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Phospholipids as Modulators of K_{ATP} Channels: Distinct Mechanisms for Control of Sensitivity to Sulphonylureas, K^+ Channel Openers, and ATP

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ABSTRACT

Recent work has established membrane phospholipids such as phosphatidylinositol-4,5-bisphosphate (PIP_2) as potent regulators of K_{ATP} channels controlling open probability and ATP sensitivity. We here investigated the effects of phospholipids on the pharmacological properties of cardiac type K_{ATP} (Kir6.2/SUR2A) channels. In excised membrane patches K_{ATP} channels showed considerable variability in sensitivity to glibenclamide and ATP. Application of the phosphatidylinositol phosphates (PIPs) phosphatidylinositiol-4-phosphate, PIP_2, and phosphatidylinositol-3,4,5-trisphosphate reduced sensitivity to ATP and glibenclamide closely resembling the native variability. Insertion of the patch back into the oocyte (patch-cramming) restored high ATP and glibenclamide sensitivity, indicating reversible modulation of K_{ATP} channels via endogenous PIPs-degrading enzymes. Thus, the observed variability seemed to result from differences in the membrane phospholipid content. PIP_2 also diminished activation of K_{ATP} channels by the K^+ channel openers (KCOs) cromakalim and P1075. The properties mediated by the sulphonylurea receptor (sensitivity to sulfonylureas and KCOs) seemed to be modulated by PIPs via a different mechanism than ATP inhibition mediated by the Kir6.2 subunits. First, polycations abolished the effect of PIP_2 on ATP inhibition consistent with an electrostatic mechanism but only weakly affected glibenclamide inhibition and activation by KCOs. Second, PIP_2 had clearly distinct effects on the concentration-response curves for ATP and glibenclamide. However, PIPs seemed to mediate the different effects via the Kir6.2 subunits because a mutation in Kir6.2 (R176A) attenuated simultaneously the effects of PIP_2 on ATP and glibenclamide inhibition. Finally, experiments with various lipids revealed structural features necessary to modulate K_{ATP} channel properties and an artificial lipid (dioleoylglycerol-succinyl-nitriloacetic acid) that mimicked the effects of PIPs on K_{ATP} channels.

K_{ATP} channels are heteromultimers formed by association of an inwardly rectifying K^+ channel, Kir6.2/Kir6.1, and an ATP binding cassette protein, the sulfonylurea receptor SUR1/SUR2 (Inagaki et al., 1995; Clement et al., 1997; Babenko et al., 1998). K_{ATP} channels show complex regulation by intracellular factors such as ATP and ADP and pharmacological compounds such as sulfonylureas and K^+ channel openers. These agents interact with the channel at different subunits and alter channel activity by different mechanisms (Ashcroft, 1988; Babenko et al., 1998; Seino, 1999; Baukrowitz and Fakler, 2000b).

ATP and ADP have opposing effects on K_{ATP} channel activity. ATP interacts with the Kir6.2 subunit and inhibits channel activity. The exact mechanism for ATP inhibition and the location of the ATP binding site are presently unknown. However, several studies indicate that ATP may reduce the open probability of K_{ATP} channels by stabilizing a closed channel state (Alekseev et al., 1998; Trapp et al., 1998; Enkvetchakul et al., 2000). Mutagenesis work has uncovered three regions in the cytoplasmic N and C termini of Kir6.2 that are possibly involved in the binding of ATP (Tucker et al., 1997; Drain et al., 1998; Trapp et al., 1998). MgADP antagonizes ATP inhibition and thereby effectively activates K_{ATP} channels under physiological concentrations of ATP. The action of MgADP is mediated by the nucleotide binding domains of the SUR subunit (Nichols et al., 1996; Gribble et

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ABBREVIATIONS: K_{ATP}, ATP-sensitive K^+ channel; SUR, sulphonylurea receptor; Kir, inward-rectifier potassium channel; KCO, K^+ channel opener; PIPs, phosphatidylinositol phosphates; PIP_2, phosphatidylinositol-4,5-bisphosphate; $PI(4,5)P_2$, L-α-phosphatidyl-D-*myo*-inositol-4,5-bisphosphate; PIP, phosphatidylinositiol-4-phosphate; PI(4)P, L-α-phosphatidyl-D-*myo*-inositol-4-phosphate; PI, phosphatidylinositol; $PI(3,4,5)P_3$, L-α-phosphatidylinositol-3,4,5-triphosphate, dipalmitoyl-, heptaammonium salt; $PI(3,4)P_2$, L-α-phosphatidylinositol-3,4-bisphosphate, dipalmitoyl-, pentaammonium salt; PC, phospatidylcholine (1,2-dihexadecanoyl-rac-glycero-3-phosphocholine); DOG, dioleoylglycerol (1,2-di[*cis*-9-octadecenoyl]-*sn*-glycerol); DOGS-NTA, dioleoylglycerol-succinyl-nitriloacetic acid (1,2-dioleoyl-*sn*-glycero-3-succinyl[*N*-(5-amino-1-carboxypentyl)iminodiacetic acid], ammonium salt); CR, concentration-response; wt, wild-type.

al., 1997; Shyng et al., 1997). The opposing effects of ATP and ADP on channel activity make the K_{ATP} channel a metabolic sensor that couples membrane excitability to the cellular metabolic state reflected by the ATP/ADP ratio. For this reason K_{ATP} channels are involved in many physiological functions such as insulin secretion (Ashcroft, 1988) and protection of cardiac myocytes during periods of metabolic impairment (e.g., ischemia) (Nichols and Lederer, 1991). Sulfonylureas (e.g., glibenclamide and tolbutamide) inhibit K_{ATP} channels and are used in treatment of diabetes because they promote secretion of insulin in pancreatic β-cells (Ashcroft, 1988; Edwards and Weston, 1993). These drugs bind to the SUR (Ashfield et al., 1999) and reduce channel open probability by a poorly understood mechanism. K^+ channel openers (KCOs) are a chemical divers group of drugs that bind to the SUR subunits (Uhde et al., 1999) and activate K_{ATP} channels. The KCOs cromakalim and P1075 act on cardiac K_{ATP} channels, whereas diazoxide is specific for pancreatic K_{ATP} channels (Edwards and Weston, 1993). The activating effect of these drugs results from their ability to antagonize inhibition of intracellular ATP by a mechanism that involves ATP binding, and, probably, hydrolysis at the nucleotide binding domains of the SUR (Gribble et al., 1997; Shyng et al., 1997; Schwanstecher et al., 1998).

