the membrane, and they provide a mechanistic framework for understanding the hallmark inhibition of these channels by ATP. Although we do not expect exact overlap of the PIP_2 and ATP binding sites, a negative heterotropic cooperativity is expected, so that individual residues may contribute to the binding of each ligand. Intact cell channel activity is reduced in the $K_{ir}6.2$[R176A] mutant, demonstrating the physiological relevance of this finding. Membrane PIP composition may vary physiologically (*16*), and this may explain the wide variability in ATP sensitivity of native K_{ATP} channels (*17*). It has long been recognized that activation of K_{ATP} channels occurs under conditions where the cytoplasmic concentration of ATP is much higher than that required to inhibit channels in excised membrane patches (*1, 6, 9*). The profound effects of PIP_2 on ATP sensitivity would suggest that as membrane PIP levels increase, K_{ATP} channels will be rendered insensitive to ATP, providing a mechanism for physiological activation.

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7. COSm6 cells were plated at ~2.5×10^5 cells per well (30-mm six-well dishes) and cultured in Dulbecco's modified Eagle's medium plus 10 mM glucose (DMEM-HG) with fetal calf serum (FCS, 10%). Cells were transfected by incubation for 4 hours at 37°C in DMEM medium plus 10% Nuserum; diethylaminoethyl-dextran (0.4 mg/ml); 100 μM chloroquine; and 5 μg each of pCMV6b-$K_{ir}6.2$, pECE-SUR1, and pECE-GFP (green fluorescent protein) cDNA. Cells were incubated for 2 min in phosphate-buffered saline buffer containing DMSO (10%) and returned to DMEM-HG plus 10% FCS. Patch-clamp experiments were made at room temperature in a chamber (*6*) that allowed rapid change of the bathing solution. Micropipettes were filled with K-INT solution (below) electrode resistance ~0.5 to 1 MΩ). Patches were isolated from cells that fluoresced green under ultraviolet illumination and then voltage-clamped (Axopatch 1B, Axon, Foster City, CA). The standard bath (intracellular) and pipette (extracellular) solution (K-INT) contained 140 mM KCl, 10 mM K-Hepes, and 1 mM K-EGTA (pH 7.3). PIP_2 was sonicated in ice for 30 min before application to inside-out patches. ATP sensitivity was assessed by brief exposures to ATP-containing solutions without PIP_2 (Fig. 1, A and B). Inward currents (shown as upward deflections) were measured at –50 mV, filtered at 0.5 to 3 kHz, digitized at 22 kHz (Neurocorder, Neurodata, NY,), and stored on videotape for offline analysis with the Microsoft Excel and Solver programs. Data are presented as the mean ± the standard error of the mean.
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12. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
13. Mutant constructs were prepared by overlap extension at the junctions of the relevant residues by sequential polymerase chain reaction (PCR). Before transfection, PCR products were subcloned into pCMV6b vector and sequenced. $K_{ir}6.2$-C was constructed by fusing a DNA fragment containing $K_{ir}6.2$ 170-390 to pGEX2T vector. GST and $K_{ir}6.2$-C, expressed in *Escherichia coli*, were purified with glutathione-agarose beads and dialysis in K-INT solution (*7*)
14. Cells were incubated for 24 hours in culture medium containing ^{86}Rb Cl (1 μCi/ml) 2 to 3 days after transfection. Before measurement of Rb efflux, cells were incubated for 30 min at 25°C in Krebs' Ringer solution with metabolic inhibitors [oligomycin (2.5 μg/ml) plus 1 mM 2-deoxy-D-glucose]. At selected time points, the solution was aspirated and replaced. The $^{86}Rb^+$ in the aspirated solution was counted.
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PIP_2 and PIP as Determinants for ATP Inhibition of K_{ATP} Channels

