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Differential Phosphoinositide Binding to Components of the G Protein-Gated K^+ Channel

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Abstract. The regulation of ion channels and transporters by anionic phospholipids is currently very topical. G protein-gated K^+ channels from the Kir3.0 family are involved in slowing the heart rate, generating late inhibitory postsynaptic potentials and controlling hormone release from neuroendocrine cells. There is considerable functional precedent for the control of these channels by phosphatidylinositol 4,5-bisphosphate. In this study, we used a biochemical assay to investigate the lipid binding properties of Kir3.0 channel domains. We reveal a differential binding affinity to a range of phosphoinositides between the C termini of the Kir3.0 isoforms. Furthermore, the N terminus in addition to the C terminus of Kir3.4 is necessary to observe binding and is decreased by the mutations R72A, K195A and R196A but not K194A. Protein kinase C phosphorylation of the Kir3.1 C-terminal fusion protein decreases anionic phospholipid binding. The differential binding affinity has functional consequences as the inhibition of homomeric Kir3.1, occurring after M3 receptor activation, recovers over minutes while homomeric Kir3.2 does not.

Key words: K^+ channel — Inward rectifier — Anionic phospholipid — Phosphatidylinositol 4,5-bisphosphate — Protein-gated

Introduction

Inwardly rectifying potassium (Kir) channels play an important role in controlling cell excitability by stabilizing the resting membrane potential. Some of their physiological roles include the regulation of insulin secretion, vascular tone and heart rate (Reimann and Ashcroft, 1999). The focus of this study is the Kir3.0 family, which is involved in the regulation of electrical excitability in atrial cardiac cells, neurons and neuroendocrine cells. The Kir3.0 channels are generally heterotetramers composed of two different subunits. In the heart, the channel is composed of Kir3.1 and Kir3.4 and acts to slow the heart rate, whereas in the brain it is composed of Kir3.1 and Kir3.2 (Yamada, Inanobe & Kurachi, 1998). Stimulation of a G protein-coupled ($G_{i/o}$) receptor by an agonist causes activation of Kir 3.0. Inhibition of the channel results from activation of other G protein-coupled receptors ($G_{q/11}$-coupled) and the subsequent activation of phospholipase C (PLC) (Kobrinsky et al., 2000). PLC acts by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG), which activates protein kinase C (PKC) (Toker, 1998). In recent years, PIP_2 has been identified as playing a variety of important roles within the cell. It is involved in cytoskeletal regulation, and its further phosphorylation by phosphatidylinositol 3-kinases (PI-3Ks) allows the generation of second messengers (Toker, 1998). Additionally, it has been shown to directly regulate various ion channels and transporters including the Na^+/Ca^{2+} exchanger (Hilgemann & Ball, 1996) and Kir channels (Huang, Feng & Hilgemann, 1998; Sui, Petit-Jacques & Logothetis, 1998). Both depletion of PIP_2 levels in the membrane on activation of PLC and activation of PKC can lead to Kir3.0 channel inhibition (Leaney, Dekker & Tinker, 2001; Rohacs et al., 2002).

Studies on different Kir family members have shown that there is direct interaction between the channel and PIP_2 in both its N- and C-terminal cytoplasmic domains. (Huang et al., 1998; Zhang et al., 1999; Schulze et al., 2003). Additionally, Kir3.0 channels require the presence of $G_{\beta\gamma}$ (Logothetis et al., 1987) or intracellular Na^+ ions (Sui, Chan & Logothetis, 1996) in order for activation of the channel via PIP_2 to occur. It is thought that the presence of these factors stabilizes the channel-PIP_2 interaction (Huang et al., 1998; Zhang et al., 1999). In

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this study, we demonstrate that different Kir3.0 members have different affinities for anionic phospholipids and that this has functional sequelae. Furthermore, both the N- and C-terminal cytoplasmic domains can participate in the binding. We also used site-directed mutagenesis to identify crucial residues that allow phosphoinositide binding. Additionally, we show that these domains can be phosphorylated by PKC and this subsequently interferes with the ability of the domain to bind anionic phospholipids.

Materials and Methods

Chemicals

All enzymes for molecular biology were from New England Biolabs (Herts, UK) or Roche Molecular Biochemicals (Lewes, UK). All primers for sequencing and polymerase chain reaction (PCR) were obtained from Sigma Genosys (Cambridge, UK). ^{32}P-γ-adenosine triphosphate (ATP) was obtained from Amersham Biosciences (Bucks, UK). All other fine chemicals were from Sigma Aldrich (Dorset, UK).

Molecular Biology

The program ScanProsite (Swiss Institute of Bioinformatics, Geneva, Switzerland) was used to determine the cytoplasmic and membrane boundaries of Kir3.1 (accession L25264), Kir3.2A (accession U51124) and Kir3.4 (accession L35771). These regions were amplified using standard PCR techniques and cloned into the pMALc2x vector (New England Biolabs) to create N-terminal fusion proteins with maltose binding protein (MBP). Insertion of the desired section of DNA into the vector was verified by sequencing (Cytomyx, Cambridge, UK). The pMALc2x + Kir3.4N + C clone was created by linking the N and C termini with a glycine linker (six glycine residues) using splicing by overlap extension PCR. The QuikChange Site-directed Mutagenesis kit (Stratagene, Amsterdam, The Netherlands) was used in order to generate mutations in pMALc2x + Kir3.1C and pMALc2x + Kir3.4N + C as well as to create a stop codon in the pMALc2x vector after the MBP gene, to prevent expression of part of the *lacZ* gene. The presence of the desired mutations was confirmed by sequencing (Cytomyx).

Expression and Purification of MBP and MBP Fusion Proteins

Analysis of MBP and MBP fusion protein expression was carried out in *Eschericia coli* BL21 (DE3)-competent cells using standard methods. Induction of protein expression was achieved with 0.3 mM isopropylthiogalactoside (IPTG) and overnight growth at 25°C. Cells were harvested by centrifugation (4,000 × *g* for 30 min at 4°C). The resulting pellet was resuspended in buffer A (20 mM Tris [pH 7.5], 200 mM NaCl, 1 mM dithiothreitol [DTT], 1 mM ethylenediaminetetraacetic acid [EDTA]) plus protease inhibitors (EDTA-free) (Roche Molecular Biochemicals) and stored at –20°C. Cells were thawed and the membranes lysed by sonication (5 × 60 s) followed by centrifugation (10,000 × *g* for 30 min at 4°C). The pellet was further resuspended in buffer A containing protease inhibitors and subjected to sonication and centrifugation as above. The resulting supernatants were pooled and diluted fourfold before loading onto amylose resin preequilibrated with buffer A (New England Biolabs). About 1 ml of resin binds 3 mg of expressed protein. The column was washed with 12 column volumes (CVs) of buffer A. Elution of the desired protein was achieved by washing the column with 2 CVs of buffer A containing 10 mM maltose. Fractions containing protein were pooled and dialyzed against buffer B (50 mM Tris [pH 7.5], 200 mM NaCl, 1 mM DTT, 1 mM EDTA). Protein was concentrated to ≥ 5 mg/ml using centricon YM-30 centrifugal filter units (Millipore, Bedford, MA). Protein concentration was determined using Bio-Rad (Richmond, CA) protein assay reagent. Purity was determined by subjecting the protein to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Protein-lipid Overlay Assay

