Multiple PIP_2 binding sites in Kir2.1 inwardly rectifying potassium channels

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Abstract Inwardly rectifying potassium channels require binding of phosphatidylinositol-4,5-bisphosphate (PIP_2) for channel activity. Three independent sites (aa 175–206, aa 207–246, aa 324–365) were located in the C-terminal domain of Kir2.1 channels by assaying the binding of overlapping fragments to PIP_2 containing liposomes. Mutations in the first site, which abolished channel activity, reduced PIP_2 binding of this fragment but not of the complete C-terminus. Point mutations in the third site also reduced both, channel activity and PIP_2 binding of this segment. The relevance of the third PIP_2 binding site provides a basis for the understanding of constitutively active Kir2 channels.

Key words: Inward rectifier potassium channel; Voltage clamp; Kir2.1; Lipid–protein interaction; Binding assay; Phosphatidylinositol-4,5-bisphosphate

1. Introduction

Inwardly rectifying potassium channels (Kir channels) play an important role in regulating cell membrane excitability. They stabilize the resting membrane potential and mediate K^+ transport across membranes. These channels are composed of four subunits, each comprising two transmembrane segments (Fig. 1). Since they have no intrinsic gating structures like voltage-gated K^+ channels, the open-probability of these channels is regulated in indirect ways such as by the voltage-dependent channel block by intracellular cations, by association with GTP-binding proteins or with ATP binding sulfonyl-urea receptors (for review see [1]). In addition, recently it has been shown that these channels, like some other ion channels and transporters, are regulated by the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2) [2].

PIP_2 is a quantitatively minor membrane component, although its local concentration may be relatively high. It is a key signaling phospholipid, its hydrolysis by PLC as well as its phosphorylation by PI3 kinases generates important second messengers. PIP_2 itself plays an important role in such processes as the organization of actin cytoskeleton and vesicular transport [3,4].

Biochemical experiments have demonstrated that PIP_2 binds directly to Kir channels [5]. Apparently, bound PIP_2 is required to stabilize the open state. The activity of Kir2.1 (IRK1) channels seems to depend only on the presence of PIP_2 in the membrane [6,7]. Other Kir channels have additional regulators that influence their dependence on PIP_2. Kir1.1 (ROMK1) channels are regulated by protein kinase A through phosphorylation that seems to enhance the interaction of these channels with PIP_2 [8]. Kir3.1/4 (GIRK1/4) channels are regulated by the βγ-complex of GTP-binding proteins (Gβγ) and intracellular Na^+. Both regulatory molecules possibly strengthen the interaction of the channel with PIP_2 [5,9]. PIP_2 also reduces the ATP-sensitivity of K_{ATP} channels (Kir6.x) [10–12]. These findings show that PIP_2 may serve as a final regulator of several Kir channel types, coupling their activity to cellular signaling pathways.

The molecular mechanism of PIP_2-dependent regulation of Kir channels is hypothesized as follows: the negatively charged phosphate groups of the lipid interact with positively charged amino acids in the Kir protein. Since PIP_2 interacts probably with all Kir channels it seems likely that these positively charged residues are somehow conserved. A good candidate for such a conserved PIP_2 binding site is the cytoplasmic region distal of the second transmembrane segment (residues 175–206 in Kir2.1, sections C1–C2 in Fig. 1), containing a stretch of positively charged amino acid residues (PKKR motif, residues 186–189). The arginine residue R189 within this region is highly conserved among the members of the Kir family. Mutations of the homologous arginines in Kir1.1, Kir6.2, and Kir5.1 significantly influence the effect of PIP_2 [5,11,13].