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Adenosine triphosphate (ATP)–sensitive potassium (K_{ATP}) channels couple electrical activity to cellular metabolism through their inhibition by intracellular ATP. ATP inhibition of K_{ATP} channels varies among tissues and is affected by the metabolic and regulatory state of individual cells, suggesting involvement of endogenous factors. It is reported here that phosphatidylinositol-4,5-bisphosphate (PIP_2) and phosphatidylinositol-4-phosphate (PIP) controlled ATP inhibition of cloned K_{ATP} channels ($K_{ir}6.2$ and SUR1). These phospholipids acted on the $K_{ir}6.2$ subunit and shifted ATP sensitivity by several orders of magnitude. Receptor-mediated activation of phospholipase C resulted in inhibition of K_{ATP}-mediated currents. These results represent a mechanism for control of excitability through phospholipids.

Modulation of K_{ATP} channels by activation of metabotropic receptors and cell metabolism is an important pathway for regulation of cell excitability (*1*). A common feature of these regulatory effects is that inhibition of K_{ATP} channels by ATP can be antagonized and activation can be mimicked by so-called K channel openers (*2*). These drugs are known to activate K_{ATP} channels even in the presence of millimolar concentrations of ATP (*3*) and involve the sulfonylurea receptor (SUR) to exert their effect (*4*, *5*).

The effect of the K channel opener diazoxide on the current mediated by K_{ATP} channels in response to voltage steps in giant inside-out patches from *Xenopus* oocytes expressing $K_{ir}6.2$ and SUR1 subunits is demonstrated in Fig. 1A (*6*). Inhibition of the K_{ATP}-mediated current by an initial application of 100 μM ATP was partly reversed by the addition of 100 μM diazoxide. After wash-out of both ATP and diazoxide, the current amplitude rapidly recovered to the level present before ATP application. This protocol was repeated four times after the patch had been intermittently exposed to the phospholipid PIP_2. Besides its known effect of inhibiting run-down (*7*, *8*), exposure to 5 μM PIP_2 reduced the inhibitory effect of ATP and removed activation of channel activity by diazoxide ($n = 3$). Patch excision into Mg-ATP–free solution resulted in substantial run-down of K_{ATP} channel activity (Fig. 1B). This phenomenon is known for a variety of native and cloned K_{ir} and K_{ATP} channels (*7–10*) and has recently been shown to be induced by wash-out of phospholipids such as PIP_2 and PIP (*8*). Run-down of K_{ATP} channels was accompanied by a marked increase in ATP sensitivity ($n = 6$). Immediately after patch excision, 10 μM ATP

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blocked about 30% of the current. This block increased to about 70% after several minutes, suggesting that the change in ATP sensitivity results from wash-out of phospholipids (Fig. 1B). The effect of PIP_2 on ATP inhibition was further characterized in experiments with single channels (Fig. 1C) and metabolites of PIP_2 (Fig. 1, D to F). Prolonged application of 10 μM PIP_2 completely removed inhibition of K_{ATP} channels by 1 mM ATP (Fig. 1D; $n = 3$). The current amplitude in the absence of ATP remained unchanged in this type of experiment, indicating that the PIP_2-mediated decrease in ATP sensitivity can be separated from the effect on run-down. Moreover, PIP_2 had no effect on the single-channel amplitude but increased the channels' open probability with respect to that observed after

