channel proteins bind $G_{\beta\gamma}$ subunits along with the C-terminal domains (Kunkel & Peralta, 1995; Huang et al., 1995; Huang et al., 1997). In Kir3.1 and Kir3.4 channel proteins, the aminoacid residues in both domains have been identified that are important for $G_{\beta\gamma}$-mediated Kir3.4 or Kir3.1/Kir3.4 channel activity (He et al., 2002). Stoichiometry of ATP and $G_{\beta\gamma}$ binding to the Kir channel tetrameres is suggested to be 1 molecule per 1 channel subunit (Corey & Clapham, 2001; Vanoye et al., 2002). This would probably mean that several regions in the cytoplasmic domains of ATP- and $G_{\beta\gamma}$–regulated channel proteins cooperate forming one ligand-binding site per channel subunit.

In the constitutively acitve Kir2.1 channel protein, cytoplasmic N- and C-termini contain regions interacting with A-kinase anchoring protein AKAP79 (Dart & Leyland, 2001). In the Kir1.1 channel the residues involved in regulation of this channel by intracellular protons have been identified both in N- and C-termini.

The data on Kir1.1 pH regulation were used to propose a gating model of the Kir1.1 channel implicating interaction between aminoacid residues in the cytoplasmic regions corresponding to the Kir6.2 protein fragments designated as N2, N5 and C6 regions in our study (reviewed by Ruppersberg, 2000). Studies of the Kir6.2 protein revealed that its N- and C-termini interact via regions conserved between different Kir channels (Tucker & Ashcroft, 1999; Jones et al., 2001). The Kir6.2 N-terminal region (approximately

corresponding to fragments N2-3 and beginning of fragment N4 in our study, see Fig.26) interacts physically with the proximal C-terminal region (fragments C1-3) and the C-terminal region overlapping with Kir6.2 fragments designated in our study as C5-6 and beginning of fragment C7 (Jones et al., 2001). This interaction is most probably necessary for channel opening, as a mutation in the N-terminal interaction region (G40D), impairing its *in vitro* binding to the C-terminal domain, and mutation of the homologous residue from Kir2.1 protein (G52D), result in formation of non-functional channels (Tucker & Ashcroft, 1999).

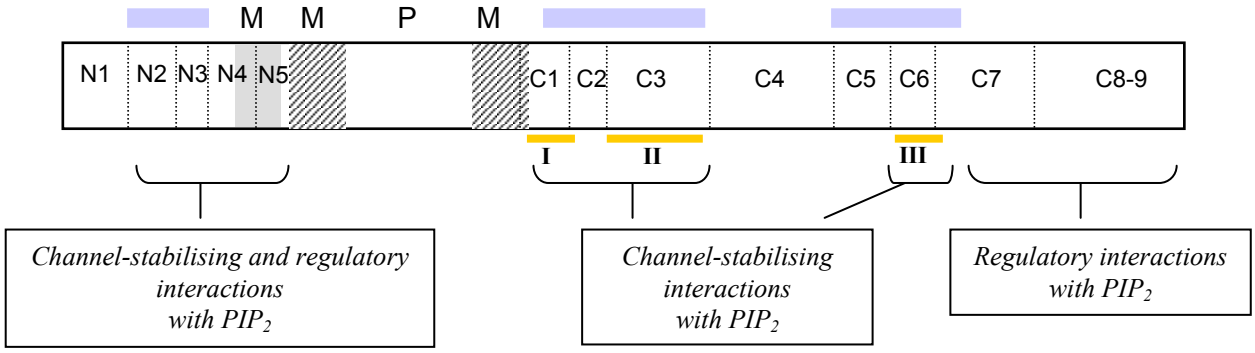


**Fig. 26.** Interactions of a Kir channel protein with PI(4,5)$P_2$. M1, M2 – transmembrane domains, M0 – membrane-buried domain, P – pore domain; N1-N5, C1-C8-9 – sections of Kir proteins used in our study. I, II, III – regions of the cytoplasmic C-terminus containing residues involved in Kir channel's activation by PI(4,5)$P_2$ *in vivo* (see Fig.3). Upper bars indicate the regions involved in the interaction between N- and C-termini of Kir channels (according to Tucker & Ashcroft, 1999; Jones et al., 2001).