How does a well-conserved binding site explains the observation that some channels only require PIP_2, whereas others need additional factors to stabilize the interaction of PIP_2 with the channel? Interestingly, PIP_2 was found to bind more weakly to the C-terminus of Kir3.1 than to the C-terminus of Kir2.1 [5]. The Kir2.1 region from amino acid residues 206–245 has been identified to be important for its higher PIP_2 affinity. R218 and R228 and also L222 within this region are required for the channel–PIP_2 interactions (Fig. 1) [7,14]. These studies highlight the relevance of regions apart

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Abbreviations: PIP_2, phosphatidylinositol-4,5-bisphosphate; PC, phosphatidylcholine; Rh-PE, rhodamine-phosphatidylethanolamine; PI3Kγ, phosphatidylinositide 3-kinase γ; GST, glutathione *S*-transferase

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from the PKKR motif, but still leave several open questions. Is the region 206–245 in Kir2.1 a modulator of the PKKR binding site or does it form a second, independent binding site? How many binding sites should we consider for Kir2.1 and other channels? Also the direct role of R189 and other residues within the Kir2.1 PKKR motif for the Kir2.1–PIP_2 interaction has not yet been shown.

2. Materials and methods

2.1. Molecular biology and recombinant proteins

The full-length mouse Kir2.1 (accession NM008425) was subcloned into pBluescript KS (Stratagene, La Jolla, CA, USA). Point mutations were generated by overlap extension PCR [15]. All the constructs were verified by sequencing. Capped mRNA was synthesized in vitro with T3 RNA polymerase (mMessage mMachine kit, Ambion, Austin, TX, USA).

To generate the glutathione *S*-transferase (GST) fusions of Kir2.1, the relevant gene fragments were generated as PCR products flanked by *Bam*HI and *Xho*I restriction sites and cloned in-frame with the GST-encoding sequence in pGEX-5x-2 (Amersham-Pharmacia GmbH). Expression of fusion proteins and GST alone was induced by 0.1 mM IPTG in *Escherichia coli* BL-21. Bacterial cultures were grown at 22°C for 4–16 h, depending on the length of the constructs. The proteins were purified using glutathione–4B-Sepharose beads as described by the manufacturer. The purity and concentration of the proteins was estimated on an SDS–PAGE gel together with a series of BSA dilutions with known concentration. The samples were diluted to 0.4–0.8 mg/ml of the GST-fused protein in a 50% Sepharose slurry. Sepharose with immobilized proteins was stored at −20°C in protein storage buffer (in mM: 50 HEPES, 50 NaCl, 5 $MgCl_2$, 1 DTT pH 7.4, 50% glycerol). For PIP_2 binding assays the proteins were washed three times with lipid binding buffer (in mM: 10 HEPES, 100 KCl, pH 7.4).

The cloning and expression of GST fusion of full-length phosphatidylinositide 3-kinase γ (PI3Kγ, residues 4–1068) has been published [16]. The fusion protein was expressed in Sf9 cells during 3 days after transfection. The cells were harvested and resuspended in lysis buffer (in mM: 50 Tris, 150 NaCl, 5 EDTA, 50 NaF, 2 PMSF, 10 benzamidine, 1 sodium orthovanadate, 0.02 leupeptin, pH 7.5, 1% (w/w) Triton X-100). The GST–PI3Kγ fusion protein was bound to glutathione–4B-Sepharose and washed three times in lysis buffer.

2.2. Electrophysiological measurements

Stage V oocytes were prepared from *Xenopus laevis* as described previously [17] and 50 nl of mRNA (200–250 ng/μl) were injected. Currents were measured at 20–23°C, 20–24 h after injection with a two-electrode voltage clamp (Turbo-TEC 10CD amplifier, NPI electronic, Tamm, Germany). The bath solution contained (in mM): 90 KCl, 3 $MgCl_2$, 10 HEPES (pH 7.2 with KOH). Experiment control including pulse generation and data recording was performed with Pulse+PulseFit software (HEKA Elektronik, Lambrecht, Germany).

2.3. Preparation of liposomes

Mixed liposomes were prepared from PIP_2 (from bovine brain, Sigma), phosphatidylcholine (PC) (from chicken egg, Sigma) and rhodamine-phosphatidylethanolamine (Rh-PE, Avanti Polar Lipids, Alabaster, AL, USA) that was used for fluorescent labeling of liposomes. Phospholipids dissolved in chloroform:methanol:H_2O:1 N HCl (20:9:1:10) were mixed to obtain the desired phospholipid composition of 2, 5, 10, 20, and 35 mol percent PIP_2 and 97, 94, 89, 79, and 64 mol percent PC, respectively. Rh-PE ratio was always 1 mol percent. Control liposomes without PIP_2 were taken to determine PIP_2-independent binding. 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 homogenizing and vortexing. The mixture was left at room temperature for 30 min, then homogenized 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 immobilized proteins.