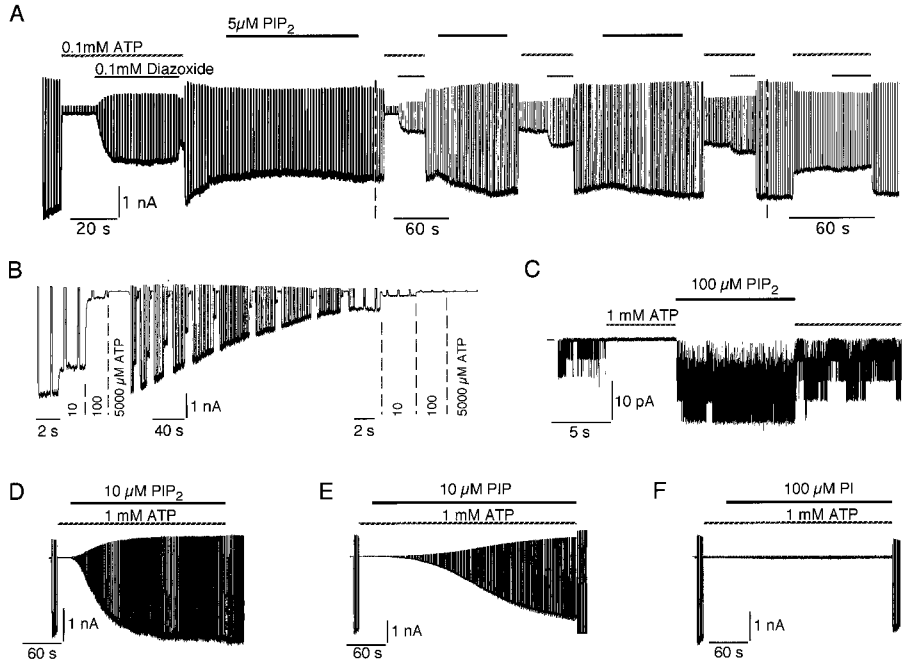

Fig. 1. Polyphospholipids act as K channel openers. (**A**) Responses to voltage steps from −80 to 20 mV in a giant inside-out patch with $K_{ir}6.2$/SUR1 channels. Solutions were exchanged with a multibarrel application system. One hundred micromolar ATP (in K-$Int_{1.0Mg}$) blocked almost all channel activity (left). Addition of 100 μM diazoxide activated channels in the presence of Mg-ATP. Wash-out of diazoxide and Mg-ATP recovered current to the level present before Mg-ATP application before run-down continued. Application of 5 μM PIP_2 stopped current run-down and slightly increased current. Reapplication of Mg-ATP produced less current inhibition, and diazoxide was less effective in antagonizing ATP inhibition (middle panel). Three subsequent applications of PIP_2 further decreased ATP sensitivity and finally abolished the diazoxide effect (right). Time scales are as indicated. (**B**) Repetitive dose-response measurements after patch excision with ATP concentrations as indicated. The first and last dose responses are shown on an expanded time scale. The first application of 10 μM ATP produced about 30% block, whereas the last application produced about 70% block. (**C**) Inside-out patch with four active channels at −80 mV. Initial application of 1 mM ATP (in K-Int_{0Mg}) blocked channel activity, application of 100 μM PIP_2 for 10 s increased channel activity, and reapplication of 1 mM ATP produced only partial channel inhibition. PIP_2 had no obvious effect on the single-channel amplitude. (**D** to **F**) Application of 10 μM PIP_2 (D), 10 μM PIP (E), or 100 μM PI (F) on inside-out patches with K_{ATP} channels in the presence of 1 mM ATP (in K-Int_{0Mg}). PIP_2 and PIP recovered K_{ATP}-mediated currents, whereas PI failed.