PIP strips (Invitrogen, Abingdon, UK) containing 100 pmol of various phosphoinositides were blocked in TBST (10 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Tween-20) + 3% nonfatty acid bovine serum albumin (BSA, blocking buffer). Strips were then incubated overnight at 4°C in blocking buffer containing 1 μg/ml of the desired MBP fusion protein. PIP strips were then washed three times for 10 min with TBST before incubation with anti-MBP antiserum (1:1,000 dilution in blocking buffer; New England Biolabs) for 2-3 h at room temperature. The anti-rabbit immunoglobulin G (IgG) antibody from the enhanced chemiluminescence Western blotting kit (Amersham Biosciences) was used to detect any binding of protein to the strips.

Phosphorylation of MBP Fusion Protein

Firstly, 1 mg of MBP fusion protein was bound to 100 μl of amylose resin by rotation overnight at 4°C. The amylose-protein complexes were washed five times with 1 ml 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (50 mM HEPES [pH 7.4], 1 mM EDTA, 10% glycerol, 1 mM DTT, protease inhibitors). Phosphorylation of the protein was carried out by adding 20 ng of catalytically active PKC subunit (Calbiochem, San Diego, CA), 5 × buffer (50 mM HEPES [pH 7.4], 1 mM ATP, 50 mM $MgCl_2$), HEPES buffer (10 μl), 50 mM HEPES + 1.5% Triton (13 μl) and 2 μCi of ^{32}P-γ-ATP. The sample was incubated at 37°C for 2 h and then washed five times with 1 ml of HEPES buffer. The sample was subjected to SDS-PAGE and autoradiography.

Determination of Binding to Phosphoinositides of Phosphorylated Protein

Phosphorylated and nonphosphorylated MBP + Kir3.1C, prepared as described above, were eluted from the amylose resin using 10 mM maltose (2 × 60 μl). The eluted protein was diluted (1/10) in blocking buffer (TBST + 3% nonfatty acid BSA) and subsequently incubated with PIP strips (preblocked) overnight at 4°C. Strips were briefly washed with TBST before being subjected to autoradiography.

Electrophysiology

Currents were recorded using whole-cell patch clamp under symmetrical K^+ conditions (~140 mM). Bath solution contained (in mM) 140 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES (pH 7.4) and pipette solution: 107 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 ethyleneglycoltetraacetic acid (EGTA), 5 HEPES, 2 MgATP, 0.3 Na_2GTP (KOH to pH 7.2, ~140 mM total K^+, calculated free Ca^{2+} 18 nM). Currents were recorded with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA), and data were acquired using a Digidata 1322A and pClamp8 software (Axon Instruments) as

previously described (Leaney, Milligan & Tinker, 2000). Drugs were applied using a gravity-driven perfusion system. Data were analyzed at –60 mV unless otherwise stated. For all current-voltage (I-V) investigations, cells were held at 0 mV and stepped to potentials between –100 and + 50 mV in 10-mV increments for 100 ms.

For cell-attached and inside-out patch recordings, patch pipettes, pulled from filamented borosilicate glass (Harvard Apparatus, Dover, MA), had resistance of 8-10 MΩ. Prior to filling, tips of patch pipettes were coated with a Parafilm/mineral oil suspension. The compositions of bath and pipette solutions are described below. Currents were recorded with an Axopatch 200B patch-clamp amplifier, filtered at 1 kHz, digitized at 5 kHz; and data were acquired using the Digidata 1322A and pClamp8 software. The pipette solution used in inside-out single-channel experiments was identical to the whole-cell bath solution as described above (except pH 7.2), while the inside-out single-channel bath solution contained (in mM) 140 KCl, 2.6 $CaCl_2$, 1 $MgCl_2$ and 5 HEPES (pH 7.4). ATP and guanosine triphosphate (GTP) supplementation is indicated in the legend. For cell-attached experiments, both pipette and bath solution were identical to whole-cell bath solution. Drugs were bath-applied using either a gravity-fed system or a localized application system (MSC160; Bio-Logic, Claix, France). Data are presented as mean ± standard error of the mean (SEM), and one-way analysis of variance (ANOVA) followed by Bonferroni's posttest was used to test for statistical significance.

Results

Purification of MBP Fusion Proteins

Eukaryotic membrane proteins, such as ion channels, are generally difficult to obtain both in large quantities and in a soluble form in order for biochemical studies to be carried out. In order to overcome this problem, a bacterial expression system, which involves tagging the protein of interest at its N terminus with MBP, was used (di Guan et al., 1988; Bedouelle & Duplay, 1988). Additionally, as we were interested only in the cytoplasmic domains of the three subunits, Kir3.1, Kir3.2A and Kir3.4, the boundaries between the transmembrane domains and the N- and C-terminal cytoplasmic domains were determined using ScanProsite (Fig. 1). The N- and C-terminal cytoplasmic domains were identified to be Kir3.1 (N = aa 1-85, C = aa 180-501), Kir3.2A (N = aa 1-96, C = aa 194-425) and Kir3.4 (N = aa 1-91, C = aa 189-419); and subsequent fusion proteins of only these domains were created. The addition of the N-terminal MBP tag to these C-terminal domains resulted in ≥50% soluble protein as judged by SDS-PAGE (Fig. 2, lane 4). This may be due to the large size of the MBP (42 kDa) that is fused to the N terminus as larger tags have been shown to increase solubility due to the promotion of correct folding of the protein within the cell (Baneyx, 1999). The one-step purification resulted in fusion proteins that had ≥ 95% purity (Fig. 2, lane 6). From 500 ml culture, 5-20 mg of MBP fusion protein was obtained.

Unfortunately, although the N-terminal cytoplasmic domains of each subunit could be correctly inserted into the vector, no visible expression of the desired protein was achieved. It is possible that the N-terminal domain was unstable even when fused to MBP, resulting in its degradation, as only a band corresponding to the expected size of MBP was observed on SDS-PAGE (*data not shown*).

