

Table 1. Transformation of incisors to molars. Incisors do not develop as well as molar tooth germs in kidney capsules, despite being cultured as pairs, with two out of three (70%) forming cysts at E10 and E11. After E11, incisor tooth germs develop well in kidney capsules. Some secondary molars also developed alongside primary molars in the Noggin/BSA presumptive molar regions.

Gestation	Presumptive incisor region		Presumptive molar region treated with Noggin or BSA
	Treated with Noggin	Treated with BSA	
E10	1 incisor and 4 molars out of 12 pairs	4 incisors and 0 molars out of 9 pairs	35 primary molars out of 36
E11	5 incisors and 0 molars out of 16 pairs	8 incisors and 0 molars out of 15 pairs	29 primary molars out of 32

At E9.5, Fgf8 beads can induce ectopic *Barx-1* expression in distal regions of the mandible, indicating that all neural crest-derived ectomesenchymal cells of the mandibular arch are equally responsive to epithelial signals. This implies that the neural crest cells that populate the mandibular arch are not pre-specified but are patterned by contact with epithelial signals. The evidence for a pre-patterning of cranial neural crest cells is limited to the proximal cells of the mandibular arch; our results are consistent with data showing that distal mandibular arch cells have a different axial origin than proximal cells (15).

The antagonistic effects of Fgf8 and Bmp4, which act to establish the distal boundary of *Barx-1* expression, are similar to those reported for *Pax-9* (16). *Barx-1* is, however, expressed earlier than *Pax-9* and, unlike *Pax-9*, is not induced at localized sites that underlie all developing teeth. This antagonistic signaling interaction thus has at least two roles in the specification of mandibular mesenchymal fates: an early role in proximal-distal axis specification, which determines whether mesenchymal cells have presumptive molar or incisor fates, and a later role in determining the sites of tooth bud initiation.

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- Mandibular arch explants were dissected and cultured as described (3). Beads were also prepared as described (3, 4), with Fgf8 and BSA (white) and BMP4 and Noggin (blue) beads at a concentration of 100 μ g/mg. Whole-mount digoxigenin (DIG) and sectioned 35 S in situ hybridization were carried out as described (3, 4).
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- E10 and E11 mandibular arch explants were cultured with beads that were placed in the distal (presumptive incisor) mesenchyme for 48 hours in vitro. These were then dissected into incisor and molar regions; the incisor regions were dissected as pairs. Single incisor and molar regions from each mandibular arch were transferred into opposite ends of a kidney, under the kidney capsule, in adult male mice. These regions were then left to develop for 14 days in vivo. The resulting teeth were photographed, placed in Bouin's fixative for 3 days, dehydrated, and embedded. Sections were stained with alcian blue and chlorontine fast red.
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Membrane Phospholipid Control of Nucleotide Sensitivity of K_{ATP} Channels

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Adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channels couple cell metabolism to electrical activity. Phosphatidylinositol phosphates (PIPs) profoundly antagonized ATP inhibition of K_{ATP} channels when applied to inside-out membrane patches. It is proposed that membrane-incorporated PIPs can bind to positive charges in the cytoplasmic region of the channel's $K_{ir}6.2$ subunit, stabilizing the open state of the channel and antagonizing the inhibitory effect of ATP. The tremendous effect of PIPs on ATP sensitivity suggests that in vivo alterations of membrane PIP levels will have substantial effects on K_{ATP} channel activity and hence on the gain of metabolism-excitation coupling.

K_{ATP} channels (1) are formed from a sulfonylurea receptor (SURx) and an inward rectifier ($K_{ir}6.x$) subunit (2). Evidence is accumulating that the $K_{ir}6.x$ subunit forms the pore and controls the hallmark inhibition by ATP (3), although the mechanism of this inhibition remains elusive. Recent reports indicate that membrane PIPs bind K_{ATP} and other K_{ir} channels, stabilizing them in an active conformation (4, 5). Both the inhibitory effect of adenosine nucleotides and the stimulatory effects of PIPs increase with the number of phosphate groups in the molecule (1, 6), which suggests that PIP activation and ATP inhibition may be related phenomena. We specifically hypothesized that PIPs and ATP might compete for binding to the K_{ATP} channel, stabilizing open and closed channels, respectively.