If a uniform gating mechanism for Kir channels is assumed, the PI(4,5)$P_2$-binding regions in the N-terminus and about the first 150 aminoacid residues of the cytoplasmic C-terminus would form a structure promoting the open channel conformation (Fig.26). This would explain the importance of PI(4,5)$P_2$ for Kir channel opening and the influence of mutations in the proximal C-terminus on Kir channel function. The distal regions interacting with PI(4,5)$P_2$ in three Kir proteins which we have studied (Kir1.1, Kir2.1, Kir6.2 and Kir3.1) are located downstream of the regions that are involved in channel assembly and interaction with the N-terminus and, thus, would probably not take part in the formation of the gating complex. This means that interaction of these regions with PI(4,5)$P_2$ will be without a strong effect on channel opening, but instead could modulate the already opened channels. In the regulated Kir channels, this region could play a role in

coordinating the effect of $PI(4,5)P_2$ and other channel-binding ligands, like ATP or $G_{\beta\gamma}$ subunits.

### 4.4. Cytoplasmic $PI(4,5)P_2$-binding regions of Kir proteins and Kir-channel connected diseases.

As it has been already mentioned in the "Introduction", Kir proteins' cytoplasmic C-terminal regions denoted in this study as region I, II and III contain residues involved in $PI(4,5)P_2$-regulation of some Kir channels and also sites affected by Bartter's or Andersen's syndrome mutations in Kir1.1 and Kir2.1 proteins, respectively. We find that isolated Kir protein fragments involving regions I and II from Kir2.1 and Kir6.2 bind $PI(4,5)P_2$ in the biochemical assay. The purified fragments containing region III do not bind $PI(4,5)P_2$, but according to the literature data might be involved in formation of the gating complex with other regions in the cytoplasmic domains. Thus, the disease-causing mutations in these regions can alter channel gating by disturbing the channel's interaction with $PI(4,5)P_2$ or other anionic membrane phospholipids.

The mutations in the region coding for the distal cytoplasmic C-terminus of the Kir1.1 protein have been identified in some cases of antenatal Bartter's syndrome. Two of them are frameshifts leading to truncations of the distal C-terminus. According to our data, this region is also potentially able for interactions with $PI(4,5)P_2$ possibly increasing the affinity of the whole channel towards this phospholipid. Disruption of this interaction could be one of the reasons for the functional defect caused by C-terminal truncations of the Kir1.1 protein.

The N-terminal domains of the Kir2.1 and Kir1.1 proteins are also affected by disease-causing mutations. An Andersen's syndrome mutation and one of the Bartter's syndrome mutations affect highly conserved aspartic acid residues in the M0 region. Other two Bartter's syndrome mutations affect the residues in the proximal N-terminus possibly involved in interaction with the cytoplasmic C-terminus (see "Introduction", Table 3). As our data suggest that these regions bind $PI(4,5)P_2$, the mutations could destabilise the channel by altering its interactions with membrane phospholipids. In support of our speculations, the group of D.E.Logothetis has reported very recently that several disease-causing mutations in the N- and C-terminal domains of Kir2.1 and Kir1.1 proteins possibly decrease the interaction of these channels with anionic phospholipids (Lopes et al., 2002).

## 5. Conclusions.

1. The biochemical binding assay of purified protein fragments with mixed fluorescently labelled liposomes, utilised in our study, provides a useful tool for studying direct interactions of Kir protein cytoplasmic domains with phospholipids of interest. Interaction of Kir protein fragments with liposomes is a sum of electrostatic and hydrophobic forces, and depends on the distribution of charged aminoacid residues within the protein fragments.

2. Cytoplasmic C-terminal domains of constitutively active Kir2.1 and ATP-regulated Kir6.2 channel proteins contain two consequtive conserved regions proximal to the second transmembrane domain able to bind independently $PI(4,5)P_2$. For Kir2.1 protein fragments, this interaction includes a significant electrostatic component, but is possibly affected by other types of interactions or conformational constraints. Mutations reducing $PI(4,5)P_2$ affinity of the first Kir2.1 fragment inhibit Kir2.1 function without influencing the channel plasma membrane localisation as verified in a mammalian expression system.

3. The Kir2.1 and Kir6.2 protein fragments containing the downstream conserved region of the cytoplasmic C-terminal domain did not bind independently $PI(4,5)P_2$. As this region has been reported to play a role in the Kir6.2 channel $PI(4,5)P_2$ response, we suggest that this region is involved in $PI(4,5)P_2$ binding via interaction with other cytoplasmic regions.

4. Kir2.1 and pH-regulated Kir1.1 channel proteins characterised by high $PI(4,5)P_2$ affinity *in vivo*, contain $PI(4,5)P_2$ binding regions in their distal C-termini. The homologous region from the Kir6.2 protein reported to play a role in the Kir6.2 channel ATP-inhibition does not substantially bind $PI(4,5)P_2$. The influence of mutations inhibiting $PI(4,5)P_2$ binding of the Kir2.1 distal fragment on the channel function depends on the expression system, possibly due to variations in the content of $PI(4,5)P_2$ in the plasma membrane of cells from various organisms. The extreme C-terminal region of the Kir6.2 protein determining its intracellular retention binds $PI(4,5)P_2$ presumably by an electrostatic mechanism.