2.4. PIP_2 binding assay

For performing PIP_2 binding assays 0.1 ml of gluthathione–4B-Sepharose with immobilized GST-fusion proteins or GST alone (40–80 μg protein/assay) were mixed with 0.1 ml of fluorescently labeled liposomes and incubated with agitation at 37°C for 1 h. Sepharose was spun down and washed three times with lipid binding buffer. Liposome binding to the immobilized proteins was quantified by fluorescence measurement in 96-well plates (Fluoroscan II, Laborsystems GmbH, Frankfurt, Germany) using an excitation and emission wavelength 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 PIP_2 binding of each protein sample.

3. Results and discussion

3.1. Mutations in the PKKR motif reduce the expression of functional Kir2.1 channels

To determine the physiological significance of the PKKR motif in Kir2.1 channels, we tested the effects of the following mutations in this motif on the functional expression of Kir2.1 in *Xenopus* oocytes: P186A, K188Q, R189Q, and K188Q•R189Q. As shown in Fig. 2A, oocytes expressing mutant channels showed smaller currents than those expressing the wild-type channels. At −100 mV the wild-type showed an average current of -6.2 ± 1.0 μA (mean ± S.E.M.). Mutant P186A yielded -1.4 ± 0.4 μA and mutant K188Q only -0.09 ± 0.05 μA. Mutants R189Q and K188Q•R189Q yielded no Kir-specific current at all. Thus, the studied mutations in the PKKR motif reduce the expression of functional Kir2.1 channels in the following order: K188Q•R189Q, R189Q > K188Q > P186A > wild-type.

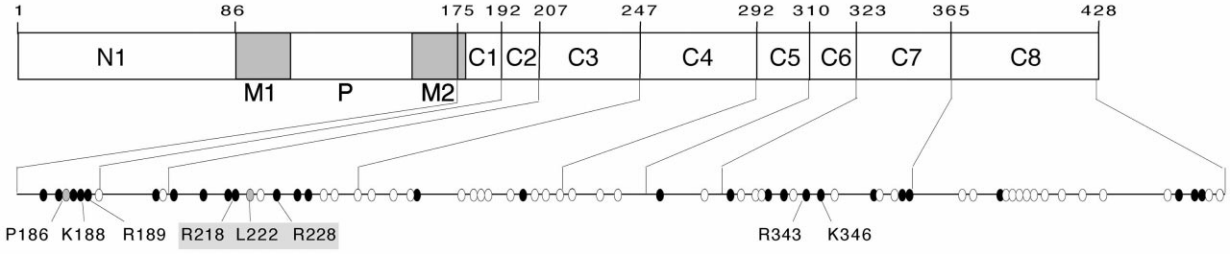

Fig. 1. Schematic representation of the Kir2.1 protein. Numbers indicate the amino acid positions. M1, M2 – transmembrane domains; P – pore domain; N1, C1–C8 – sections of Kir2.1 used in this study. Bottom: Distribution of positively (black) and negatively (white ovals) charged residues. Numbers indicate residues that have been tested for PIP_2 binding in this study and by [7] (gray). The hydrophobic residues that have been tested for their influence on PIP_2 binding are indicated as gray ovals.