To test this hypothesis, we recorded cur-

rents in inside-out patches from COSm6 cells expressing cloned ($K_{ir}6.2$ +SUR1) K_{ATP} channels (7). After patch excision, a slow run-down of channel activity occurred over the following minutes, and this run-down could be avoided by addition of phosphatidylinositol-4,5-bisphosphate (PIP_2) (Fig. 1A). Open probability in the absence of ATP (P_{open}) was ~ 0.4 before application of PIP_2 but increased to ~ 0.85 (Fig. 1C), which is consistent with the approximate doubling of current in macroscopic patches that followed treatment with PIP_2 (Fig. 1E) and demonstrates that PIP_2 activates K_{ATP} current by increasing P_{open} . ATP sensitivity (7) immediately after patch excision could be fit by a sigmoid relation with a half-maximal inhibition concentration ($K_{1/2}$) of 12.1 μ M and a Hill coefficient (H) of 1.3 ($n = 26$), which is similar to previous reports (2), but it decreased by orders of magnitude after PIP_2 application (Fig. 1, A and B). The rate of this decrease was variable from patch to patch (Fig. 1D), probably because of variable diffusion distances (8), but correlated with the time course of increase in ATP-independent

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channel activity (Fig. 1F). ATP sensitivity decreased 340 times (from $K_{1/2}$ of $10.5 \pm 0.1 \mu\text{M}$ to $3.6 \pm 0.3 \text{ mM}$) after 10 min of exposure to PIP_2 ($5 \mu\text{g/ml}$) ($n = 18$). This shift in ATP sensitivity is far greater than any previously reported effects of potassium channel-opening drugs (9). After PIP_2 application, channel activity became very stable, with run-down time constants of $>30 \text{ min}$, and the effect on ATP sensitivity remained, at least within 20 min of PIP_2 withdrawal (Fig. 2A). The general applicability of the phenomenon is illustrated in Fig. 2, C and D; prolonged exposure of K_{ATP} channels to PIP_2 in either

cardiac myocyte or β -cell (HIT-T15) membrane patches resulted in 100- to 700-fold increases of $K_{1/2}$.

Phosphatidyl inositol-4-phosphate (PI-4-P) also stimulated channel activity and reduced ATP sensitivity, although it did so less effectively than PIP_2 (Fig. 2B). Phosphatidyl inositol-3,4,5-triphosphate (PIP_3) was as effective as PIP_2 . Neither phosphatidyl choline (PC) nor inositol triphosphate (IP_3) altered P_{open} (4) or ATP sensitivity (Fig. 2B). Therefore, a negatively charged head and a lipid tail are necessary both to stimulate ATP-independent activity (4) and reduce ATP sen-

sitivity. Polycations have been shown to inhibit K_{ATP} channels by screening negative charges (10), and this action is a substantial cause of channel run-down in excised patches. As shown in Fig. 3A, application of polylysine or Ca^{2+} or spermine (11) after stimulation by PIP_2 caused rapid reversal of both the increased P_{open} and the reduced ATP sensitivity.

There have been indications that the COOH-terminus of K_{ir} channels is involved in PIP_2 activation (4, 5) and in the ATP sensitivity of K_{ATP} channels (3). According to the above hypothesis, mutations that re-