Binding of Phosphoinositides to KIR3.0 C-Terminal Cytoplasmic Domains

The regulation of various Kir subunits by the probable binding of PIP_2 to their cytoplasmic domains has been well documented (Huang et al., 1998; Zhang et al., 1999; Schulze et al., 2003). Here, we investigated the binding of the cytoplasmic C-terminal domains of the various Kir3.0 to anionic phospholipids using a protein-lipid overlay assay. Purified fusion proteins were incubated with PIP strips as described in Materials and Methods. PIP strips are nitrocellulose membranes that have 100 pmol of various phospholipids and phosphatidylinositols spotted onto them. Only the MBP-Kir3.1 C-terminal domain was shown to be able to bind to some of the lipids on the membrane (Fig. 3A, ii). In fact, significant binding was only observed to phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 5-phosphate (PI5P) (Fig. 3A, ii, and B). Some binding to phosphatidylinositol 3-phosphate (PI3P) and phosphatidylinositol 3,4-bisphosphate ($PI3,4P_2$) was also observed (Fig. 3A, ii, and B). However, binding of the MBP-Kir3.1 C-terminal domain to PI3P was not significant due to the high variability observed between experiments (Fig. 3B). In comparison, no binding to either the MBP-Kir3.2 or the MBP-Kir3.4 C-termini was observed for any of the phospholipids or phosphatidylinositols present (Fig. 3A, iii and iv, and B). Interestingly little binding to PIP_2 was observed.

The Presence of the N Terminus of KIR3.4 Affects the Binding Profile

Some residues involved in the binding of PIP_2 and related species have been identified in the N-terminal cytoplasmic domain (Schulze et al., 2003). Therefore, we created a fusion protein containing the N terminus of Kir3.4 (aa 1-90) linked to the Kir3.4 C terminus (aa 189-419) via six glycine residues. The glycine residues offer an inert linker, which should allow flexibility and not interfere with the binding profile. The binding of MBP-Kir3.4N+C is significantly altered in comparison to that of MBP-Kir3.4C alone (Fig. 4A, compare iii to ii, and B). The presence of the N-cytoplasmic domain allows the protein to bind to PI3P, PI4P and PI5P. Additionally some binding is observed to phosphatidylinositol 3,5-bisphosphate

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                                N-terminal cytoplasmic domain
mKir3.2A  MTMAKLTESMTNVLEGDSMDQDVESPVAIHQP-KLPKQARDDLPRHISRDR----TKRKI 55
rKir3.4   --MAGDSR--------NAMNQDMEIGVTSQDHKKIPKQARDYIPIATDRTRLLPEGKKPR 50
rKir3.1   --MSALRR---------KFGDDYQVVTTSSSGSGLQPQGPGQGPQQQLVPK------KKR 43

                                                              *
mKir3.2A  QRYVRKDGKCNVHHGNVR-ETYRYLTDIFTTLVDLKWRFNLLIFVMVYTVTWLFFGMIWW 114
rKir3.4   QRYMEKTGKCNVHHGNVQ-ETYRYLSDLFTTLVDLKWRFNLLVFTMVYTITWLFFGFIWW 109
rKir3.1   QRFVDKNGRCNVQHGNLGSETSRYLSDLFTTLVDLKWRWNLFIFILTYTVAWLFMASMWW 103

mKir3.2A  LIAYIRGDMDHIEDPSWTPCVTNLNGFVSAFLFSIETETTIGYGYRVITDKCPEGIILLL 174
rKir3.4   LIAYVRGDLDHVGDQEWIPCVENLSGFVSAFLFSIETETTIGYGFRVITEKCPEGIILLL 169
rKir3.1   VIAYTRGDLNKAHVGNYTPCVANVYNFPSAFLFFIETEATIGYGYRYITDKCPEGIILFL 163

                              *    C-terminal cytoplasmic domain
mKir3.2A  IQSVLGSIVNAFMVGCMFVKISQPKKRAETLVFSTHAVISMRDGKLCLMFRVGDLRNSHI 234
rKir3.4   VQAILGSIVNAFMVGCMFVKISQPKKRAETLMFSNNAVISMRDEKLCLMFRVGDLRNSHI 229
rKir3.1   FQSILGSIVDAFLIGCMFIKMSQPKKRAETLMFSEHAVISMRDGKLTLMFRVGNLRNSHM 223

mKir3.2A  VEASIRAKLIKSKQTSEGEFIPLNQSDINVGYYTGDDRLFLVSPLIISHEINQQSPFWEI 294
rKir3.4   VEASIRAKLIKSRQTKEGEFIPLNQTDINVGFDTGDDRLFLVSPLIISHEINEKSPFWEM 289
rKir3.1   VSAQIRCKLLKSRQTPEGEFLPLDQLELDVGFSTGADQLFLVSPLTICHVIDAKSPFYDL 283

mKir3.2A  SKAQLPKEELEIVVILEGMVEATGMTCQARSSYITSEILWGYRFTPVLTLEDGFYEVDYN 354
rKir3.4   SRAQLEQEEFEVVVILEGMVEATGMTCQARSSYMDTEVLWGHRFTPVLTLEKGFYEVDYN 349
rKir3.1   SQRSMQTEQFEVVVILEGIVETTGMTCQARTSYTEDEVLWGHRFFPVISLEEGFFKVDYS 343

mKir3.2A  SFHETYETSTPSLSAKELAELANRAEVPLSWSVSSKLNQHAELETEE------------ 401
rKir3.4   TFHDTYETNTPSCCAKELAEMKRNGQLLQSLPSPPLLGGCAEAEKEA------------ 396
rKir3.1   QFHATFEVPTPPYSVKEQEEMLLMSSPLIAPAITNSKERHNSVECLDGLDDISTKLPSKL 403

mKir3.2A  -EEKNPEELTERNGDVANLENESKV---------------------------------- 425
rKir3.4   -EAEHDEEEEPNGLSVSRATRGSM---------------------------------- 419
rKir3.1   QKITGREDFPKKLLRMSSTTSEKAYSLGDLPMKLQRISSVPGNSEEKLVSKTTKMLSDPM 463

mKir3.2A  ------------------------------------
rKir3.4   ------------------------------------
rKir3.1   SQSVADLPPKLQKMAGGPTRMEGNLPAKLRKMNSDRFT 501
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Fig. 1. Alignment of Kir3.0 subunits. Alignment of rat Kir3.1, mouse Kir3.2A and rat Kir3.4 produced using ClustalW v.1.82. The boundaries of the N- and C-terminal cytoplasmic domains are indicated. Conserved residues that may be involved in anionic phospholipid binding to the channel are boxed.

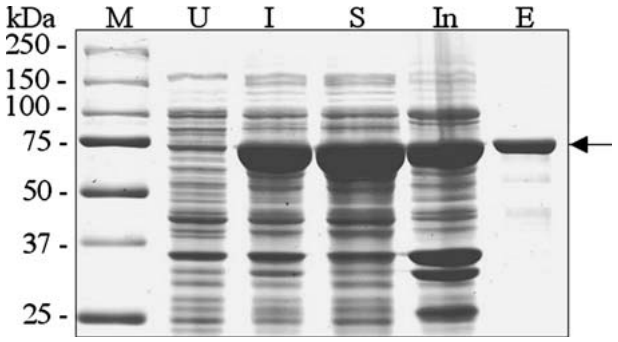

Fig. 2. Purification of MBP-Kir3.4 C-terminal domain. A 10% SDS-PAGE showing purification of MBP-Kir3.4C fusion protein. M, Bio-Rad Precision plus broad-range SDS standards; U, uninduced cells; I, induced cells; S, soluble protein; In, insoluble protein; E, eluted protein (2 μg). Marker sizes are indicated in kilodaltons.