Recent work has uncovered a new class of regulatory molecules for K_{ATP} channel gating (Hilgemann, 1997; Baukrowitz and Fakler, 2000b). Membrane phosphatidylinositol phosphates (PIPs) such as PIP_2 and PIP were found to interact with K_{ATP} channels, resulting in increased open probability (Furukawa et al., 1996; Hilgemann and Ball, 1996; Fan and Makielski, 1997) and profoundly reduced ATP sensitivity (Baukrowitz et al., 1998; Shyng and Nichols, 1998; Fan and Makielski, 1999). The effect of PIPs on ATP inhibition seems to involve electrostatic interactions because the negatively charged phosphate groups at the inositol ring seem to be critical for the phospholipid effect on ATP inhibition. Highly negatively charged phosphatidylinositol-3,4,5-trisphosphate and PIP_2 are very effective in reducing ATP inhibition, whereas PIP is less potent and PI has no effect (Baukrowitz et al., 1998; Shyng and Nichols, 1998). Moreover, polycations such as polylysine and neomycin, which are known to bind to phospholipids and neutralize their negative charges, abolish the effect of PIPs on ATP inhibition (Deutsch et al., 1994; Shyng and Nichols, 1998). Furthermore, PIP_2 diminishes the potency of the KCO diazoxide to antagonize ATP inhibition (Baukrowitz et al., 1998; Koster et al., 1999) and reduces inhibition of K_{ATP} channels by tolbutamide (Koster et al., 1999).

Most studies on the modulation of K_{ATP} by PIPs have focused on open probability and ATP sensitivity, whereas little is known about the effects of PIPs on the channel's pharmacological properties. To gain further insight into the interference of PIPs with the pharmacological properties of K_{ATP} channels we address in this study the following questions: Do PIPs contribute to the variability reported for K_{ATP} channels in respect to inhibition by sulfonylureas? What are the structural features critical for PIPs to modulate the pharmacological properties of K_{ATP} channels? Do PIPs modulate the pharmacological properties of K_{ATP} channels and ATP-sensitivity by the same mechanisms?

Materials and Methods

Mutagenesis and Expression of K_{ATP} Channels. Murine $K_{ir}6.2$ and rat SUR2A (Inagaki et al., 1995; Inagaki et al., 1996) were used in this study. Site-directed mutagenesis of Kir6.2 (K185Q, R176A) was carried out as described previously (Baukrowitz et al., 1999). For oocytes expression, constructs were subcloned into the pBF expression vector, which provides the 5′- and 3′-untranslated regions of the *Xenopus laevis* globin gene (Baukrowitz et al., 1999). Capped cRNAs specific for $K_{ir}6.2$ and SUR2A were synthesized in vitro using SP6 polymerase (Promega, Heidelberg, Germany) and stored in stock solutions at −70°C.

X. laevis oocytes were surgically removed from adult females and manually dissected. About 50 nl of a solution containing cRNA specific for SUR2A and $K_{ir}6.2$ subunits was injected into Dumont stage VI oocytes. Oocytes were treated with collagenase type II (0.5 mg/ml; Sigma, St. Louis, MO) for 15 min and incubated at 19°C for 1 to 3 days before use.

Electrophysiology. Giant patch recordings (Baukrowitz et al., 1999) in inside-out configuration under voltage-clamp conditions were made at room temperature (approximately 23°C) 3 to 7 days after injection. Polylysine [poly(L-lysine), hydrobromide, M_r 30,000–70,000], neomycin, heparin, ATP, glibenclamide, and cromakalim were purchased from Sigma and P1075 was kindly provided by Dr. U. Quast (Department of Pharmacology, University of Tübingen, Tübingen, Germany). Pipettes used were made from thick-walled borosilicate glass, had resistances of 0.2 to 0.4 MΩ (tip diameter of 20–30 μm) and were filled with 120 mM KCl, 10 mM HEPES, and 1.8 mM $CaCl_2$ (pH adjusted to 7.3 with KOH). Currents were recorded with an EPC9 amplifier (HEKA Electronics, Lamprecht, Germany) and sampled at 1 kHz with analog filter set to 3 kHz (−3 dB). Solutions were applied to the cytoplasmic side of excised patches via a multibarrel pipette and had the following composition (K_{int}): 100 mM KCl, 10 mM HEPES, 2 mM K_2EGTA (total K^+ concentration was 120 mM, pH adjusted to 7.3 with KOH). Solutions with P1075, cromakalim, and glibenclamide had 1.4 mM $MgCl_2$. Computational work was done on Macintosh PowerPC 7600/132 Mhz using commercial software (IGOR; WaveMetrics, Lake Oswego, OR) and Excel 98 for the Macintosh (Microsoft, Redmond, WA).

Preparation of Lipid Solutions. L-α-Phosphatidyl-D-*myo*-inositol-4,5-bisphosphate [PI(4,5)P_2 from bovine brain] and L-α-phosphatidyl-D-*myo*-inositol-4-phosphate [PI(4)P, from bovine brain] was purchased from Roche Diagnostics GmbH (Mannheim, Germany); 1,2-dihexadecanoyl-rac-glycero-3-phosphocholine, synthetic (PC), L-α-phosphatidylinositol (PI, from bovine liver), and 1,2-di[*cis*-9-octadecenoyl]-*sn*-glycerol (DOG) from Sigma; L-α-phosphatidylinositol-3,4,5-triphosphate, dipalmitoyl-, heptaammonium salt [PI(3,4,5)P_3] and L-α-phosphatidylinositol-3,4-bisphosphate, dipalmitoyl-, pentaammonium salt [PI(3,4)P_2] from Calbiochem-Novabiochem GmbH (Bad Soden, Germany); and DOGS-NTA (1,2-dioleoyl-sn-glycero-3-succinyl[*N*(5-amino-1-carboxypentyl)iminodiacetic acid], ammonium salt) from Avanti Polar Lipids (Alabaster, AL). All lipids were stored as stocks at −20°C; PI(4)P, PI(4,5)P_2, PI(3,4)P_2, PI(3,4,5)P_3, DOG (1 mM), and DOGS-NTA (0.5 mM) in K_{int}, PI at concentration of 1 mM in dimethyl sulfoxide, and PC at concentration of 1 mM in ethanol (99%); lipid stocks were sonicated for 15 min before storage. For experiments lipids were diluted in K_{int} solution to final concentrations and sonicated for 30 min and used within 6 h.