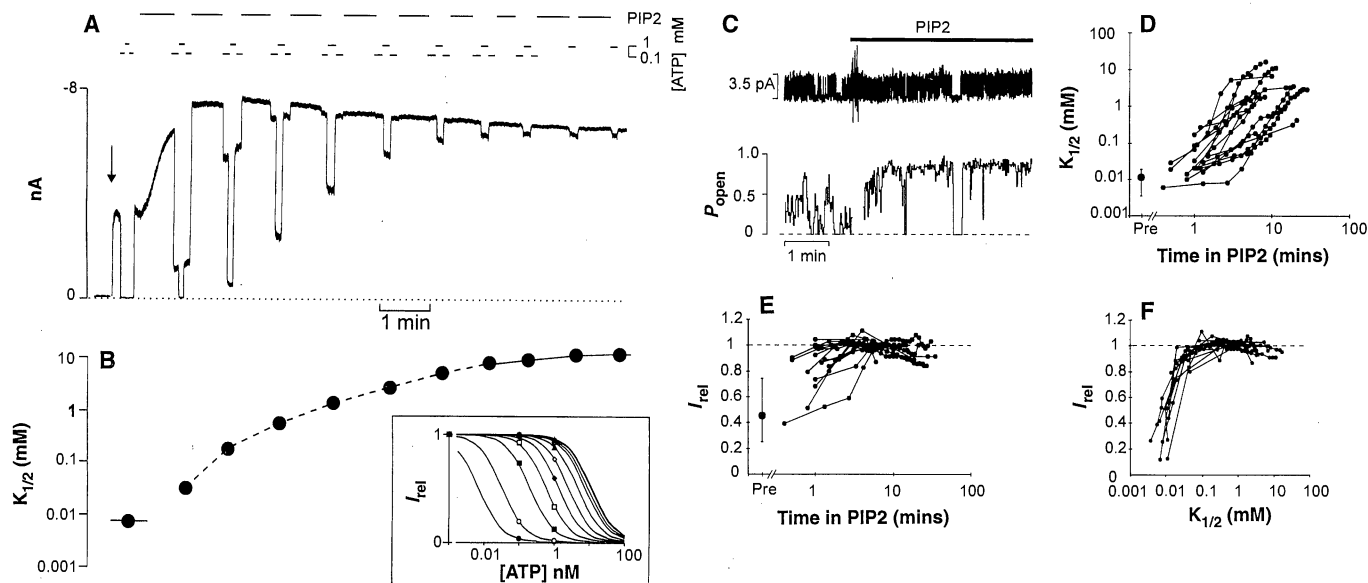


Fig. 1. (A) Representative WT (SUR1+ $\text{K}_{\text{ir}}6.2$) K_{ATP} channel currents in an inside-out patch (7). The patch was isolated at the arrow and exposed to differing ATP concentrations or to $5 \mu\text{M}$ PIP_2 , as indicated. The dotted line indicates zero current. (B) $K_{1/2}$ [estimated from fits of the Hill equation $I_{\text{rel}} = 1/(1 + ([\text{ATP}]/K_{1/2})^H)$, with I_{rel} being the current relative to the peak current after PIP_2 and H fixed at 1.3; see inset] as a function of time for the record in (A). (C) Single WT K_{ATP} channel

current (top) and P_{open} (1-s bins) (bottom). The patch was isolated immediately before the onset of the record and exposed to $5 \mu\text{M}$ PIP_2 , as indicated. (D) $K_{1/2}$ from individual experiments like those in (A) versus time after onset of exposure to $5 \mu\text{M}$ PIP_2 . Averaged $K_{1/2}$ ($\pm \text{SEM}$) before application of PIP_2 (Pre) is indicated by the larger symbol. (E) I_{rel} versus time after onset of exposure to PIP_2 . (F) I_{rel} [from (E)] versus $K_{1/2}$ [from (D)].