($PI3,5P_2$) and phosphatidylinositol 3,4,5-triphosphate ($PI3,4,5P_3$) (Fig. 4A, iii, and B). This result indicates that there are amino acid residues present in both the N and C cytoplasmic domains that are important in the binding of phosphoinositides. The presence of both cytoplasmic domains may also be important for phosphoinositide binding to MBP-Kir3.2N+C; however, we were unable to clone this construct into the pmalc2x vector.

Identification of Possible Residues Involved in Binding of Phosphoinositides

Previous studies with Kir1.1, Kir2.1 and Kir6.2 have identified positively charged arginine and lysine residues in the N and C termini that may be involved in the interaction of the channel with PIP_2 (Huang et al., 1998; Shyng & Nichols, 1998; Soom et al., 2001; Schulze et al., 2003). In order to investigate the effect of some of these residues on the binding of phosphoinositides, the following mutations were created in MBP-Kir3.4N+C: K194A, K195A and R196A. These residues make up an important KKR motif (Fig. 1). The loss of K194 in

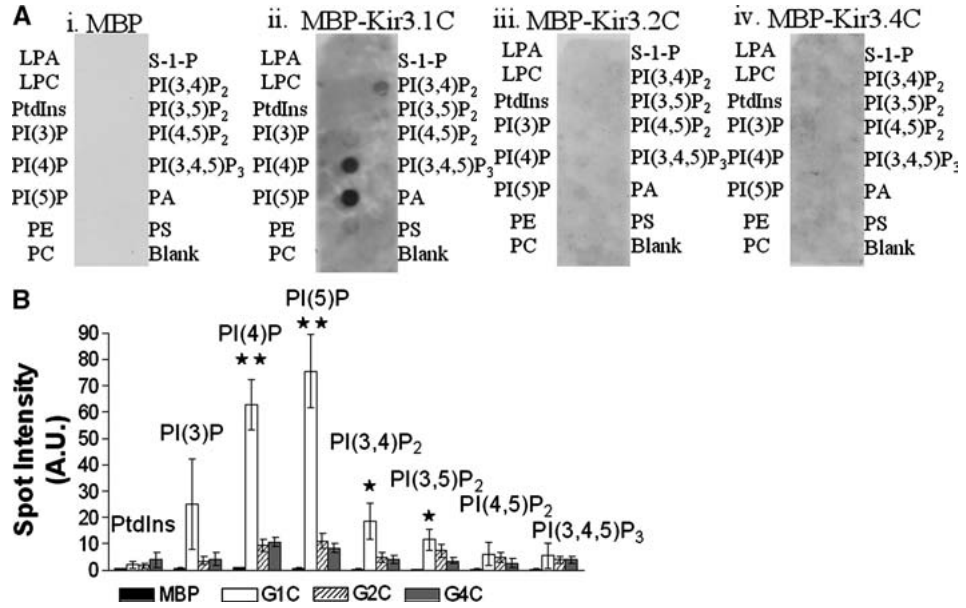

Fig. 3. Protein-lipid overlay assay with Kir3.0 C-terminal MBP proteins. (*A*) PIP strips with the various MBP fusion proteins bound: *i*, MBP only; *ii*, MBP-Kir3.1C; *iii*, MBP-Kir3.2C; *iv*, MBP-Kir3.4C. LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S-1-P, sphingosine-1-phosphate; PA, phosphatic acid; PS, phosphatidylserine. (*B*) Graph showing the average spot intensity ($n = 4$) for the indicated phosphoinositides determined for each protein. Statistical significance was determined using one-way ANOVA and Dunnett's posttest and is represented as follows: $*P < 0.05$, $**P < 0.01$.

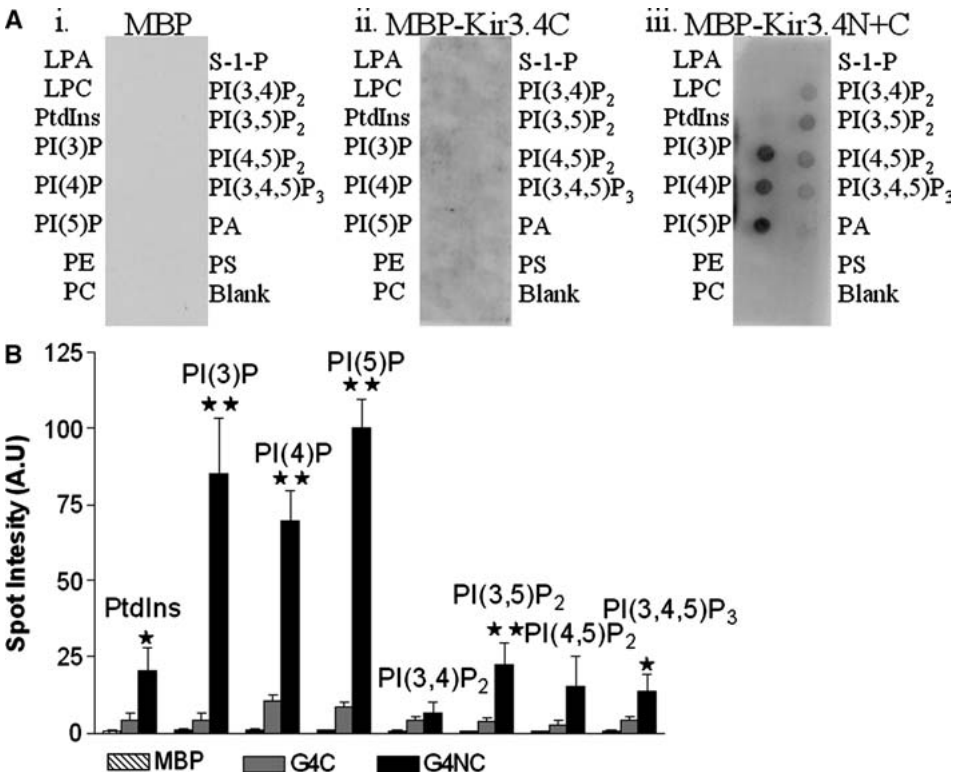

Fig. 4. Protein-lipid overlay assay with Kir3.4 N and C-terminal MBP proteins. (*A*) PIP strips with the various MBP fusion proteins bound: *i*, MBP only; *ii*, MBP-Kir3.4C; *iii*, MBP-Kir3.4N + C. (*B*) Graph showing the average spot intensity ($n = 3$) for the indicated phosphoinositides for each protein. Statistical significance is represented as follows: $*P < 0.05, **P < 0.01$.