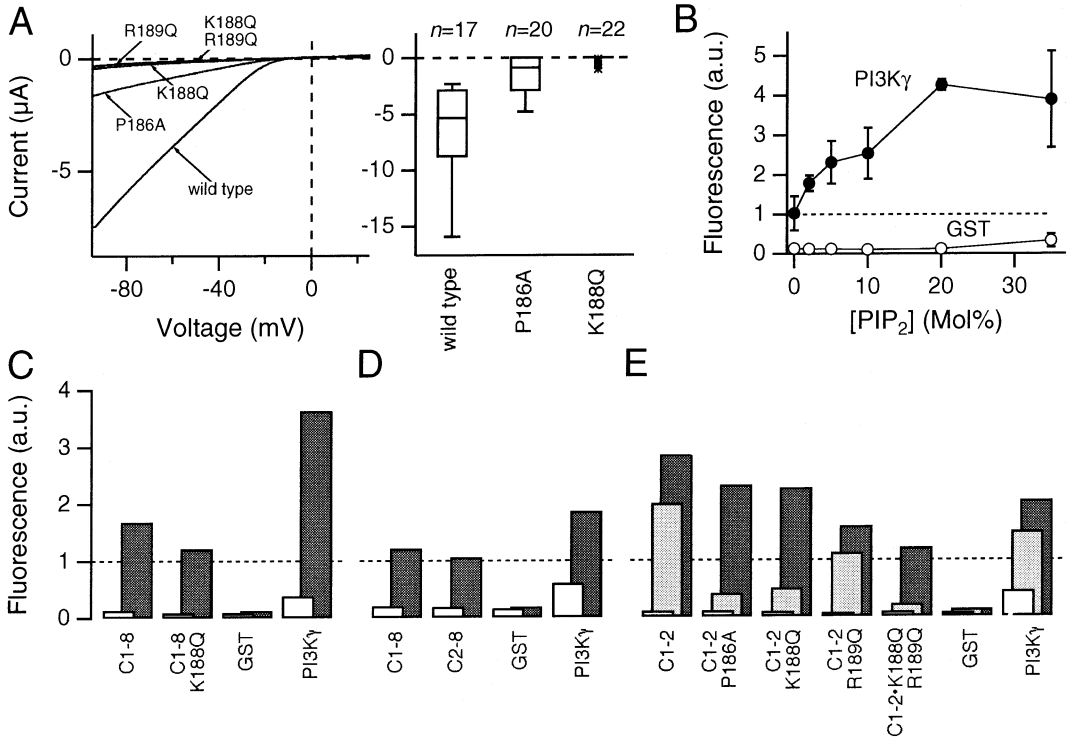

Fig. 2. Evaluation of the PKKR motif for channel expression and PIP_2 binding. A: Effects of point mutations in the PKKR-motif on the expression of Kir2.1 channels. Left: Current traces in response to voltage ramps (−100 to +50 mV in 2 s) from oocytes expressing wild-type (wt) and the mutants P186A, K188Q, R189Q, and K188Q•R189Q. Right: Box plots representing the statistics for the currents measured at −100 mV. Linear leak correction was performed assuming that the current at +50 mV was leakage current with a reversal potential of 0 mV; *n* indicates the number of oocytes. The center-line indicates the median, the boxes indicate the second and third quartile; whiskers and asterisks indicate extreme values and outliers, respectively. B: Averaged binding data of GST alone and GST–PI3Kγ (PI3Kγ) to liposomes as a function of the PIP_2 concentration. Error bars indicate ±S.D., the dashed line the threshold for PIP_2 binding. C–E: Independent PIP_2 binding determinations to the indicated constructs. In C and D data are shown for liposomes containing 35 mol percent PIP_2 (dark gray) and for liposomes without PIP_2 (white). In E, additional experiments at 5 mol percent PIP_2 (light gray) are shown.

3.2. *Effect of mutations in the PKKR motif on Kir2.1–PIP_2 binding*

To correlate the effects of the mutations on Kir2.1 current and their binding to PIP_2 we measured PIP_2 binding of the corresponding GST-fused proteins. As a control we tested the binding of GST alone and GST–PI3Kγ, which is known to use PIP_2 as one of its substrates [18], to the liposomes containing different concentrations of PIP_2 (Fig. 2B). At 35 mol percent PIP_2 the fluorescence value for GST was 0.34 ± 0.17 (mean ± S.D.), and for GST–PI3Kγ 3.9 ± 1.2. Therefore, we chose as a threshold value for PIP_2 binding detection 1 arbitrary fluorescence unit, which is about the GST value plus three standard deviations. In the following experiments we considered all fluorescence values below 1 as absence of PIP_2 binding.