Results

Variability for Inhibition by ATP and Glibenclamide Is Linked to Membrane Phospholipids. For cardiac K_{ATP} channels large variability in sensitivity to ATP and sulfonylureas has been reported (Findlay and Faivre, 1991). We here investigated this variability for heterologously expressed cardiac-type (Kir6.2/SUR2A) K_{ATP} channels in inside-out

patches from *X. laevis* oocytes. As shown in Fig. 1A, in some patches K_{ATP} channels showed rather weak sensitivity to inhibition by ATP and glibenclamide, whereas in others high sensitivity was observed. Current inhibition by 100 μM ATP and 10 μM glibenclamide ranged from 98 to 40% measured in 54 patches excised from five different batches of oocytes. For each patch ATP inhibition closely matched glibenclamide inhibition. This observation was quantified in Fig. 1B, where relative glibenclamide inhibition is plotted against ATP inhibition. The linear dependence indicates a direct link between ATP and glibenclamide sensitivity for each patch. The variability observed for ATP inhibition has been related to differences in membrane concentrations of phosphatidylinositol phosphates such as PIP_2 and PIP (Baukrowitz et al., 1998; Shyng and Nichols, 1998). To test for an effect on glibenclamide inhibition, PIP_2 was applied to the intracellular side of patches and inhibition by glibenclamide and ATP was monitored. As shown in Fig. 1C glibenclamide inhibition was reduced by successive application of 10 μM PIP_2 and this reduction occurred in parallel to the reduction of ATP inhibition (Fig. 1D). Thus, the close correlation between inhibition by ATP and glibenclamide, which characterizes the endogenous variability, was preserved for the effect of exogenously applied PIP_2. Furthermore, we tested whether the effects of PIP_2 on K_{ATP} channels could be reversed when the patch was brought back into contact with the cytoplasm of the oocyte. For this purpose an inside-out patch was first exposed to PIP_2, resulting in large reduction of ATP and glibenclamide sensitivity, and then crammed back into the oocyte and subsequently excised again to assay sensitivity to glibenclamide and ATP (Fig. 1E). Every round of cramming successively restored sensitivity to glibenclamide and ATP (Fig. 1, E and F). We presume that contact of the patch with the cytoplasm enables either membrane-bound or cytoplasmic enzymes (e.g., lipases or PIPs-phosphatases) to break down PIP_2 and thereby reduce the PIP_2 content of the membrane toward the original level before PIP_2 application.

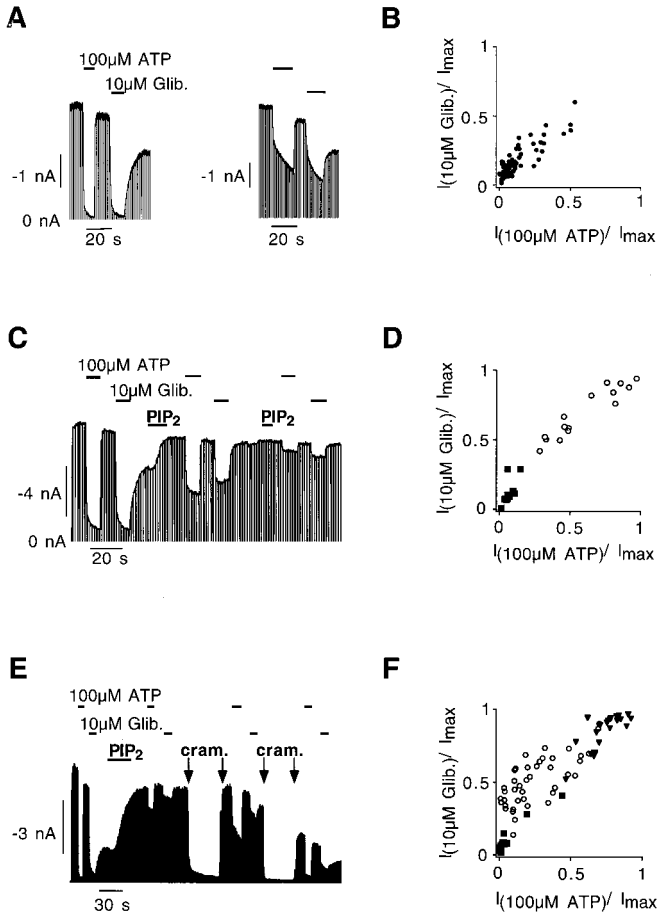

Fig. 1. Variability of sensitivity of K_{ATP} channels to ATP and glibenclamide is linked to PIPs. A, variable inhibition of currents mediated by Kir6.2/SUR2A channels in response to application of ATP and glibenclamide shown for two inside-out patches. Currents were recorded at membrane potentials of −80 mV stepped to 60 mV for 200 ms every second; only currents at −80 mV are shown as upward deflection. Patches were exposed to ATP and glibenclamide (Glib.) as indicated. B, relative (rel.) current at 10 μM glibenclamide ($I_{(10\ \mu M\ Glib.)}/I_{max}$) plotted against the rel. current at 100 μM ATP ($I_{(100\ \mu MATP)}/I_{max}$) from experiments as in A, plot represents summary of n = 54 experiments. C, change in inhibition by ATP and glibenclamide upon application of 10 μM PIP_2 as indicated. D, rel. current with glibenclamide plotted against the rel. current with ATP from experiments as in C; ■ (rel. currents before PIP_2) and ○ (rel. currents after PIP_2), plot represents summary of $n = 10$ experiments. E, reversal of the PIP_2 effect on ATP and glibenclamide inhibition upon insertion of the patch into the oocyte (cramming), application of 10 μM PIP_2 and duration of cramming as indicated (cramming caused reversible current inhibition due to high concentrations of ATP in the oocyte). F, rel. current with glibenclamide plotted against rel. current with ATP from experiments as in E; ■ (current before PIP_2), ▼ (current after PIP_2), and ○ (current after cramming), plot represents summary of $n = 22$ experiments.