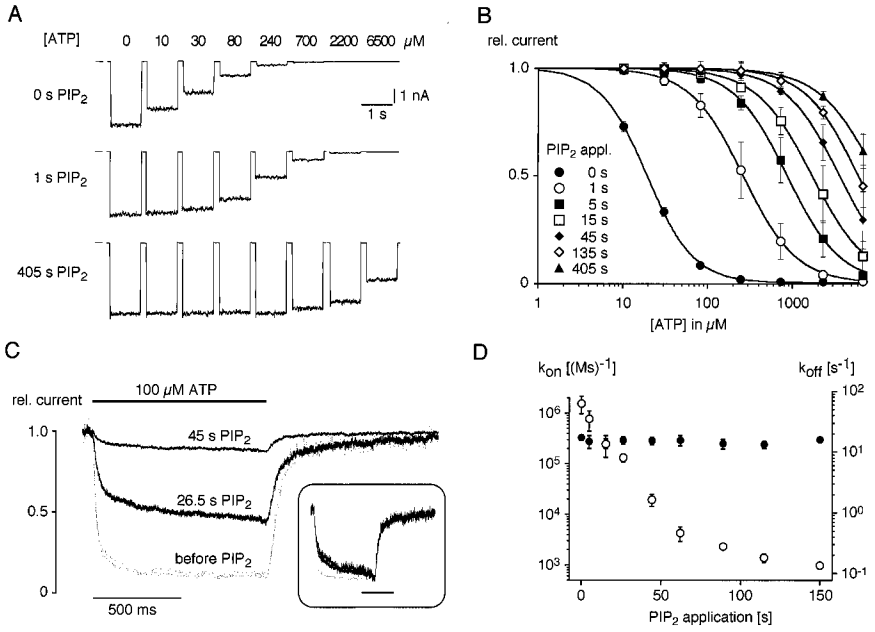

Fig. 2. Effect of PIP_2 on steady state and time course of ATP inhibition. (**A**) Dose-response measurements of ATP inhibition (ATP concentrations as indicated, voltage steps from 0 to −80 mV) in an inside-out patch before (top) and 1 s (middle) or 405 s (bottom) after PIP_2 (100 μM) application. (**B**) Dose-response curves from experiments as in (A); data points are mean ± SD from three experiments. Continuous lines represent fit of a Hill equation: $I/I_{max} = 1/[1+([ATP]/IC_{50(ATP)})^n]$, where I is the current in the presence of ATP, I_{max} is the current amplitude in the absence of ATP, $IC_{50(ATP)}$ is the concentration for half-maximal inhibition, and n is the Hill coefficient. PIP_2 (cumulative application time as indicated) shifted ATP sensitivity of K_{ATP} channels without affecting the Hill coefficient. (**C**) On and off kinetics of ATP inhibition measured with a piezo-driven application system allowing solution exchange at an inside-out patch in less than 2 ms. Time course of block and block release by 100 μM ATP (in K-Int_{0Mg}) as measured before and after application of 10 μM PIP_2 (duration as indicated). Each trace is the average current from five subsequent ATP applications. Currents were normalized to the current preceding ATP application. Time course was slowed and steady state of ATP inhibition was decreased by PIP_2, whereas block release remained unchanged. (**Inset**) Current traces normalized to their amplitude for better comparison of time courses for block and unblock. (**D**) Time dependence of PIP_2 effect on ATP inhibition kinetics. Off-rates were calculated from the time constants obtained by monoexponential fits to the release of ATP inhibition (τ_{off}) as $k_{off} = 1/\tau_{off}$. Apparent on-rates were calculated from k_{off}, steady-state block (b), and ATP concentration ([ATP]) as $k_{on} = (bk_{off})/\{(1 - b)[ATP]\}$; this procedure was used because fit of the on-reaction required more than a single exponential. The last four data points were obtained with 1 mM ATP to obtain sufficient current inhibition. ○, k_{on}; ●, k_{off}. Data points are mean ± SD from two to four experiments.

run-down of channel activity (Fig. 1C). Similar to PIP_2, PIP was also able to reduce ATP inhibition, although on a slower time scale (Fig. 1E; $n = 6$), whereas phosphatidylinositol (PI) had no obvious effect on K_{ATP} channels even at a 10 times higher concentration (Fig. 1F; $n = 3$). Furthermore, phospholipids were only active when applied to the cytoplasmic side of the membrane; addition of 50 μM PIP_2 into the pipette had no effect on ATP sensitivity in inside-out patches (*11*).