MBP-Kir3.4N + C had no impact on the binding of phosphoinositides, especially PI4P and PI5P (Fig. 5A, ii, and B). The mutation of K195 and R196 to alanine residues resulted in decreased binding to PI4P and PI5P, more so for R196A (Fig. 5B). If both of these residues, K195 and R196, are lost from this protein, does this have an effect on the binding? It seems that, indeed, the loss of both

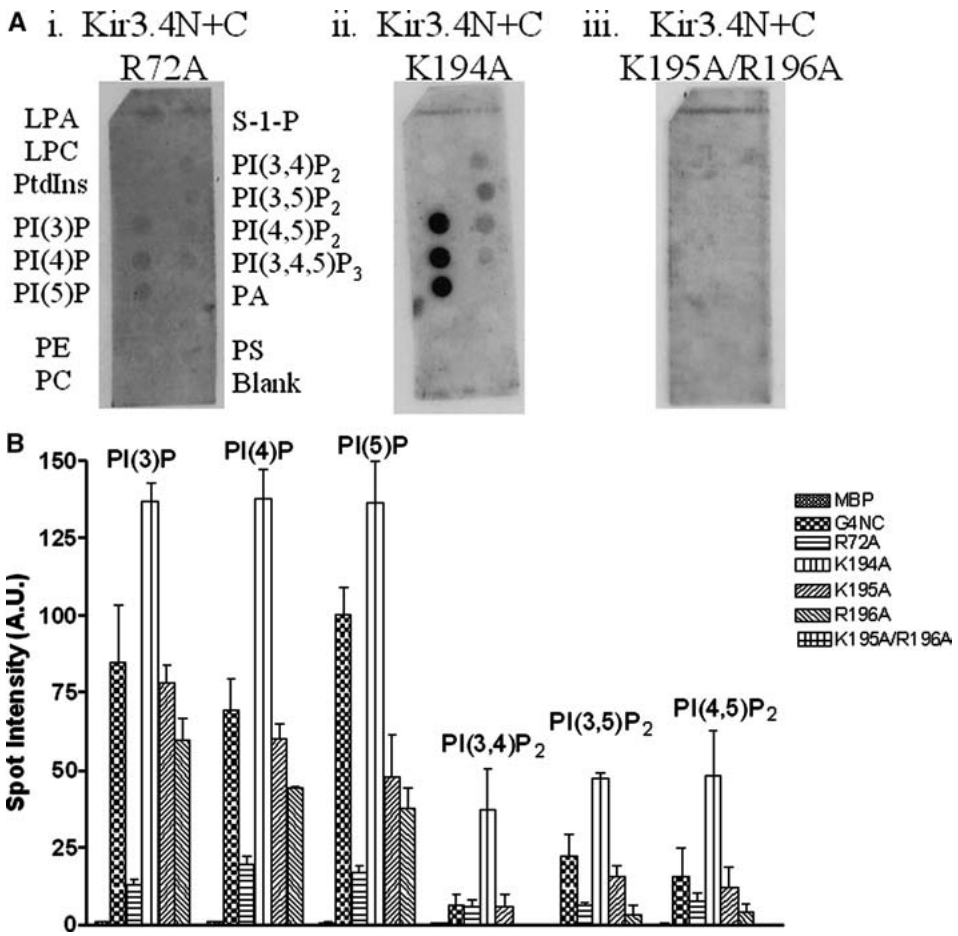

Fig. 5. Protein-lipid overlay assay with Kir3.4N + C-terminal MBP mutant proteins. (*A*) PIP strips incubated with MBP-Kir3.4N + C mutant proteins: *i*, R72A; *ii*, K194A; *iii*, K195A/R196A. (*B*) Graph showing the average spot intensity for indicated phosphoinositides for all of the MBP-Kir3.4N + C mutant proteins. As $n = 2$, no statistical significance was determined.

of these residues results in the complete loss of binding to any of the phosphoinositides present (Fig. 5A, iii, and B). Therefore, loss of only one of these residues decreases the ability of MBP-Kir3.4N + C to bind to phosphoinositides, but the combined loss of both completely abolishes binding. The N-terminal residue thought to be involved in binding, R72 was also mutated to an alanine (Fig. 1). The loss of this residue completely abolished the ability of MBP-Kir3.4N + C to bind to phosphoinositides (Fig. 5A, i, and B). This indicates that residues present in both the N and C terminal domains are important for phosphoinositide binding.

The homologous residues present in MBP-Kir3.1C were also mutated to alanine, in particular, the conserved KKR motif (K188A, K189A, R190A). Incubation of these mutant proteins with PIP strips indicated a similar pattern to that observed for the KKR motif in MBP-Kir3.4N + C. Again, there was no decrease in the ability of the protein to bind phosphoinositides in the absence of K188. However, the loss of K189 and R190 separately contributed to a decrease in the ability of the mutant proteins to bind PI4P and PI5P in particular (*data not shown*).

Phosphorylation and Effect on Phosphoinositide Binding

Inhibition of Kir3.0 channels by PLC-coupled hormones may occur via PIP_2 depletion or by activation of PKC. Here, we investigated whether MBP fusion proteins containing the C termini of these channels can be phosphorylated. We have previously shown that the C termini of Kir3.1, but not Kir3.2, can be phosphorylated by PKC (Brown et al., 2005). We now compared this with Kir3.4. In Figure 6A, radioactive ^{32}P-ATP was incubated in the presence or absence of PKC with all of the fusion proteins. However, in the presence of PKC, only MBP-Kir3.1C, MBP-Kir3.4C and MBP-Kir3.4N + C were phosphorylated. MBP was not phosphorylated, so any incorporation must have occurred on the cytoplasmic domains of the channel. MBP-Kir3.1C appeared to be heavily phosphorylated, and this phosphorylation was clearly visible after 6 h exposure. Phosphorylation of MBP-Kir3.4C and MBP-Kir3.4N + C was visible only after 24-h exposure and was much weaker. No phosphorylation was visible for MBP-Kir3.2C even after 72-h exposure.

Does phosphorylation of the cytoplasmic domains affect the ability of the domains to bind to