Structural Requirements of Lipids for Inference with Glibenclamide and ATP Inhibition. To inquire for the structural determinants of the lipids to modulate glibenclamide inhibition and ATP inhibition, lipids of different charge and structure were tested. Application of 50 μM phosphatidylcholine (PC), 50 μM phosphatidylinositol (PI), and 50 μM dioleoylglycerol (DOG) for 45 s had no effect on ATP or glibenclamide inhibition, whereas application of 10 μM PI(4)P for 45 s virtually abolished inhibition by 100 μM ATP and 10 μM glibenclamide inhibition (Fig. 2A). The negatively charged PIPs PI(4)P, $PI(4,5)P_2$, $PI(3,4)P_2$, and $PI(3,4,5)P_3$ were tested in an assay as described in Fig. 1, C and D, and found to reduce glibenclamide inhibition with similar potency than ATP inhibition (Fig. 2C). Furthermore, the synthetic anionic lipid dioleoylglycerol-succinyl-nitriloacetic acid (DOGS-NTA) was tested, which lacks the inositol head group of PIPs but carries a negatively charged NTA head group instead (structure shown in Fig. 2B). DOGS-NTA reduced sensitivity of K_{ATP} channels to inhibition by 100 μM ATP and 10 μM glibenclamide in parallel similar to PIPs (Fig. 2, C and D). In summary, neutral or weakly negatively charged lipids (PC, DOG, and PI) had no effect on neither ATP nor glibenclamide inhibition, whereas the highly negatively charged PIPs and DOGS-NTA reduced both properties with similar potency.

PIP_2 Modulates Sensitivity to Glibenclamide and ATP by Distinctive Mechanisms. To learn about the mechanism underlying the modulation of glibenclamide inhibition by PIP_2, glibenclamide concentration-response (CR) curves were obtained before and after application of PIP_2. Before PIP_2 application glibenclamide inhibited about 80% of the K_{ATP} current with an inhibitory constant (K_i) of 15 nM and a Hill coefficient of 1, whereas 20% of the K_{ATP} channels were insensitive to glibenclamide ($n = 5$). Application of PIP_2 successively increased the fraction of glibenclamide insensitive channels, but had little effect on the K_i value and the Hill coefficient of the glibenclamide-sensitive fraction (Fig. 3A;

$n = 7$). The effect of PIP_2 on the glibenclamide CR curve was strikingly different from the effect on the ATP CR curve from Baukrowitz et al. (1998), which is shown in Fig. 3B for comparison. PIP_2 reduced ATP inhibition by gradually shifting the ATP CR curve toward higher concentrations without a change in steepness or indication for multiple components.

Polycations such as polylysine and neomycin have been shown to abolish the effect of PIP_2 on current amplitude and ATP inhibition (Deutsch et al., 1994; Fan and Makielski, 1997; Shyng and Nichols, 1998). We therefore tested whether polycations also reverse the effect of PIP_2 on glibenclamide inhibition. As shown before, application of PIP_2 increased K_{ATP} current amplitude and largely reduced inhibition by ATP and glibenclamide (Fig. 3C). Subsequent application of polylysine (100 μg/ml) induced partial channel run down and completely reversed the effect of PIP_2 on ATP inhibition resulting in channels that are again highly sensitive to inhibition by ATP. In contrast, polylysine treatment had only minor effects on glibenclamide inhibition. As shown in Fig. 3D, the relative current measured in the presence of 10 μM glibenclamide changed from 0.83 ± 0.03 to 0.62 ± 0.05 (n = 9), whereas the relative current observed with 100 μM ATP changed from 0.75 ± 0.05 to 0.04 ± 0.02 (n = 9) upon application of polylysine. To remove polylysine again from the patch, heparin was used, which is a polyanion and known to bind polylysine. Consistently, application of a solution containing 100 μM/ml heparin reversed the effect of polylysine on ATP inhibition and current amplitude resulting in K_{ATP} channels with again low ATP sensitivity. Very similar results were obtained with the polycation neomycin (Fig. 3E). For these experiments ATP was applied in the presence of 500 μM neomycin because the effect of neomycin was readily reversible upon washout. Furthermore, polylysine also abolished the effect of DOGS-NTA on ATP inhibition but not on glibenclamide inhibition (Fig. 3F). In summary, the effect of different negatively charged lipids (PIP_2 and DOGS-NTA) on ATP inhibition is characterized by a high sensitivity to polycations (polylysine and neomycin), whereas the effect on glibenclamide inhibition is almost insensitive in respect to treatment with polycations.

R176A Attenuates the Effect of PIP_2 on Glibenclamide and ATP Inhibition. Recently a conserved C-terminal arginine (R181 in Kir1.1 and R176 in Kir6.2) has been identified that contributes to PIP_2 binding (Fan and Makielski, 1997; Huang et al., 1998). Mutating arginine 176 to alanine (R176A) in Kir6.2 reduced the ability of PIP_2 to affect ATP inhibition (Baukrowitz et al., 1998; Shyng and Nichols, 1998). Therefore, we tested whether R176A also reduced the potency of PIP_2 to attenuate glibenclamide inhibition. Figure 4 shows the time course for attenuation of inhibition produced by 100 μM ATP (Fig. 4A) and 10 μM glibenclamide (Fig. 4B) upon application of 10 μM PIP_2 for wild-type (wt) channels (n = 5) and R176A mutant channels (n = 14). The experiments for wt and mutant channels were performed on the same day with the same batch of oocytes and the same solutions to minimize differences in the experimental conditions. We found that inhibition by ATP and glibenclamide is reduced more slowly by PIP_2 for R176A channels compared with wt channels. The differences are not large but are significant ($p < 0.002$, unpaired t test) for every single time point. Thus, the mutation R176A attenuated the effect of PIP_2 on ATP and glibenclamide inhibition consistent with R176 contributing to a PIP_2 binding site that modulates both properties.