For a more quantitative investigation of the phospholipid effect, PIP_2 was applied to giant inside-out patches for increasing intervals, and the ATP sensitivity of channels was determined. The dose-response curves for ATP inhibition were gradually shifted toward higher concentrations, without changes in their steepness (Fig. 2, A and B). This result suggests that increasing concentrations of PIP_2 gradually change ATP sensitivity of individual channels rather than render channels ATP insensitive in an all-or-none manner. To analyze the underlying mechanism, we determined the kinetics of ATP inhibition before and after exposure to 10 μM PIP_2, using a piezo-controlled application system (*12*). The release of ATP inhibition exhibited a monoexponential time course (time constant: 69.1 ± 10.4 ms; $n = 5$) that did not change with PIP_2 application (Fig. 2C). The steady-state ATP inhibition, however, was markedly reduced by the phospholipid (Fig. 2, B and C). As a consequence, the apparent blocking rate constants (k_{on}) calculated from steady-state inhibition and release rates (k_{off}) changed by as much as three orders of magnitude in response to the PIP_2 exposure, whereas K_{off} remained unchanged (Fig. 2D). This change suggests that PIP_2 decreases the probability of ATP binding to its receptor site without affecting the stability of the ATP-receptor interaction.

This raised the question of which subunit of the K_{ATP} channel complex is involved in action

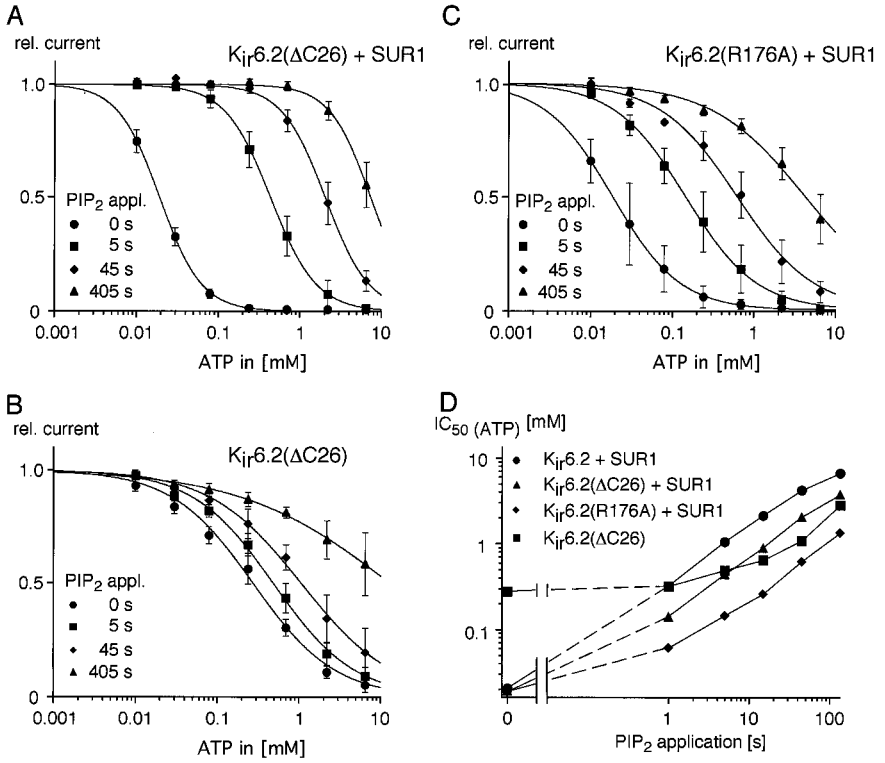

Fig. 3. Contribution of K_{ATP} subunits to ATP inhibition and PIP_2 effect. (**A** to **C**) Dose-response curves from experiments as in Fig. 2A with $K_{ir}6.2(\Delta C26)$ channels in the presence (A) and absence (B) of SUR1 or with $K_{ir}6.2$(R176A)/SUR1 channels (C). Data points are mean ± SD from four experiments; cumulative application time of PIP_2 is as indicated. (**D**) Dependence of IC_{50} for ATP inhibition as a function of PIP_2 application time for the channels indicated. Data points are taken from the results shown in Fig. 2B and (A) to (C).