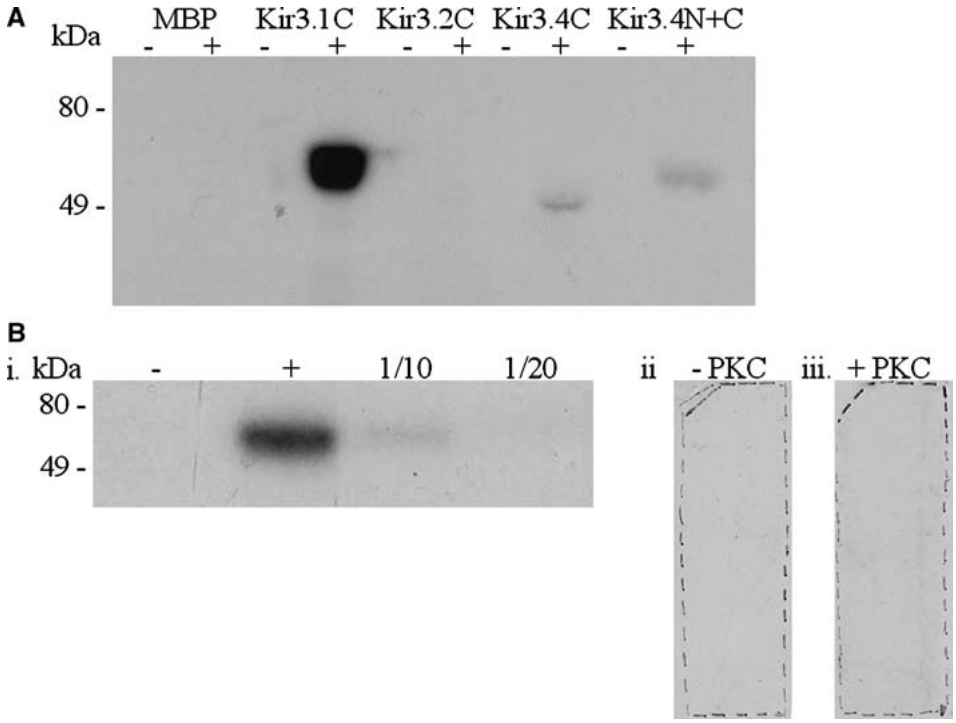

Fig. 6. Phosphorylation of MBP fusion proteins. (*A*) Autoradiograph after 24 h showing phosphorylation of MBP alone, MBP + Kir3.1 C termini, MBP + Kir3.2A C-termini, MBP + Kir3.4 C-termini, MBP + Kir3.4 N- and C-termini. Protein was incubated in the absence (−) or presence (+) of PKC. (*B*) Autoradiograph showing the phosphorylation of MBP + Kir3.1 C termini and subsequent binding to PIP strips. (*i*) Determination of phosphorylation of MBP + Kir3.1C in the absence (-) or presence (+) of PKC. Dilutions of 1/10 and 1/20 of phosphorylated MBP + Kir3.1C are also indicated in order to show limitations of the assay. PIP strips incubated with 1/10 dilution of nonphosphorylated (−PKC) and phosphorylated (+PKC) MBP + Kir3.1C are shown in *ii* and *iii*, respectively.

PI4P or PIP_2? This was tested using the MBP + Kir3.1C fusion protein as this was shown to be heavily phosphorylated (Fig. 6A) and to have a high affinity for these phosphoinositols. The non-phosphorylated and phosphorylated forms of MBP + Kir3.1C were incubated with PIP strips and subsequently subjected to autoradiography. Interestingly, phosphorylated MBP + Kir3.1C did not bind to any of the phospholipids on the PIP strips (Fig. 6B, iii). In order to confirm that detection of phosphorylation was still possible when the protein was diluted, samples of phosphorylated protein at this dilution were subjected to SDS-PAGE. Autoradiography was able to detect a band in the undiluted phosphorylated protein and in the sample diluted 1:10 (Fig. 6B, i). Therefore, protein bound to phospholipid spots should be detectable using this method. This result therefore suggests that phosphorylation of the Kir3.1 C-terminal cytoplasmic domain reduces the ability of the domain to bind to phosphoinositides.

The Functional Effects of Differential Phospholipid Affinity

We investigated whether the differential phospholipid sensitivity between Kir3.1 and Kir3.2 has any functional sequelae. The most direct method to examine this would be to pull inside-out patches and directly apply phospholipids to the inside of the membrane. For example, we have shown that Kir6.2 runs down rapidly in inside-out patches and the application of water-soluble PIP_2 reverses this run-down. In contrast, Kir6.1 has a much higher affinity for PIP_2 and does not so readily run down (Quinn et al., 2003). Thus, we attempted analogous experiments with the homomeric channels, namely Kir3.1 with point mutation F137S to render it functional (Vivaudou et al., 1997) and Kir3.2C, which expresses significant GIRK currents when expressed alone in our hands. In cells overexpressing the mutant channel subunits together with $G_{\beta 1\gamma 2}$ in cell-attached patches, there were substantial inward currents. However, on excision into solutions replete with ATP and GTP, these currents rapidly declined and activity did not recover with the application of PIP_2 (Fig. 7). In contrast, studies on heteromeric channel complexes in similar conditions showed basal activity on excision and an increase in channel activity on application of PIP_2 (Brown et al., 2005).

We previously established that it is possible to obtain an indirect measure of this by examining how quickly channel inhibition recovers after M3 receptor activation (Quinn et al., 2003; Brown, Tinker &

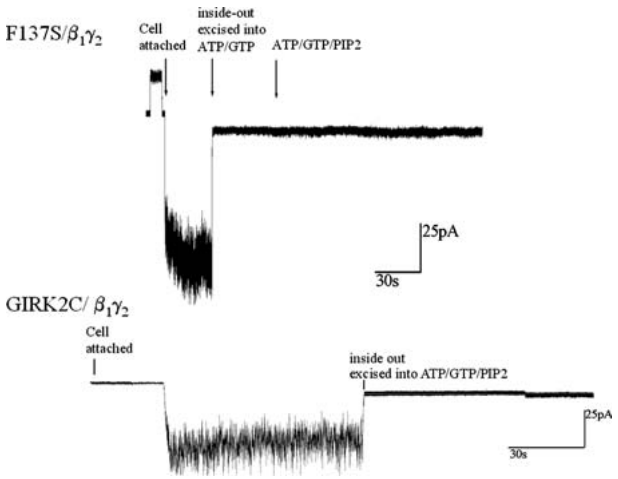

Fig. 7. Irrecoverable rundown of homomeric channels. In cell-attached patches both Kir3.1(F137S) and Kir3.2C expressed substantial inward currents when coexpressed with $G_{\beta1\gamma2}$ and held at a holding potential of –60 mV. Excision into a bath solution containing 2 mM ATP and 0.1 mM GTP resulted in loss of channel activity, which was not recovered with application 10 μM DiC8 PIP_2 with both homomeric channels.

Leaney, 2004). The rationale is that PIP_2 replenishment is relatively slow in the whole-cell configuration with our pipette solutions. Channels with high affinity return to basal activity more quickly than those with low affinity (Quinn et al., 2003). We compared the homomeric channels, namely Kir3.1 with point mutation F137S to render it functional (Vivaudou et al., 1997) and Kir3.2C, which expresses significant GIRK currents when expressed alone in our hands. One complication with the latter is that to see significant basal currents it is necessary to overexpress the G protein subunits $G_{\beta1}$ and $G_{\gamma2}$. In both the presence of $G_{\beta1}$ and $G_{\gamma2}$ and without their expression, Kir3.1(F137S) was inhibited (after an initial activation) and this recovered on agonist withdrawal (Fig. 8). In contrast, Kir3.2C did not recover from inhibition over an 8-min period (Fig. 9).