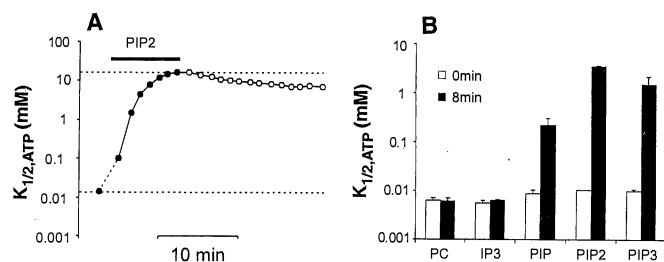
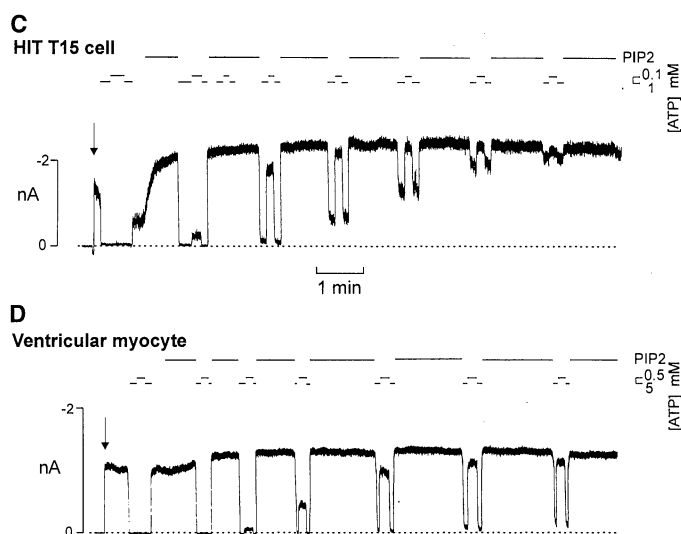


Fig. 2. (A) $K_{1/2}$ as a function of time for an experiment like that in Fig. 1A. PIP_2 was applied only during the period indicated. (B) $K_{1/2}$ from patches ($n = 3$ to 6) exposed to $5 \mu\text{M}$ PC, IP_3 , PI-4-P , PIP_2 , or PIP_3 for 0 min (open bars) or 8 (7.3 to 9.1) min (solid bars). (C and D) Representative K_{ATP} channel currents in inside-out patches from a HIT T15 cell (C) and a mouse ventricular myocyte (D). Patches were isolated at the arrows and exposed to ATP or to $5 \mu\text{M}$ PIP_2 , as indicated. In all experiments with HIT T15 cells and ventricular myocytes, $K_{1/2}$ increased from $9 \pm 1 \mu\text{M}$ and $19 \pm 4 \mu\text{M}$ to $2.1 \pm 0.3 \text{ mM}$ and $5.8 \pm 0.8 \text{ mM}$, respectively ($n = 4$ and 7 patches), after $5.5 \pm 0.3 \text{ min}$ and $9 \pm 0.5 \text{ min}$ of PIP_2 exposure.



duce ATP binding to $K_{ir}6.2$ should reduce ATP sensitivity without altering P_{open} . Neutralization of residue K185 in $K_{ir}6.2$ reduces ATP sensitivity and may contribute to ATP binding (3, 12). $K_{ir}6.2$ [K185Q] mutant channels (13) are ~ 30 times less sensitive to ATP than wild-type (WT) $K_{ir}6.2$ +SUR1 channels when first isolated, but have similar P_{open} values (11). ATP sensitivity shifts in parallel for both WT and K185Q mutant channels after PIP_2 treatment, with the $K_{1/2}$ of K185Q mutant channels rising to 39 ± 7 mM after

6.9 ± 1.0 min of PIP_2 treatment (six patches were tested). Conversely, mutations that reduce PIP_2 sensitivity should have a low P_{open} but show a saturating P_{open} and ATP sensitivity similar to those of WT channels after PIP_2 treatment. Residues R176 and R177 of $K_{ir}6.2$ are two positively charged amino acids that might contribute to PIP_2 binding (4, 5); neutralization of residue R188 in $K_{ir}1.1$ (equivalent to R177 in $K_{ir}6.2$) reduces PIP_2 binding to these channels (5). Both $K_{ir}6.2$ [R176A] and $K_{ir}6.2$ [177A] mutations