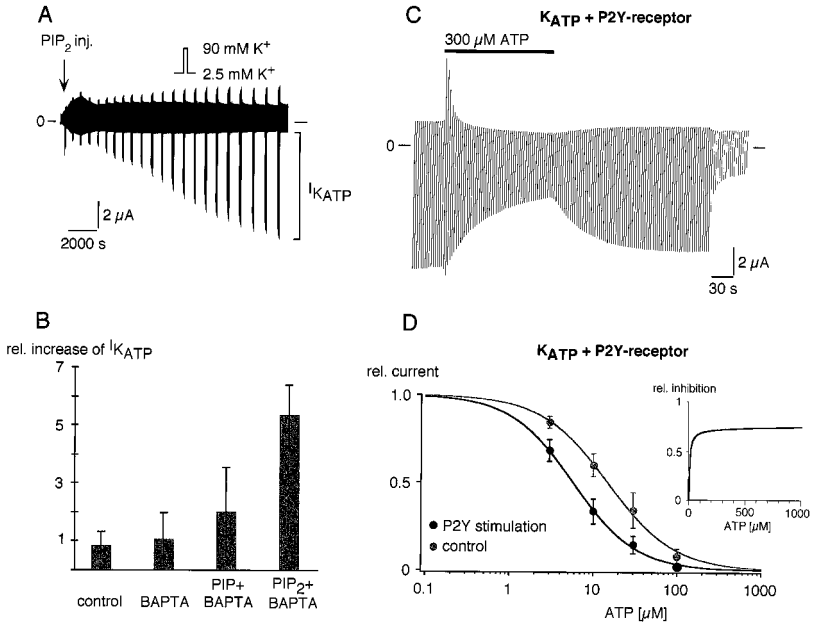

Fig. 4. Modulation of K_{ATP} currents by PIP_2 injection or stimulation of a coexpressed P2Y receptor. (**A**) Two electrode voltage-clamp measurements from oocytes expressing cloned K_{ATP} channels in response to voltage ramps from −120 to 50 mV. The external solution was intermittently changed from Ext_{90K} to $Ext_{2.5K}$ to monitor both leakage and K_{ATP} current. Injection of 50 nl of 10 mM PIP_2 plus 10 mM BAPTA produced an initial transient increase of the leakage current (visible at 2.5 mM K^+) and a delayed but continuous increase in K_{ATP}-mediated ($I_{K_{ATP}}$) current from about 1 to about 8 μA over 4.5 hours. (**B**) Relative increase of K_{ATP}-mediated current measured 10 hours after injection of either 10 mM BAPTA, 1 mM PIP and 10 mM BAPTA, or 1 mM PIP_2 and 10 mM BAPTA. Bars represent mean ± SD of three to five experiments. (**C** and **D**) Stimulation of a coexpressed P2Y receptor decreased K_{ATP}-mediated currents by increasing ATP sensitivity. (C) Responses to voltage ramps from −120 mV to 50 mV in 2.5 s in Ext_{90K} (traces in black) or $Ext_{2.5K}$ (traces in grey) to which 100 nM staurosporine was added. Application of ATP evoked a transient increase in outward current followed by a reversible decrease in K_{ATP}-mediated current. Measurements of K_{ATP}-mediated currents in whole oocytes were enabled by high expression levels of the channel protein. (D) Dose response for ATP inhibition of K_{ATP} channels measured in patches from oocytes before (control) and after stimulation of P2Y receptors (P2Y stimulation). Data points are mean ± SD from five experiments. (**Inset**) Relative inhibition (calculated from the ratio of the two dose responses) of K_{ATP} currents by P2Y receptor stimulation was independent of intracellular ATP for concentrations > 100 μM.