In comparison with GST and GST–PI3Kγ we now tested the whole C-terminus of Kir2.1 (C1–8) in the wild-type form and containing the single-site mutation K188Q in the PKKR motif (Fig. 2C). In an independent experiment the PIP_2 binding to C1–8 was compared with a fragment lacking the PKKR motif (C2–8) (Fig. 2D). In both cases we did not find qualitative changes in PIP_2 binding compared to the wild-type C1–8 fragment. In other words, neither the point mutation in the PKKR motif, nor the complete absence of this region abolished in vitro PIP_2 binding to the Kir2.1 C-terminus. This strongly suggests that other PIP_2 binding regions must exist in the Kir2.1 C-terminus, compensating for the inactivation of the PKKR motif and making it impossible to detect differences in PIP_2 binding in the background of the full-length C-terminus.

Therefore, short C-terminal segments (C1–2) of the wild-type and mutants were assayed with liposomes containing 0, 5, and 35 mol percent PIP_2 (Fig. 2E). The wild-type fragment was binding PIP_2 already at 5 mol percent PIP_2. At the same concentration, K188Q•R189Q showed almost no binding. PIP_2 binding of mutants P186A and K188Q was stronger, but still below threshold. Mutant R189Q was significantly binding PIP_2, but about 1.5 times weaker than the wild-type. At 35 mol percent PIP_2 all of the constructs were binding PIP_2 to a significant degree, so we could not distinguish them in the qualitative PIP_2 binding assay.

These experiments showed that the mutations decrease the PIP_2 affinity of the region C1–2 in the following order: K188Q•R189Q > P186A, K188Q > R189Q > wild-type. The reduction of PIP_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 PIP_2 binding does not correlate in all cases. Such correlation is observed for mutants K188Q and K188Q•R189Q, as they both lead to significant reduction in Kir2.1 expression and PIP_2 binding in vitro. In the case of mutants P186A and R189Q the situation is reversal. Mutant P186A still allows for formation of functional channels, but significantly reduces PIP_2 binding in vitro. Mutant R189Q completely abolishes Kir2.1 expression in oocytes, but binds PIP_2 containing liposomes. This indicates the existence of factors that could influence the bio-

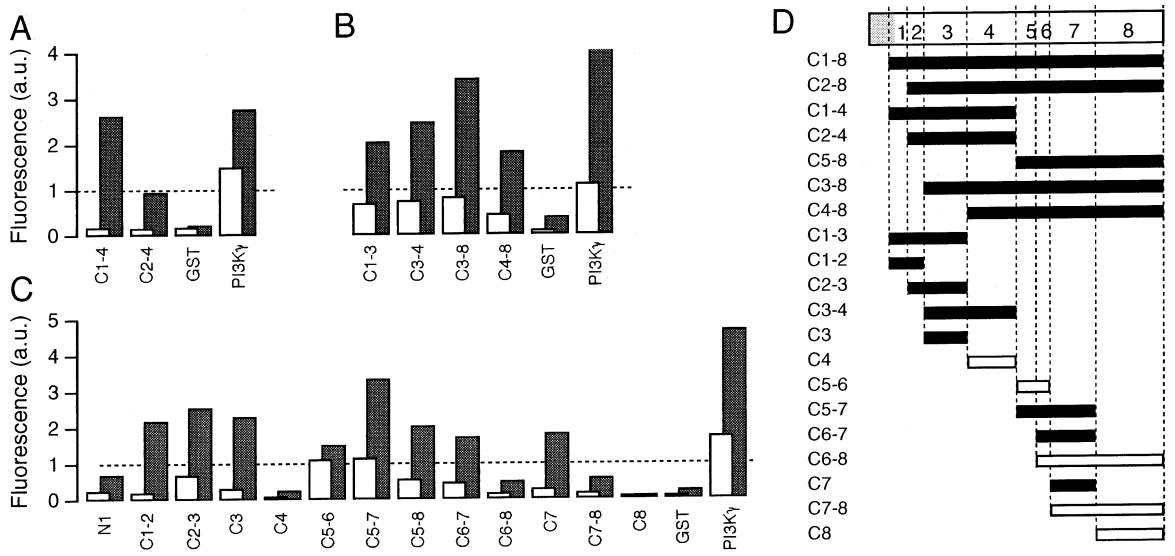

Fig. 3. Identification of PIP_2 binding domains. A–C: Each panel shows the data from one PIP_2 binding experiment. The labeling of the protein constructs and liposomes is as in Fig. 2. D: Summary of the data from experiments on in vitro PIP_2 binding of GST-fused C-terminal Kir2.1 fragments. Black bars indicate fragments that bind to liposomes containing 35 mol percent PIP_2 above the threshold; white bars indicate fragments without significant PIP_2 binding.

chemical characteristics of the PIP_2 binding region C1–2 in vivo.

