MPS1 integration construct made by polymerase chain reaction to introduce Xho I and Eco RI sites at either end of the *NmycMPS1* fragment, allowing it to be subcloned into pDK20) were grown overnight in yeast-extract peptone (YEP) containing 2% raffinose, arrested by exposure to 4% galactose for 5 hours, and prepared for immunofluorescence as described (Fig. 2C) (*8*). ELW200 cells (integrated *GAL1-NmycMPS1*) were grown in YEP containing 2% dextrose or shifted into YEP with 3% galactose for 6 hours and prepared for flow cytometry as described (Fig. 2D) (*21*). The DNA stained by propidium iodide in 5000 cells per sample was detected on a Becton Dickinson FACScan flow cytometer.

25. Unbudded cells containing integrated copies of *GAL-MPS1* were picked individually and placed on slabs containing 4% galactose (Fig. 3A). The cells were grown at 30°C, and the number of cells that had divided and rebudded were counted at the times shown. Each point is an average from at least 50 cells. Strains are wild type (KH153), *mad1Δ.1* (KH155), *mad2-1* (KH157), *mad3-2* (KH150), *bub1Δ* (KH161), *bub2-1* (KH163), and *bub3Δ* (KH165). Cells of the strains listed above were grown in YEP with 2% raffinose to mid-log phase, collected, and incubated in fresh medium for 90 min with the addition of alpha factor to a final concentration of 10 μM. The cells were collected and resuspended in YEP containing 2% raffinose, 10 μM alpha factor, and 3% galactose and incubated for 2 hours. Finally, cells were collected, rinsed once in medium without alpha factor, and released into YEP containing 2% raffinose and 3% galactose. Timing began at the release from mating factor arrest, and samples were taken every 20 min. Cells were fixed with 70% ethanol and examined microscopically to determine the fraction of large budded cells as described (*3*). Cells of the previously mentioned strains were grown overnight in YEP with 4% raffinose and then galactose was added to a final concentration of 4%. Samples were taken after 3 and 6 hours of growth at 30°C. Control (0) cells were grown for 3 hours in dextrose (Fig. 3C).

26. We thank A. Straight for the integrating *GAL1-MPS1* construct, T. Giddings for help with the EM analysis, A. Hoyt and B. T. Roberts for *bub* strains and deletion constructs, and all the members of our labs for their advice and encouragement. pNZ2 was provided by G. N. Zecherle of the University of Washington, Seattle, and pDK20 was provided by D. Kellogg of the University of California, Santa Cruz. K.G.H. and F.C.L. are Fellows of the Leukemia Society of America and E.W.W. was a trainee of NIH. This work was supported by grants to A.W.M. from NIH, the March of Dimes, and the David and Lucile Packard Foundation; and to M.W. from NIH, the American Cancer Society, and the Pew Scholars Program in the Biomedical Sciences.

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Regulation of Cardiac Na^+,Ca^{2+} Exchange and K_{ATP} Potassium Channels by PIP_2

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Cardiac Na^+,Ca^{2+} exchange is activated by a mechanism that requires hydrolysis of adenosine triphosphate (ATP) but is not mediated by protein kinases. In giant cardiac membrane patches, ATP acted to generate phosphatidylinositol-4,5-bisphosphate (PIP_2) from phosphatidylinositol (PI). The action of ATP was abolished by a PI-specific phospholipase C (PLC) and recovered after addition of exogenous PI; it was reversed by a PIP_2-specific PLC; and it was mimicked by exogenous PIP_2. High concentrations of free Ca^{2+} (5 to 20 μM) accelerated reversal of the ATP effect, and PLC activity in myocyte membranes was activated with a similar Ca^{2+} dependence. Aluminum reversed the ATP effect by binding with high affinity to PIP_2. ATP-inhibited potassium channels (K_{ATP}) were also sensitive to PIP_2, whereas Na^+,K^+ pumps and Na^+ channels were not. Thus, PIP_2 may be an important regulator of both ion transporters and channels.