Negatively Charged Lipids Render K_{ATP} Channels Insensitive to KCOs by a Polycation-Insensitive Mechanism. For the pancreatic type of K_{ATP} channels (Kir6.2/SUR1) we and others have recently shown an attenuating effect of PIP_2 on the potency of the KCO diazoxide (Baukrowitz et al., 1998; Koster et al., 1999). The interference of PIP_2 with cardiac-type K_{ATP} channels (Kir6.2/SUR2A) was investigated using the KCOs cromakalim and P1075. As shown in Fig. 5A, application of 10 μM P1075 activated 77% of the current inhibited by 100 μM ATP (n = 7). To test for the effect of PIP_2, patches were treated with PIP_2 until ATP sensitivity was reduced about 10-fold so that 1 mM ATP was

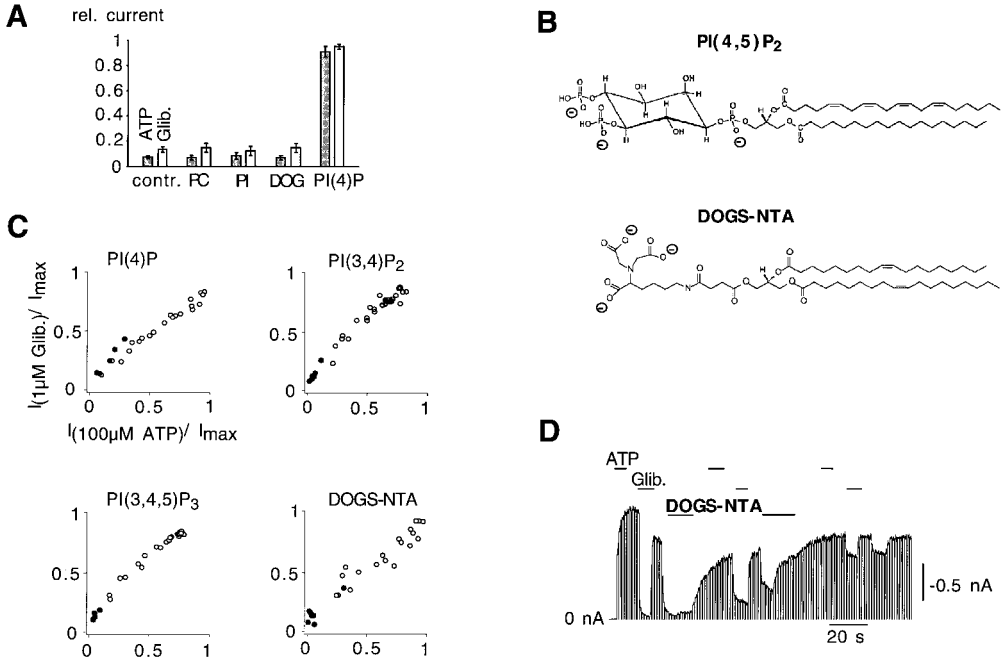

Fig. 2. Lipid specificity for modulation of ATP and glibenclamide inhibition. A, effect of 50 μM PC (n = 3), 50 μM PI (n = 6), 50 μM DOG (n = 3), and 10 μM PI(4)P (n = 5) on ATP and glibenclamide sensitivity. Lipids were applied for 45 s and subsequently inhibition by 100 μM ATP and 10 μM glibenclamide was plotted as columns; mean ± S.E.M.; control (contr.) represents inhibition before lipid application (n = 16). B, structure of $PI(4,5)P_2$ and DOGS-NTA. C, change in inhibition by ATP and glibenclamide upon application of PI(4)P (10 μM), $PI(3,4)P_2$ (10 μM), $PI(3,4,5,)P_3$ (10 μM), and DOGS-NTA (10 μM) obtained from experiments as in D plotted as rel. currents as described in Fig. 1D. D, currents from a patch exposed to 100 μM ATP, 10 μM glibenclamide, 10 μM DOGS-NTA, and 100 μg/ml polylysine (poly-K) as indicated.

necessary to produce inhibition comparable with that of 100 μM ATP before PIP_2 treatment. Application of 10 μM P1075 to those patches failed to reverse ATP inhibition (Fig. 5B; n = 8). Very similar results were obtained for the KCO cromakalim (before PIP_2 activation was 85 ± 1%, subsequent to PIP_2 activation was 12 ± 5%, n = 9, data not shown). In control experiments (no PIP_2) P1075 only weakly activated K_{ATP} channels in the presence of 1 mM ATP (Fig. 5C). This outcome is not surprising because KCOs are thought to antagonize ATP inhibition by an apparent competitive mechanism. Thus, the apparent ability of KCOs to overcome ATP inhibition ceases with increasing inhibition by ATP (Thuringer and Escande, 1989). Consistently, when ATP sensitivity was reduced (about 10-fold, comparable with that of PIP_2-treated patches in Fig. 5B) via a mutation in Kir6.2 (K185Q, K_i = 137 ± 20, n = 8; Tucker et al., 1997) 10 μM P1075 potently activated K_{ATP} channels inhibited by 1 mM ATP (Fig. 5D; n = 6). Thus, the experimental conditions in Fig. 5B are appropriate to assess the impact of PIP_2 on the activation of K_{ATP} channels by KCOs.

In a next set of experiments the effect of PIP_2 was tested for its sensitivity to polylysine. Therefore patches were treated with PIP_2 as before and subsequently exposed to 100 μg/ml polylysine, which reversed the effect of PIP_2 on ATP inhibition. K_{ATP} channels in these patches showed again high ATP sensitivity similar to that before PIP_2; however, P1075 still failed to activate the channels (Fig. 5E; n = 15). Very similar results were obtained when ATP sensitivity of K_{ATP} channels was reduced by application of DOGS-NTA instead of PIP_2 (Fig. 5E). We conclude that negatively charged lipids impair the ability of K_{ATP} channels to respond to KCOs by a polycation-insensitive mechanism.

The lipids PC, PI, and DOG were tested to inquire further on the lipids' structural prerequisites to modulate the ability of K_{ATP} to respond to KCOs. We found that application 50 μM PC, 50 μM PI, and 100 μM DOG for 45 s had no effect on the potency of 10 μM P1075 to activate K_{ATP} channels inhibited by 100 μM ATP (Fig. 5F). Thus, neutral and weakly negatively charged lipids (PC, PI, and DOG) are ineffective, whereas the highly negatively charged lipids PIP_2 and DOGS-NTA abolish the sensitivity of K_{ATP} channels to KCOs.