3.3. *Kir2.1 has three C-terminal PIP_2 binding regions*

These data suggested that additional PIP_2 binding regions could exist in the C-terminal part of Kir2.1 channels, and, in fact, the region between aa 206 and 245 had been already shown to be important for the high PIP_2 affinity of Kir2.1 [7]. For a further screen of the Kir2.1 C-terminus for minimal PIP_2 binding sites we constructed GST-fusion proteins with overlapping Kir2.1 fragments. Region C4–8 (Fig. 3B) that lacks the putative PIP_2 binding region C3 still bound PIP_2 containing liposomes. Judging from the distribution of charged amino acids in the Kir2.1 C-terminus (Fig. 1) we suspected that there could be one more PIP_2 binding site in the region C7.

Fig. 3A–C shows data from three independent experiments, in which overlapping C-terminal fragments were tested for PIP_2 binding. Assays were performed at 35 mol percent PIP_2 to distinguish better between PIP_2 binding and not binding regions. The GST-fused segment N1 (see Fig. 1) was used as a control; consistent with the data reported by others [5] it bound PIP_2 below threshold (Fig. 3C, leftmost bar). GST–PI3Kγ usually showed a stronger binding to the control liposomes without PIP_2 than the Kir2.1 fusions, reflecting its lower specificity towards phospholipids. Two Kir2.1 fragments, C5–6 and C5–7, also bound unspecifically to the control liposomes (Fig. 3C). 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 PIP_2 affinity of these proteins by the difference between binding to control and experimental (PIP_2 containing) liposomes. For the fragment C5–6 this difference was 0.4 and for C5–7 2.2 a.u., so we considered them as not binding and binding PIP_2, respectively. For comparison, the equivalent values for GST and C5–8 were, 0.2 and 1.5 a.u., respectively.

Several regions bound PIP_2 significantly over the threshold. The summary of PIP_2 binding data from all experiments is given in Fig. 3D. The shortest PIP_2 binding regions other than C1–2 were C3 (207–246) and C7 (324–365). In the study of [7] regions 246–270 and 292–310 could not been tested for their importance for PIP_2 binding. According to our data the fragments containing these regions (C4, C5–6) do not bind PIP_2, but the latter one may influence PIP_2 binding by region C7. Indeed, some fragments containing this last PIP_2 binding site (C6–8 and C7–8) did not show PIP_2 binding, whereas other

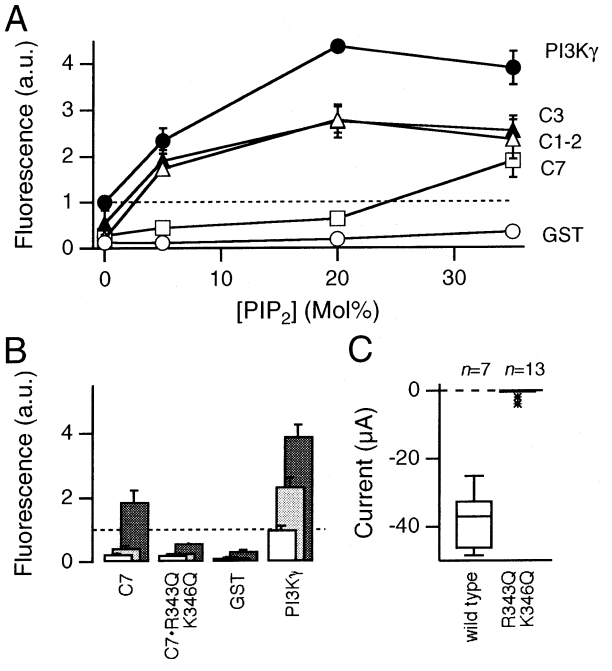

Fig. 4. Comparison of three PIP_2 binding sites. A: Binding of GST fusion proteins was assayed as a function of PIP_2 concentration (mean ± S.E.M.). B: Influence of mutation R343Q•K346Q on PIP_2 binding. The error bars indicate S.E.M. values for at least two independent experiments. C: Box plots representing the statistics for the currents measured at −100 mV in oocytes expressing the wild-type and the mutant R343Q•K346Q. Data are presented as in Fig. 2.