Cardiac Na^+,Ca^{2+} exchange activity can be enhanced by several acidic lipids (*1*, *2*) that may occur in domains in cell membranes (*3*). In cardiac membrane patches treated with ATP, acidic lipids are generated on the cytoplasmic side of the membrane in parallel with a stimulation of Na^+,Ca^{2+} exchange current (*2*, *4*). The underlying mechanism might be (i) an ATP-dependent transport of phosphatidylserine (PS) from the extracellular to the cytoplasmic side by an amino phospholipid "flippase" (*5*), (ii) the phosphorylation of diacylglycerol (DAG) to form phosphatidic acid (PA) (*6*), or (iii) the phosphorylation of PI to form PIP and PIP_2 (*7*). We used specific phospholipases and phospholipid vesicles to modify the lipid composition of giant cardiac membrane patches (*8*) and determined that the major mechanism is the generation of PIP_2 from PI.

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Outward Na^+,Ca^{2+} exchange current was increased by addition of Mg-ATP to the cytoplasmic side of inside-out giant cardiac membrane patches (Fig. 1A) (*9*). The current was first activated by application of 90 mM Na^+ to the cytoplasmic side of the patch with 2 mM extracellular (pipette) Ca^{2+}. With the free cytoplasmic Ca^{2+} concentration used (0.5 μM) the current inactivated (decreased) by about 80% over 15 s. Subsequent application of Mg-ATP (2 mM) for 40 s increased the current sixfold, and after ATP was removed the current remained stimulated for 100 s, after which it was turned off by removal of Na^+.

The record in Fig. 1A is a control experiment from a randomized series of patches, one-half of which were treated for 4 min with a phospholipase C that specifically hydrolyzes PI (PI-PLC) (*10*). The PI-PLC treatment (0.6 U/ml) did not significantly decrease the current before application of ATP (*11*) (Fig. 1B), and PI-PLC had no effect after the current had been stimulated by ATP (*12*). However, the treatment decreased the ATP effect by 96% ($P < 0.001$). PIP_2 (50 μM) strongly activated the exchange current, although ATP did not (Fig. 1B). Pure PI vesicles (0.3 mM) were applied for 60 s to other treated patches that failed to respond to ATP (Fig. 1C). PI had no effect by itself, but it restored the capacity of ATP to stimulate the exchange current.

The effect of ATP was reversed by a recombinant PIP_2-specific phospholipase C, PLC-β1, that is fully activated by 0.5 μM free Ca^{2+} under standard assay conditions (Fig. 2A) (*13*). This PLC-β1 was histidine-tagged, expressed in Sf9 cells, purified by Ni^{2+}-chelate affinity chromatography, and dialyzed against the solution used in the experiments. Reversal of the ATP effect after ATP removal was very slow (Fig. 2A). However, upon application of PLC-β1 (0.2 mg ml^{-1} with a maximal specific activity of 100 μmol min^{-1} mg^{-1}), the current declined to its original value within 40 s (in three similar experiments). PLC-β1 had no effect when it was applied to patches in which the exchange current had been stimulated by PS rather than ATP (*12*).

High concentrations of cytoplasmic free Ca^{2+} induced a fast reversal of the ATP effect, probably mediated by an endogenous Ca^{2+}-dependent PLC (Fig. 2B). After ATP was applied and removed, 20 μM free Ca^{2+} was applied. At first, the exchange current was slightly stimulated because cytoplasmic Ca^{2+} activates the exchanger by an intrinsic regulatory mechanism (*14*). Thereafter, the exchange current declined rapidly over 30 s, and it declined to below its original level when free Ca^{2+} was reduced back to 0.5 μM (*15*). To determine the Ca^{2+} dependence of endogenous cardiac membrane–associated PLC, a crude membrane fraction was prepared from guinea-pig myocytes, and PLC activity was measured as inositol trisphosphate (IP_3) released from exogenous vesicles containing [^{3}H]PIP_2 (*16*). The PLC activity of the cardiac membranes was slightly activated with 0.5 μM free Ca^{2+} and was maximally activated with 20 μM free Ca^{2+} (Fig. 2C), which correlates with the ability of 20

μM but not 0.5 μM free Ca^{2+} to reverse the ATP effects in patches.