of PIP_2 and PIP. Experiments as in Fig. 2B were performed with a mutant of $K_{ir}6.2$ that has been deleted by the COOH-terminal 26 amino acids [$K_{ir}6.2(\Delta C26)$] and that forms ATP-sensitive channels in the absence of the SUR subunit (*13*). $K_{ir}6.2(\Delta C26)$ channels displayed PIP_2-dependent decrease of ATP inhibition in the absence and presence of SUR1 (Fig. 3, A and B). As described before (*13*), ATP sensitivity determined before PIP_2 application was substantially lower in homomeric $K_{ir}6.2(\Delta C26)$ channels than in $K_{ir}6.2(\Delta C26)$/SUR1 channels. However, longer applications of PIP_2 were required to shift ATP inhibition (Fig. 3, A, B, and D). In addition, the PIP_2 effect was less stable in $K_{ir}6.2(\Delta C26)$ channels in the absence of SUR1 as judged from the faster wash-out of the phospholipid effect (*11*). These results indicate that PIP_2 basically exerts its effect on ATP inhibition through interaction with the $K_{ir}6.2$ subunit. Morever, SUR1 increases sensitivity of K_{ATP} channels for ATP and stabilizes binding of PIP_2. Correlation between binding affinity for PIP_2 and channel inhibition by ATP was further investigated in a $K_{ir}6.2$ mutant channel, in which arginine (R) 176 was changed to alanine (A). This and the adjacent residue have recently been shown to reduce interaction between $K_{ir}6.2$ and $K_{ir}1.1$ with PIP_2 (*8*, *10*). Longer PIP_2 applications were indeed necessary to induce a given shift in ATP sensitivity compared with $K_{ir}6.2$ wild-type channels, although the ATP inhibition measured before PIP_2 exposure was similar in both channels (Fig. 3, C and D).

To ensure that PIP_2 plays a role in the activity of K_{ATP} channels in the cellular environment, we injected PIP_2 and PIP into oocytes expressing K_{ATP} channels (*14*). Injection of about 50 nl of phospholipids at a concentration of 10 or 1 mM resulted in an increase of K_{ATP}-mediated currents (Fig. 4, A and B). PIP_2 was more effective than PIP, and intracellular calcium had no effect on the K_{ATP}-mediated current (Fig. 4B). We further tested the effect of transient stimulation of a coexpressed metabotropic purino-receptor of the $P2Y_2$ subtype, which is known to activate phospholipase C (PLC) (*15*, *16*) and thus decrease the concentration of PIP_2. Such receptor-activated stimulation of PLC reduces PIP_2 concentrations in cultured cells within minutes by about a factor of 2 (*17*). Application of 300 μM ATP to an oocyte coexpressing K_{ATP} channels and $P2Y_2$ receptors resulted in a reversible reduction of the potassium current by 59 ± 3% (Fig. 4C; $n = 2$). To exclude effects of protein kinase C, we performed these experiments in the presence of staurosporine (100 nM). The initial increase in outward current observed in the first few seconds after ATP application is due to transient activation of calcium-dependent chloride channels endogenous to *Xenopus* oocytes through the PLC-IP_3 pathway (*15*, *18*). The $P2Y_2$-mediated increase in ATP inhibition was confirmed by dose-response experiments performed with excised patches from oocytes that had been incubated with ATP (1 mM) before the measurements. As shown in Fig. 4D, $P2Y_2$ stimulation resulted in an increase in ATP inhibition by about 70%, which was independent of intracellular ATP at concentrations > 100 μM.

In conclusion, inhibition of K_{ATP} channels by intracellular ATP depends on the concentration of phospholipids such as PIP and PIP_2 in the cell membrane. At low concentrations of phospholipids, K_{ATP} channels are blocked by micromolar concentrations of ATP, whereas prolonged application of PIP_2 renders channels ATP insensitive. This observation might underlie the variability observed for ATP sensitivity of K_{ATP} channels in various tissues and studies (*19*, *20*) and may contribute to the mechanism or mechanisms by which K_{ATP} channels can overcome the high physiological concentrations of intracellular ATP.