5. The distal C-termini of the $G_{\beta\gamma}$-activated Kir3.1 and Kir3.2 channel proteins differ in their affinity towards $PI(4,5)P_2$. The Kir3.2 distal C-terminus, presumably containing the $G_{\beta\gamma}$ binding region does not bind $PI(4,5)P_2$. In the distal C-terminus of the Kir3.1 channel

protein the $G_{\beta\gamma}$ interaction region overlaps with the region able for *in vitro* $PI(4,5)P_2$ binding. Binding to $G_{\beta\gamma}$ subunits in the biochemical assay increases $PI(4,5)P_2$ affinity of this region.

6. Both the Kir2.1 and Kir6.2 channel N-terminal cytoplasmic domains bind $PI(4,5)P_2$-containing liposomes. The Kir6.2 N-terminal domain has a higher affinity towards $PI(4,5)P_2$ and its $PI(4,5)P_2$-binding part can be narrowed down to the region of higher conservation proximal to the first transmembrane domain. Comparison of biochemical and physiological mutational analysis data suggests that this region might be part of the channel-$PI(4,5)P_2$ interaction site.

7. Based on the above observations, we suggest that the conserved proximal N- and C-terminal cytoplasmic regions of Kir channels stabilise channel open conformation by interactions with $PI(4,5)P_2$ or other anionic phospholipids, whereas the distal C-termini of some Kir channels contain $PI(4,5)P_2$-binding regions with modulatory functions. Disease-causing mutations that locate in Kir protein regions, identified in our study as $PI(4,5)P_2$ binding domains, may negatively affect Kir channel-$PI(4,5)P_2$ interactions.

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# 7. Appendix.

## Abbreviations

| | |
|---|---|
| a.a. | aminoacid |
| ATP | adenosine 5`triphosphate |
| BSA | bovine serum albumin |
| DOG | dioleoylglycerol |
| DOGS-NTA | dioleoylglycerol-succinyl-nitriloacetic acid |
| DTT | dithiothreitol |
| ECL | enhanced chemiluminescence |
| EDTA | ethylenediamine-tetraacetic acid |
| EGTA | ethylene-bis(oxyethylenenitrilo) tetraacetic acid |
| FCS | foetal calf serum |
| FV | fluoride vanadate |
| GDP | guanosine diphosphate |
| GST | glutathione-S-transferase |
| GTP | guanosine triphosphate |
| HEPES | (N-(2-hydroxyethyl) piperazine-N´-(2-ethanesulfonic acid)) |
| $IP_3$ | inositoltriphosphate |
| IPTG | isopropylthiogalaktoside |
| PA | phosphatidic acid |
| PAGE | polyacrylamide gel-electrophoresis |
| PBS | phosphate buffered saline |
| PC | phospatidylcholine |
| PDGF | platelet derived growth factor |
| PE | phosphatidylethanolamine |
| PH | pleckstrin homology |
| PI | phosphatidylinositol |
| PI3K | phosphatidylinositol 3-kinase |
| PI4K | phosphatidylinositol 4-kinase |
| PI4P(5)K | phosphatidylinositol-4-phosphate 5-kinase |
| $PI(3,4)P_2$ | phosphatidylinositol-3,4-bisphosphate |
| $PI(3,5)P_2$ | phosphatidylinositol-3,5-bisphosphate |
| $PI(3,4,5)P_3$ | phosphatidylinositol-3,4,5-triphosphate |
| PI(4)P | phosphatidylionsitol-4-phosphate |
| $PI(4,5)P_2$ | phosphatidylinositol-4,5-bisphosphate |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PLC | phospholipase C |
| PLD | phospholipase D |
| PP | pyrophosphate |
| PS | phosphatidylserine |
| PVDF | polyvinylidendiflouride |
| Rh-PE | rhodamin-phosphatidylethanolamine |
| SDS | sodiumdodecylsulfate |
| TNP-ATP | 2,4,6,-trinitrophenyl-ATP |

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**Selbständigkeitserklärung**

Ich erkläre, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

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**Erklärung zur Bewerbung**

Hiermit erkläre ich, dass ich mich mit der vorliegenden Arbeit an keiner anderen Hochschule um den akademischen Grad Doctor rerum naturalium beworben habe und dass ich weder früher noch gegenwärtig die Eröffnung eines Verfahrens zum Erwerb des o.g. akademischen Grades an einer anderen Hochschule beantragt habe.

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