(+SUR1) expressed considerably lower conductances in intact cells than did WT channels, as assessed by ^{86}Rb efflux (14) ($21 \pm 8\%$ and $\sim 0\%$, respectively, in three separate paired transfections). In inside-out patches, no currents were detected from the R177A mutant, and very small currents were observed from the R176A mutant. The low channel activity makes accurate assessment of $K_{1/2}$ very difficult and reflects an extremely low P_{open} , as confirmed by an enormous increase of current that followed PIP_2 application (Fig. 3). The current in zero ATP increased >80 -fold (the current before PIP_2 was $1.2 \pm 0.1\%$ of the current after PIP_2) and increased more slowly than in WT channels [half-time ($t_{1/2}$) = 76 ± 20 s and 32 ± 6 s, respectively], but $K_{1/2}$ nevertheless reached comparable levels (2.1 ± 0.1 mM after 9.8 ± 0.3 min of PIP_2 treatment; $n = 6$; Fig. 3C). These results are consistent with the R176A mutation lowering PIP_2 affinity, thus underlying the reduced physiological activity of $K_{ir}6.2$ [R176A] channels and providing crucial evidence for the physiological role of PIP_2 in maintaining normal channel activity.

To further examine interactions between PIP_2 , ATP, and the $K_{ir}6.2$ subunit, we engineered a protein ($K_{ir}6.2$ -C) containing the $K_{ir}6.2$ COOH-terminus (amino acids 170 through 390) fused to glutathione S-transferase (GST) (13). After PIP_2 exposure, GST was without effect, but purified $K_{ir}6.2$ -C markedly inhibited channel activity (Fig. 4). When $K_{ir}6.2$ -C was removed, channel activity recovered only slowly; this recovery, however, was greatly accelerated by exposure to ATP. $K_{ir}6.2$ -C might block the channel, acting as a "ball"-like domain similar to the NH_2 -terminus of *Shaker*-like channels (15), but the following observations suggest an alternative hypothesis. $K_{ir}6.2$ -C inhibition was relieved in the presence of PIP_2 , which suggests that rather than directly blocking the pore, positive charges on $K_{ir}6.2$ -C may bind to the negatively charged PIPs in the membrane, screening them from the channel itself and effectively reducing the membrane PIP_2 concentration that the channel itself sees. When $K_{ir}6.2$ -C is added in the presence of PIP_2 , $K_{ir}6.2$ -C is significantly bound to the micellar PIP_2 in the solution rather than to PIPs in the membrane. These experiments were performed after prolonged pretreatment with PIP_2 . Under this condition, 1 mM ATP was without significant inhibitory effect (see Fig. 4B; ATP was added at the up arrow) but augmented the inhibitory effect of subsequently applied $K_{ir}6.2$ -C (Fig. 4, B and C), as would be expected if exogenous $K_{ir}6.2$ -C lowers the effective membrane PIP_2 concentration by a charge-screening effect.

The present results show that ATP sensitivity can be changed over orders of magnitude by manipulation of the PIP content of

Fig. 3. (A) Representative WT current in an inside-out patch, isolated at the arrow and exposed to differing ATP concentrations or to $5 \mu M$ PIP_2 , as indicated. The gaps in the record are 2 and 3.5 min long. Polylysine (10 $\mu g/ml$, molecular weight ~ 1000) was applied as indicated. (B) $K_{1/2}$ as a function of time for the record in (A). (C) Representative mutant $K_{ir}6.2$ [R176A]+SUR1 currents in an inside-out patch, isolated at the arrow and exposed to differing ATP concentrations or to $5 \mu M$ PIP_2 , as indicated. Inset at left shows current immediately after patch excision, amplified 20 times.