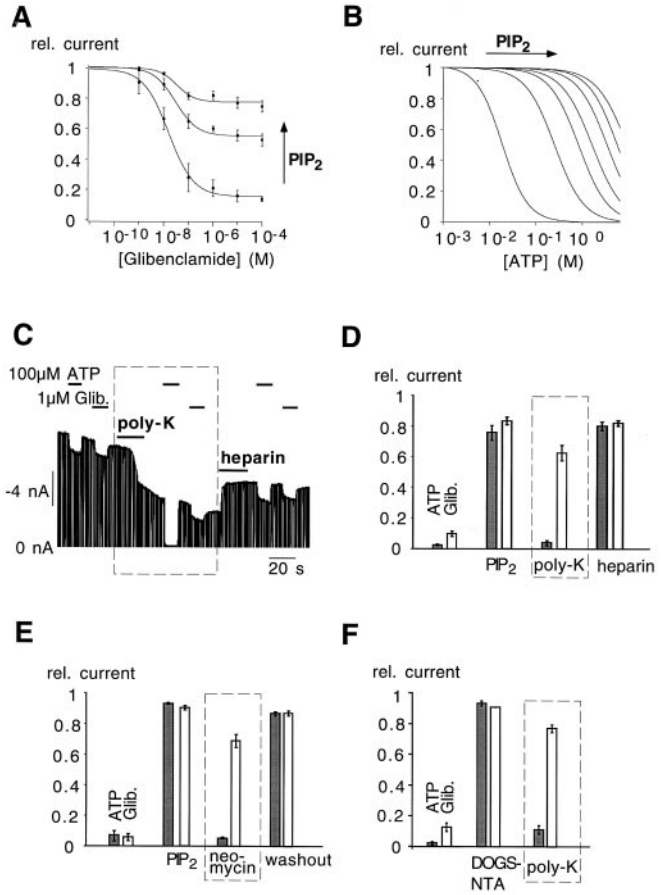

Fig. 3. Distinctive mechanisms for the effects of PIP_2 on glibenclamide and ATP sensitivity. A, CR curves for glibenclamide inhibition of K_{ATP} channels before and subsequent to application of PIP_2. To obtain mean and S.D. of each data point CR curves were pooled by the respective ATP sensitivity measured shortly before the CR curves were taken. CR curve without PIP_2 corresponds to patches with K_{ATP} channels inhibited to 80 to 90% by 100 μM ATP; CR curves succeeding application of PIP_2 correspond to K_{ATP} channels inhibited to 50 to 60% (short PIP_2 application) and 25 to 35% (prolonged PIP_2 application). Continuous lines represent fit to the Hill equation $I / I_{max} = 1 / [1 + ([ATP] / K_{i(Glib.)})^{nH}]$, where I_{max} is the current amplitude without glibenclamide, $K_{i(Glib.)}$ is the concentration for half-maximal inhibition, and n_H is the Hill coefficient. $K_{i(Glib.)}$ and n_H were 16 nM/0.9 (before PIP_2), 29 nM/1 (after PIP_2) and 33 nM/1.2 (after prolonged PIP_2). B, change of the CR curve for ATP inhibition induced by PIP_2, reproduced from Baukrowitz et al. (1998). C, polylysine abolishes the effect of PIP_2 on ATP but not on glibenclamide inhibition. The patch was preincubated with PIP_2 (data not shown) and exposed to ATP, glibenclamide, 100 μg/ml polylysine (poly-K) and 100 μg/ml heparin as indicated. D, relative (rel.) currents with 100 μM ATP and 10 μM glibenclamide from experiments as in C before and subsequent to PIP_2, polylysine and heparin treatment plotted as columns, mean ± S.E.M. of n = 6 experiments. E, effect of neomycin on ATP and glibenclamide inhibition subsequent to PIP_2 treatment; rel. currents with 100 μM ATP and 10 μM glibenclamide before and subsequent to PIP_2 and in the presence of 500 μM neomycin plotted as columns, mean ± S.E.M. of n = 6 experiments. F, rel. currents with 100 μM ATP and 10 μM glibenclamide before and subsequent to application of 10 μM DOGS-NTA and subsequent to treatment with 100 μg/ml poly-K plotted as columns, mean ± S.E.M. of n = 4 experiments.

Discussion

PIPs Modulate Sensitivity to ATP, Sulfonylureas, and KCOs by Distinctive Mechanisms. We report here that PIPs profoundly alter the sensitivity of cardiac type K_{ATP} channels to ATP, glibenclamide, and the KCOs P1075 and cromakalim. Although PIPs simultaneously desensitize K_{ATP} channels to ATP, sulfonylureas, and KCOs several findings suggest different modulatory mechanisms. First, the impact of PIP_2 on the CR curves for inhibition by ATP and

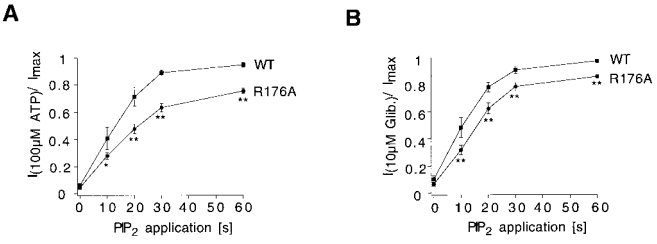

Fig. 4. R176A attenuates the effects of PIP_2 on glibenclamide and ATP inhibition. A, time course for attenuation of inhibition by 100 μM ATP for wt channels (n = 6) and R176A mutant channels (n = 17) upon application of 10 μM $PI(4,5)P_2$. B, time course for attenuation of inhibition by 10 μM glibenclamide for wt channels (n = 5) and R176A mutant channels (n = 14) upon application of 10 μM $PI(4,5)P_2$. Asterisks indicate a significant difference between wt and R176A mutant channels (*$p < 0.05$, **$p < 0.002$, unpaired t test). Prolonged application of PIP_2 (>2 min) completely abolished inhibition by glibenclamide and ATP for wt and mutant channels (data not shown).

glibenclamide are very distinct. The effect of PIP_2 on the ATP CR curve is characterized by a shift toward higher concentrations without a change in steepness or indication for multiple components. In contrast, PIP_2 abolishes glibenclamide inhibition by increasing the fraction of glibenclamide-insensitive channels without altering the CR curve of the glibenclamide-sensitive fraction. Thus, PIP_2 seems to alter glibenclamide sensitivity in an all-or-nothing manner, whereas ATP sensitivity is shifted gradually. This gradual shift suggests the existence of multiple PIP_2 binding sites, which contribute, to the overall shift in ATP sensitivity in an additive manner. The "all-or-nothing" effect of PIP_2 on glibenclamide inhibition suggests that the channel exists in two states with respect to glibenclamide sensitivity and binding of PIP_2 promotes the channel from a sensitive into an insensitive state.