fragments (C5–8, C5–7, and C6–7) did. Thus, C8 has a negative effect on PIP_2 binding by fragments C7 and C6–7. This can be explained by the high density of negatively charged residues in this region preventing electrostatic binding between the positive charges in the region C7 and PIP_2. The region C5 antagonizes this effect possibly due to its hydrophobicity that enhances the protein–lipid interactions when present in the constructs.

3.4. Characterization of the third PIP_2 binding region

We further characterized the three PIP_2 binding regions by measuring their affinity to liposomes containing different PIP_2 concentrations (Fig. 4A). Fragments C1–2 and C3 bound PIP_2 already at 5 mol percent PIP_2 and reached their maximal PIP_2 affinity at 20 mol percent. Thus, C3 harbors an independent binding site that binds PIP_2 in vitro with almost the same affinity as the PKKR containing region C1–2. PIP_2 binding by the distal fragment C7 was weaker. At 5 and 20 mol percent PIP_2, the binding was below the threshold; only at 35 mol percent PIP_2 it almost reached the PIP_2 binding strength of the first two fragments. The weaker PIP_2 affinity may reflect a higher number of negatively charged residues in this region. These data characterize fragments C1–2 and C3 as 'strong' and fragment C7 as 'weak' in vitro PIP_2 binding sites. In the study of Zhang and colleagues [7] segments 316–341 and 342–366, which split the region C7 in two, were shown to be not important for high-affinity PIP_2 binding. This could mean that an intact C7 region is necessary for PIP_2 binding and/or that this region is indeed a low-affinity PIP_2 binding segment.

The functional relevance of the third PIP_2 binding region in Kir2.1 was tested by the double mutation R343Q•K346Q. As shown in Fig. 4B, this mutation significantly reduces in vitro PIP_2 binding of C7 at 35 mol percent PIP_2. Upon expression in *Xenopus* oocytes, it gave almost no current in comparison with wild-type Kir2.1 (-0.58 ± 0.34 μA and -38.6 ± 3.4 μA, respectively) (Fig. 4C). These data show that R343 and K346 are critical for PIP_2 binding of C7 in vitro. Furthermore, they suggest that the additional PIP_2 binding site in C7 is necessary for Kir2.1 function.

All three PIP_2 binding segments of Kir2.1 are conserved among the members of the Kir2 subfamily. Comparison of these segments does not reveal close sequence homology or even a defined amino acid motif. However, a common feature of the identified regions is the presence of multiple positive charges interspersed with aromatic or aliphatic amino acids. These characteristics seem to be a conserved principle among proteins that bind phosphoinositides, but do not involve pleckstrin homology (PH) domains, such as phospholipase D, synaptotagmin or gelsolin [19,20]. Our data support the idea that phosphoinositide binding depends not only on the electrostatic, but also on hydrophobic interactions. The relevance of Kir2.1 mutations P186A (this study) and L222I [7] for PIP_2 binding and channel expression suggests that the mechanism of protein–phosphoinositide interaction could be even more complex.

The identification of multiple PIP_2 binding sites in Kir2.1 sheds light on the constitutive activity of this channel. It can be hypothesized that some of these PIP_2 interaction sites are not functional in the regulated members of the Kir family, and thus, additional factors are required to stabilize the active channel conformation. Interestingly, Kir2.1 region 316–341, which overlaps the third binding site 324–365, has been shown to cause formation of a Gβγ-insensitive Kir3.4 channel, when replacing a homologous region in Kir3.4 [21]. The Gβγ-dependent activity of Kir3.1/Kir3.4 channels could be explained in a way that Kir3.4 has a site for Gβγ binding in place of a PIP_2 binding site. The presence of additional binding sites may also confer to a reported specificity of Kir2.1 toward PIP_2 in contrast to Kir3.1/Kir3.4 [6]. Whether this principle can be extended to other members of the Kir family, such as K_{ATP} channels, will be an important question for future investigations.

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