Discussion

Using a protein-lipid overlay assay, we have shown that only the Kir3.1 C-terminal cytoplasmic domain is capable of binding phosphoinositides. Interestingly, no binding to any phosphoinositides was observed by the Kir3.4 C-terminal domain on its own; however, when the N-terminal domain was linked to the C-terminal domain by a flexible linker, the ability of the protein to bind to phosphoinositols was increased. The same may also be true for the Kir3.2 C-terminal domain, in that the presence of the N-terminal domain may facilitate the binding of phosphoinositides; however, we did not investigate this here. This differential affinity for phospholipids has functional consequences: homomeric channels recover at different rates after M3 receptor activation and agonist withdrawal.

We show here and have previously demonstrated (Quinn et al., 2003) that Kir3.0 and Kir6.0 channels bind monophosphoinositides (PI4P, PI5P) with higher affinity than PIP_2. The location of this phosphate does not appear to be important as the presence of a phosphate group in the 3′, 4′ or 5′ position does not affect binding to the C-terminal domain. Previous studies investigating whether Kir3.0 channels could be activated by PI4P and PIP_2 showed that both could activate single channels, although activation by PI4P was about fourfold less than that by PIP_2 (Rohacs et al., 1999). This reflects the results shown here as it suggests that interaction of these domains with PI4P and other monophosphoinositols is possible. It is also plausible that the monophosphoinositols bind with high affinity but the efficacy of channel activation is reduced compared to that of PIP_2 (i.e., acting in a manner analogous to a partial agonist). It is then difficult to correlate affinity with channel activation and the potential physiological role of a particular phospholipid after appropriate receptor activation. This interaction may be physiologically relevant as PI4P is present at a high concentration in the Golgi, and it is plausible that the interaction may be important for the trafficking of the channel (Roth, 2004). Additionally, a high-affinity interaction for PI4P may prevent the channel from PIP_2 binding and subsequent activation under conditions of low PIP_2 concentrations at the plasma membrane. Furthermore, Kir3.0 channels interact with PIP_2 to a lesser extent than do other inward rectifiers, e.g., Kir1.1 and Kir2.1 (Huang et al., 1998; Zhang et al., 1999).

A possible anionic phospholipid binding site would be expected to consist of positively charged amino acid residues to allow for electrostatic interactions with the negatively charged phosphate groups. Functional studies have identified putative residues that may interact with PIP_2. In this study, the KKR motif present in all Kir3.0 channels was investigated along with an N-terminal arginine. Soom et al. (2001) identified the PKKR motif in Kir2.1 (aa 186-189) to be one of the multiple binding sites required for the strong PIP_2 interaction. The Kir2.1 R189 residue is highly conserved among the Kir channels, e.g., R188 in Kir1.1 (Huang et al., 1998) and R177 in Kir6.2 (Shyng & Nichols, 1998). When mutated, these residues result in decreased PIP_2 sensitivity. This is also the case for the homologous residue in Kir3.1 and Kir3.4 (R190 and R196, respectively) investigated in this study. The mutation of this residue to an uncharged alanine reduced the ability of the protein to bind to phosphoinositides when compared to wild-type protein. Additionally,

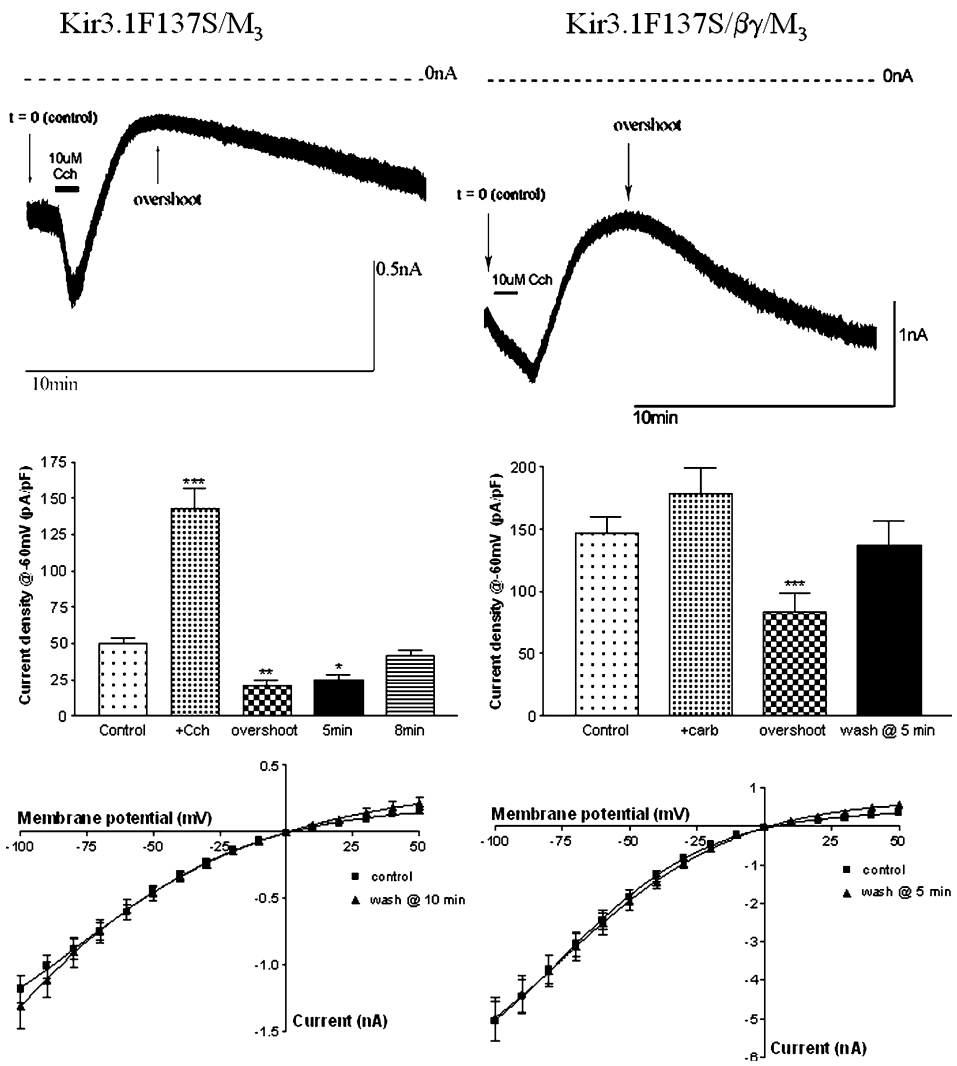

Fig. 8. Inhibition of Kir3.1(F137S) via M3 receptors. Kir3.1(F137S) was expressed with the M3 muscarinic receptor (*left panels*, $n = 12$) and with $G_{\beta 1}$ and $G_{\gamma 2}$ (*right panels*, $n = 6$). In the upper panels are representative recordings with bar graphs summarizing the recovery from inhibition (*middle panels*) and mean current-voltage relationships (*lower panels*). In both conditions, Kir3.1(F137S) recovered to baseline current density 5–8 min after removal of the agonist.

the two preceding lysine residues were investigated and only K195A showed a loss in the ability of the protein to bind phosphoinositides. Together the loss of K195 and R196 in Kir3.4 completely abolished any binding; the same result was observed for the corresponding residues in Kir3.1 (*data not shown*). This indicates that although loss of either the lysine or arginine alone can reduce the ability of the protein to bind phosphoinositides, loss of both residues results in the complete loss of binding. Additionally in Kir3.4N+C, the R72 residue was found to correspond to an arginine residue in Kir6.2 (R54) that has been implicated in the binding of PIP_2 (Schulze et al., 2003). Mutation of this residue in MBP-Kir3.4N+C resulted in the abolition of binding. Therefore, we have established that residues within both the N- and C-terminal cytoplasmic domains of the Kir3.4 channel are required for phosphoinositide interactions.