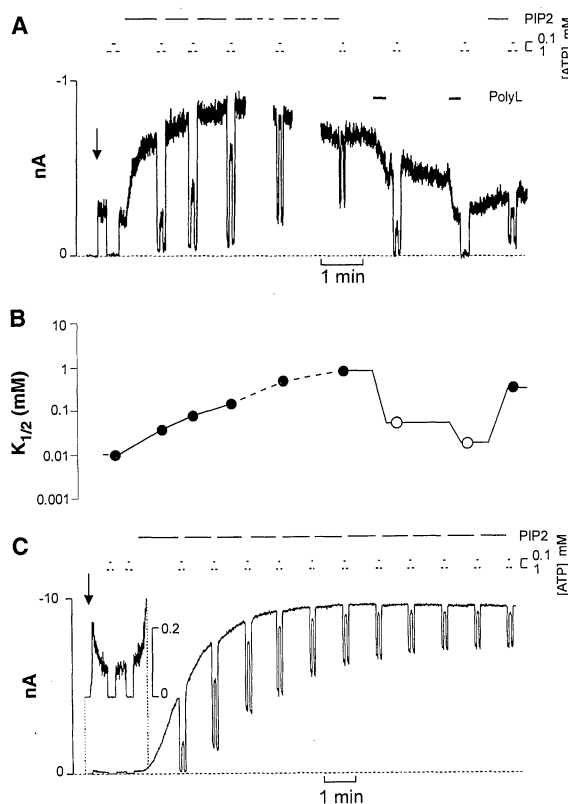
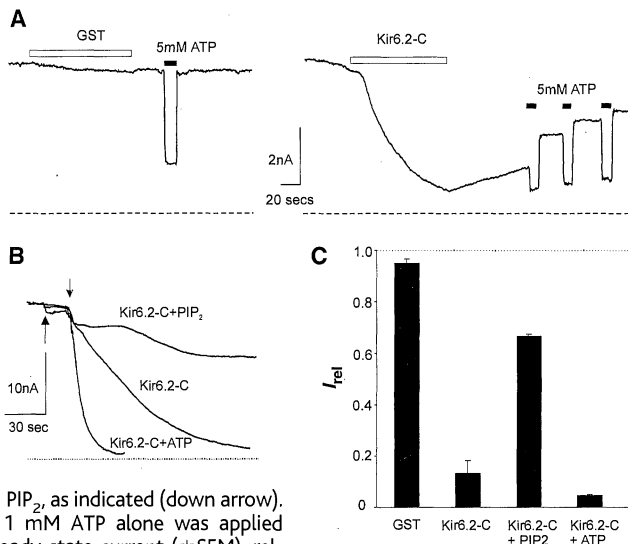


Fig. 4. (A) Representative WT current in an inside-out patch. The patch had been exposed to $5 \mu M$ PIP_2 for ~ 10 min before the first trace, and the second trace was obtained ~ 3 min after the first. The patch was exposed to 100 nM GST, 100 nM $K_{ir}6.2$ -C, or 5 mM ATP, as indicated. (B) Representative WT currents in another inside-out patch. The patch had been exposed to $5 \mu M$ PIP_2 for ~ 12 min before the first trace, and the traces were ~ 5 min apart. The patch was exposed to $K_{ir}6.2$ -C with either 1 mM ATP or $5 \mu M$ PIP_2 , as indicated (down arrow). For the $K_{ir}6.2$ -C+ATP trace, 1 mM ATP alone was applied beforehand (up arrow). (C) Steady-state current (\pm SEM), relative to the current before treatment, during application of GST or $K_{ir}6.2$ -C, alone or with PIP_2 or ATP from experiments ($n = 3$) like that in (B).



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 PIP₂ 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 PIP₂ 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 $\sim 2.5 \times 10^5$ 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 μ M chloroquine; and 5 μ g 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 M Ω). 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). PIP₂ 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 PIP₂ (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 \pm 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 ⁸⁶Rb Cl (1 μ Ci/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 μ g/ml) plus 1 mM 2-deoxy-D-glucose]. At selected time points, the solution was aspirated and replaced. The ⁸⁶Rb⁺ in the aspirated solution was counted.

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18. We are grateful to J. Shepard for technical assistance and to S. Seino and J. Nerbonne for K_{ir}6.2 and dissociated ventricular myocytes, respectively. This work was supported by grant HL45742 from NIH (C.G.N.), an Established Investigatorship from the American Heart Association (C.G.N.), a Career Development Award from the American Diabetes Association (S.L.S.), and by the Washington University Diabetes Research Training Center.

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PIP₂ 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 (PIP₂) 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 ex-

pressing 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 μ M ATP was partly reversed by the addition of 100 μ M 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 PIP₂. Besides its known effect of inhibiting run-down (7, 8), exposure to 5 μ M PIP₂ 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 PIP₂ 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 μ M ATP

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