Mechanistically, PIP_2 most likely binds to the $K_{ir}6.2$ subunit, although SUR increases binding affinity of the phospholipids. It exerts its effect either by stabilizing a state in which the channel cannot interact with ATP (*21*) or by rendering the ATP-binding site inaccessible for ATP, for example, by competitive binding. However, the finding that PIP_2 changes the apparent on-rate for ATP inhibition by several orders of magnitude without having any effect on the off-rate argues against a change in conformation of the ATP-binding site, which would also affect the off-rate.

The observation that a metabotropic receptor coupling to PLC can control activity of K_{ATP} channels might point toward the physiological role of the phospholipid effect presented here. This role is supported by the finding that ATP sensitivity of cardiac K_{ATP} channels is regulated through a G-protein–related pathway (*22*). This was observed in a membrane patch and did not involve a diffusible second messenger. Thus, phospholipid-mediated opening of otherwise ATP-blocked K_{ATP} channels might represent a new mechanism to control excitability in a wide variety of cells.

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6. *Xenopus* oocytes were surgically removed from adult females and manually dissected. About 50 nl of a solution containing complementary RNA was injected into Dumont stage VI oocytes. Oocytes were treated with collagenase type II (0.5 mg/ml; Sigma) and incubated at 19°C for 1 to 3 days before use. Experiments were done at room temperature (about 22°C) 2 to 7 days after injection. Giant patch pipettes were made from thick-walled borosilicate glass, had resistances of 0.3 to 0.6 Mohms (tip diameter of 20 to 30 μm), and were filled with 120 mM KCl, 10 mM Hepes, and 1.8 mM $CaCl_2$. Currents were recorded and corrected for capacitive transients with an EPC9 amplifier (HEKA Electronics, Lamprecht, Germany), whose analog filter was set to 3 kHz (−3 dB). Leakage correction was not performed. Solutions were applied to the cytoplasmic side of the excised patches through a motor-driven multibarrel pipette; solutions had the following compositions (pH adjusted to 7.2 with KOH) with the concentration of free Mg^{2+} ions calculated according to A. Fabiato [*Methods Enzymol.* **157**, 378 (1986)]: K-$Int_{1.0Mg}$ (120 mM KCl, 10 mM Hepes, 10 mM EGTA, and 1.44 mM $MgCl_2$), which was also used as bathing solution, and K-Int_{0Mg} (120 mM KCl, 10 mM Hepes, and 10 mM EGTA), to which K_2-ATP was added as indicated. PIP_2, PIP, and PI were dissolved by 30 min of sonification. Staurosporine, ATP, diazoxide, and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) were from Sigma, and PIP_2, PIP, and PI were from Boehringer-Mannheim.
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14. Whole-cell recording experiments with injection of BAPTA and phospholipids were done with oocytes coexpressing a tandem $K_{ir}6.2$ (two $K_{ir}6.2$ subunits linked by 10 glutamines) and SUR1. The $K_{ir}6.2$ tandem was made with standard mutagenesis techniques and used because of its large expression efficiency. No differences were detected in experiments with tandem and monomeric $K_{ir}6.2$ subunits with respect to ATP sensitivity and PIP_2 and PIP effects (*11*). Oocytes injected with $P2Y_2$ RNA were cultured in the presence of 1 mM suramine to inhibit $P2Y_2$ receptor activation by ambient ATP. Extracellular solutions for whole-cell experiments had the following compositions (pH adjusted to 7.2 with NaOH): $Ext_{2.5K}$ (2.5 mM KCl, 115 mM NaCl, 1.8 mM $CaCl_2$, and 10 mM Hepes) and Ext_{90K} (90 mM KCl, 27.5 mM NaCl, 1.8 mM $CaCl_2$, and 10 mM Hepes). Na_2-ATP was dissolved in Ext_{90K}, as indicated. Whole-cell recordings were performed with the two-microelectrode voltage clamp with a TurboTec 01C amplifier (npi, Tamm, Germany); data were digitized with an ITC16 board (HEKA, Lamprecht, Germany) and stored on hard disk.
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