The publication of the structure of the Kir3.1N+C domain (Nishida & MacKinnon, 2002) and the prokaryotic inwardly rectifying K^+ channel KirBac1.1 (Kuo et al., 2003) may give some insight into the binding of phosphoinositides. A comparison of the residues that have been identified in this study and the Kir3.1 N+C structure shows that only the R190 residue of the KKR motif is present. The Kir3.1 N+C structure contains N-terminal residues 41-63 and C-terminal residues 190-371. The corresponding R72 residue in Kir3.1 is R66, and this is not present within the structure. Additionally, R190 (corresponding to R196 in Kir3.4) is the only residue present from the KKR motif. However, the structure indicates that R190 is close to the membrane cytoplasmic interface, with the arginine side chain pointing in the direction of the lipid bilayer (Nishida & MacKinnon, 2002). Conceivably, K189 could be in a similar location with the side chain in the direction of the lipid bilayer and subsequently both R190 and K189 could be in a position to interact with anionic phospholipids. As the loss of one of these residues only decreases the binding affinity, they must be in close enough proximity to be able to interact. There is

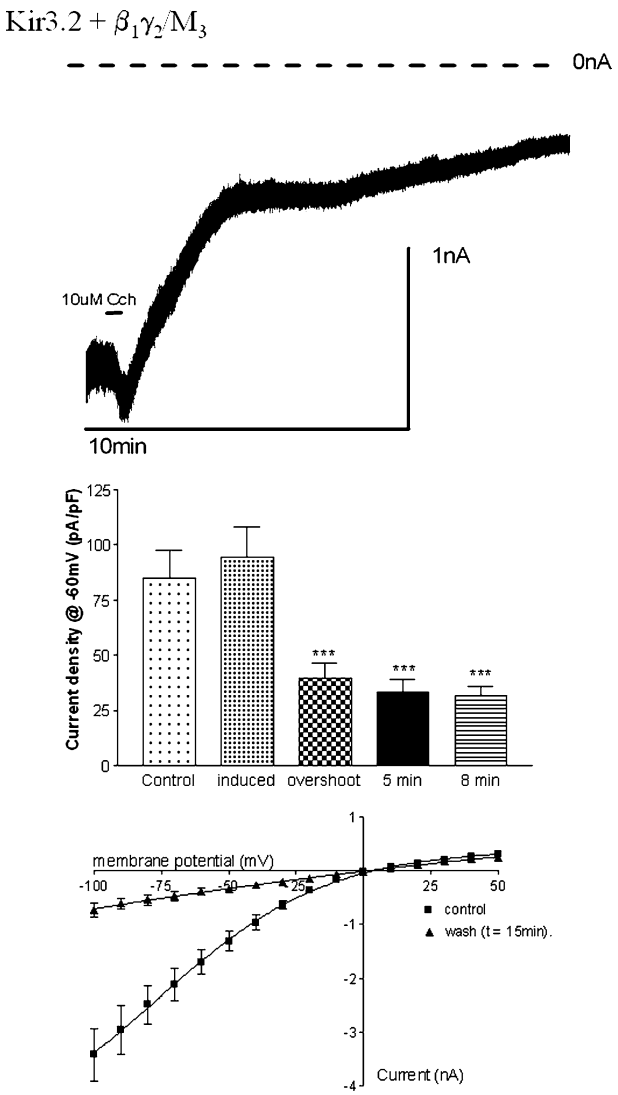

Fig. 9. Inhibition of Kir3.2C via M3 receptors. Kir3.2C was expressed with the M3 muscarinic receptor and with $G_{\beta 1}$ and $G_{\gamma 2}$ ($n = 11$). In the *upper panel* is a representative recording with a bar graph below summarizing the recovery from inhibition. Mean current-voltage relationships are shown at the *bottom* of the figure. Kir3.2 did not recover to baseline current density 8 min after agonist removal.

an interaction between the N- and C-terminal residues via a short parallel β-sheet structure (Nishida & MacKinnon, 2002); in KirBac1.1, this has been identified as residues 40-42 in the N terminus and 298-300 in the C terminus (Kuo et al., 2003). As the amino acids in the N terminus have been predicted to lie close to the membrane intracellular interface, residue R66 could be in such a position that possible interactions with phosphoinositides present in the membrane are feasible.

Kir3.0 is regulated via $G_{q/11}$-coupled receptor-mediated pathways, e.g., M1 and M3 muscarinic receptors through PKC and PIP_2 (Leaney et al., 2001; Brown et al., 2005). We have previously shown direct phosphorylation of the Kir3.1, but not Kir3.2, C-terminal domain by PKC (Brown et al., 2005). We now examined Kir3.4 and observed that the domains were phosphorylated but at a lower level compared to Kir3.1 (Fig. 6A; Kir3.1 and Kir3.2 are shown for comparison from Brown et al., 2005). In keeping with the higher level of phosphorylation observed with Kir3.1, there are a larger number of predicted serine/threonine phosphorylation sites present than for Kir3.2 or Kir3.4. Determination of the potential phosphorylation sites using Netphos 2.0 (Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark) (Blom, Gammeltoft & Brunak, 1999) demonstrated that in the C-terminal cytoplasmic domains alone Kir3.1 has 24 possible serine/threonine phosphorylation sites compared to 16 and 10 for Kir3.2 and Kir 3.4 respectively. At present, further studies need to be carried out in order to determine the exact residues of Kir3.1 and Kir3.4 phosphorylated by PKC. It has been suggested that Kir3.1/3.4 inhibition is solely dependent on the hydrolysis of PIP_2 from the membrane (Meyer et al., 2001) as is Kir6.2 (Xie, Horie & Takano, 1999; Quinn et al., 2003). Additionally, inhibition of Kir1.1 has been linked to a reduction in the membrane PIP_2 content as a result of PKC activation (Zeng et al., 2003). However, Kir6.1 has been shown to be inhibited through a PKC-dependent mechanism (Quinn et al., 2003), and Zhang et al. (1999) suggest that a direct interaction of Kir3.0 members with PIP_2 is modulated by PKC. Does phosphorylation affect the affinity of these domains for anionic phospholipids? Here, we have demonstrated that the binding of anionic phospholipids by the Kir3.1 C-terminal cytoplasmic domain is potentially decreased in the presence of phosphorylated protein, consistent with this hypothesis.

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