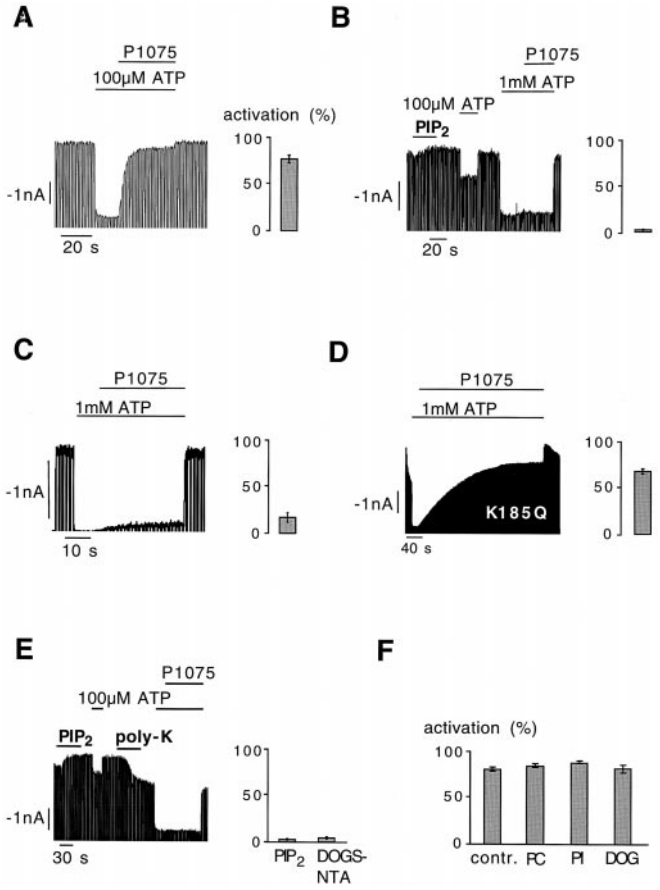

Fig. 5. Negatively charged lipids abolish sensitivity to KCOs by a polycation-insensitive mechanism. A, activation of K_{ATP} channels inhibited by ATP upon application of 10 μM P1075 as indicated. Activation was quantified as percentage of the current inhibited by 100 μM ATP that was reopened by P1075, activation (%) = $[(I_{(100\ \mu M\ ATP\ +\ 10\ \mu M\ P1075)} - I_{(100\ \mu M\ ATP)})/I_{max}]/(1 - I_{(100\ \mu M\ ATP)}/I_{max}] * 100$ and plotted as columns, mean ± S.E.M. (n = 7). B, K_{ATP} current from a patch exposed to 10 μM PIP_2, ATP, and 10 μM P1075 as indicated. Columns represent percentage of activation of the current inhibited by 1 mM ATP that was reopened by P1075, mean ± S.E.M. (n = 7). C, K_{ATP} current from a patch exposed to 1 mM ATP and 10 μM P1075 (no PIP_2). Columns represent percentage of activation of the current inhibited by 1 mM ATP that was reopened by P1075, mean ± S.E.M. (n = 9). D, activation of K185Q mutant channels by 10 μM P1075. Columns represent percentage of activation of the current inhibited by 1 mM ATP that was reopened by P1075, mean ± S.E.M. (n = 5). E, polylysine (poly-K) restored high ATP sensitivity but not activation by P1075. K_{ATP} current from a patch exposed to 10 μM PIP_2, 100 μg/ml polylysine, ATP, and 10 μM P1075 as indicated. Columns represent mean ± S.E.M. percentage of activation of the current inhibited by 1 mM ATP that was reopened by P1075 subsequent to treatment with PIP_2 and poly-K (n = 9). A similar outcome was obtained for 10 μM DOGS-NTA and subsequent poly-K application (n = 5). F, patches were exposed to 50 μM PC (n = 9), 50 μM PI (n = 7) and 100 μM DOG (n = 4) for 45 s and subsequently activation by P1075 was determined. Columns represent percentage of activation of the current inhibited by 100 μM ATP that was reopened by 10 μM P1075.

Furthermore, the effects of different negatively charged lipids (PIP_2 and DOGS-NTA) on K_{ATP} channels have in common that their impact on ATP inhibition is readily reversed by polycations (polylysine and neomycin), whereas their impact on glibenclamide inhibition and activation by KCOs is not. Thus, it is rather unlikely that PIPs affect only a single parameter in K_{ATP} channels (e.g., open probability) that subsequently causes the various effects on sensitivity to ATP, glibenclamide, and KCOs. If this were the case, polycations would reverse all properties affected by PIPs simultaneously and not ATP sensitivity preferentially. Taken together, the results presented here indicate that PIPs modulate ATP inhibition by a mechanism that is (at least in part) different from the mechanism underlying the modulation of glibenclamide inhibition and activation by KCOs.

Possible Mechanisms for the Action on PIP_2 on K_{ATP} Channel Properties. Two mechanisms have been proposed to account for the regulation of ATP sensitivity by PIPs: a competitive mechanism where PIP_2 binds in proximity to the ATP binding site on Kir6.2, reducing binding of ATP by an electrostatic mechanism (Deutsch et al., 1994; Fan and Makielski, 1999) and an allosteric mechanism where PIP_2 increases the open probability of K_{ATP} channels and thereby decreases the frequency of a closed state to which ATP binds to inhibit channel activity (Enkvetchakul et al., 2000). Furthermore, a recent article provides evidence that both mechanisms coexist and reduction in ATP sensitivity is brought about by a direct effect on ATP binding and an indirect effect involving modulation of the open probability (Ribalet et al., 2000). The effect of PIP_2 on glibenclamide inhibition and activation by KCOs is presumably allosteric because the PIP_2 interaction sites are likely on the Kir6.2 subunit, whereas sulfonylureas and KCOs bind to the SUR. In line with this concept Koster et al. (1999) recently reported correlation between the open probability of K_{ATP} channels and their sensitivity to inhibition by tolbutamide and suggested allosteric modulation of tolbutamide inhibition by PIP_2. How to explain in this context the differential sensitivity to polycations for the effect of PIP_2 on ATP, glibenclamide, and KCO sensitivity? Polycations might by neutralizing the negative charge of PIP_2 reduce electrostatic interactions necessary for a direct effect on ATP binding that, however, are less important for the allosteric effect on glibenclamide inhibition and activation by KCOs.

Structural Requirements of Lipids to Modulate K_{ATP} Channels. To elucidate the structural requirements for modulation of K_{ATP} channels lipids with different structure and charge were tested for their potency to affect ATP inhibition, glibenclamide inhibition, and activation by KCOs. The zwitterionic PC, the neutral DOG, and the weakly negatively charged PI had no effect on the sensitivity of K_{ATP} channels for ATP, glibenclamide, or P1075. In contrast, the highly negatively charged phosphatidylinositol phosphates [PI(4)P, PI(4,5)P2, PI(3,4)P2, and $PI(3,4,5)P_3$] were potent modulators of all three properties. Thus, a negatively charged head group attached to a hydrophobic tail are necessary structural

requirements for the lipids to reduce glibenclamide inhibition and activation by openers, as has been previously demonstrated for ATP inhibition (Fan and Makielski, 1997; Baukrowitz et al., 1998; Shyng and Nichols, 1998). Furthermore, we show that the structure of the negatively charged head group is less critical because the artificial lipid DOGS-NTA acts as a full substitute for PIPs but possesses a negatively charged NTA group instead of the phosphorylated inositol ring of PIPs (Fig. 2B). DOGS-NTA might serve as a useful agent for further functional and structural studies because it is considerably less expensive and more stable than PIP_2.

It is intriguing to compare the modulation of K_{ATP} channels by PIPs with the effects of PIPs on other members of the inward rectifier channel family. Kir2.1 channels interact with PIP_2 strongly and display marked preference for PIPs with a 4,5-bisphosphat head group. In contrast, Kir3.1/Kir3.4 channels bind PIP_2 only weakly and are similarly affected by PIPs with 3,4-; 3,5-; and 4,5-bisphosphat inositol head groups (Rohacs et al., 1999). Furthermore, Kir2.1 and Kir3.1/3.4 channels show distinct preferences for the acyl chains of the PIPs (Rohacs et al., 1999). These results imply that PIPs binding sites on Kir channels can markedly differ in their lipid specificity. Therefore, our finding that modulation of glibenclamide inhibition, activation by KCOs, and ATP inhibition show the same lipid specificity suggests that PIPs interact with the same sites on the channel to modulated these properties. In line with this view, a mutation in the C terminus of Kir6.2 (R176A) that had been shown to attenuate the effect of PIP_2 on ATP inhibition also reduced the effect of PIP_2 on glibenclamide inhibition.

Implications for Physiology and Pharmacology of K_{ATP} Channels. For cardiac K_{ATP} channels large variability with respect to sulfonylurea and ATP inhibition has been reported. ATP sensitivity measured in excised patches from cardiac myocytes can vary 60-fold (Findlay and Faivre, 1991). In addition, ATP sensitivity may change during different metabolic states of cardiac myocytes; e.g., metabolic stress has been demonstrated to profoundly reduce ATP sensitivity of K_{ATP} channels (Deutsch and Weiss, 1993). This mechanism might be important for the cardioprotective function of K_{ATP} channels. Intriguingly, also glibenclamide sensitivity is dramatically reduced under metabolic stress situations (Findlay, 1993a,b). Thus, it seems that sulfonylurea and ATP sensitivity of K_{ATP} channel are regulated by a shared pathway. We report here that also heterologously expressed cardiac type K_{ATP} channels show considerable variability for ATP and glibenclamide inhibition in excised patches (Fig. 1). This variability is characterized by a close correlation between ATP and glibenclamide sensitivity. Application of PIPs reduced ATP and glibenclamide sensitivity in a way closely resembling the native variability because ATP and glibenclamide sensitivity is changed in parallel. The effects of PIP_2 on K_{ATP} channels are stable (even in presence of high Mg^{2+} and Ca^{2+}, data not shown) in excised patches but can be readily reversed upon cramming of the patch back into the cytoplasm of the oocyte. Thus, exogenously applied PIP_2 can serve as substrate for endogenous PIPs-degrading enzymes. These results suggest that PIPs modulate K_{ATP} channels in a reversible manner as expected for a regulatory mechanism occurring in vivo.

In general, levels of PIPs may change as a result of altered phospholipid metabolism, which is controlled by PI-kinases, PIPs-phosphatases, and phospholipases (e.g., phospholipase C) (Baukrowitz and Fakler, 2000a). Stimulation of PI-kinases will increase PIPs levels, which is expected to desensitize K_{ATP} channels to ATP and pharmacological manipulations, whereas PIPs-phosphatases and phospholipases break down PIPs and should sensitize K_{ATP} channels to ATP, sulfonylureas, and KCOs. In line with this concept, activation of phospholipase C was shown to decrease the levels of PIP_2 in vivo (Stauffer et al., 1998) and increase ATP inhibition (Baukrowitz et al., 1998; Xie et al., 1999), whereas overexpression of a PI-kinase reduces ATP sensitivity of K_{ATP} channels (Shyng et al., 2000). Together, these findings provide good evidence that levels of PIPs can significantly change in vivo and are therefore likely to contribute to the observed variability for K_{ATP} channels in respect to ATP, KCOs, and sulfonylurea and their changing during different cellular regulatory states.

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