Occasionally, the ATP effects reversed rapidly with 0.5 μM free Ca^{2+} (5), apparently due to traces of polyvalent cations in our solutions (17). The ATP-stimulated exchange current returned nearly to baseline in 20 s when 50 μM aluminum was applied (Fig. 3A), although the calculated free aluminum concentration with 10 mM EGTA was only 5 pM (18). The current remained inhibited after aluminum removal. In contrast to the effect of Ca^{2+}, the effect of aluminum was relieved for the most part by application of fluoride (0.5 mM), which binds aluminum with very high affinity (19). Aluminum inhibits PLC activity by forming stable complexes with PIP_2 (20), and aluminum (50 μM) inhibited both cardiac membrane PLC and Sf 9-expressed PLC-β1 by >90% when our recording solutions were used in PLC activity assays (21). Although aluminum (50 μM) reversed the stimulatory effect of PIP_2 on exchange current, it did not reverse stimulation by PS, by PA, or by limited proteolysis to destroy the inactivation process (22) (Fig. 3B). Thus, aluminum may act on exchange current by forming poorly reversible aluminum-PIP_2 complexes that cannot bind to the exchanger.

To monitor aluminum-lipid interactions, vesicles of various compositions were incubated separately with each of three fluorescent membrane probes, which are sensitive to membrane charge (23). The fluorescence of each probe changed when aluminum (50 μM with 10 mM EGTA) was added to vesicles containing PIP_2, but not PS, in a 1:4 ratio with phosphatidylcholine (PC). The hydrophobic anion, 1,8 anilinonaphthalene sulfonate (ANS), fluoresces only when bound to membranes and is repelled from negatively charged membranes (24). Aluminum increased ANS fluorescence in PIP_2-containing vesicles, as would be expected if

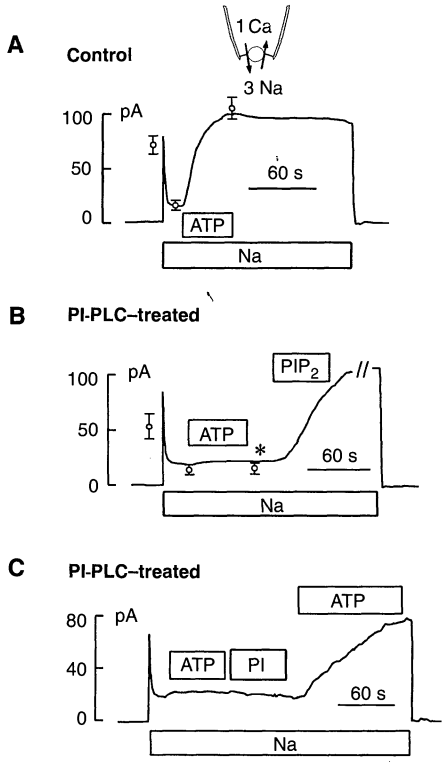

Fig. 1. Stimulation of cardiac Na^+,Ca^{2+} exchange current by cytoplasmic ATP and its dependence on PI in giant excised inside-out cardiac membrane patches. Horizontal bars indicate the time that a particular substance was applied to the cytoplasmic patch surface. Error bars give the SEM. All results are with 10 mM EGTA to buffer Ca^{2+} on the cytoplasmic side. (**A**) Outward Na^+,Ca^{2+} exchange current was activated by replacement of 90 mM CsCl with 90 mM NaCl. After current stabilized to a basal level, 2 mM Mg-ATP was applied for 40 s. Current typically remained stimulated for several minutes after removal of ATP. The current stopped when cytoplasmic Na^+ was removed. (**B**) A typical record from patches treated for 4 min with PI-PLC (1 U/ml) in the absence of Na^+. ATP (2 mM) had almost no effect, but subsequent application of 50 μM PIP_2 activated the current. (**C**) Recovery of the ATP effect by application of PI vesicles. The record is from a PI-PLC–treated patch. ATP (2 mM) had no effect initially, but after PI was applied (0.3 mM) ATP (2 mM) stimulated exchange current.

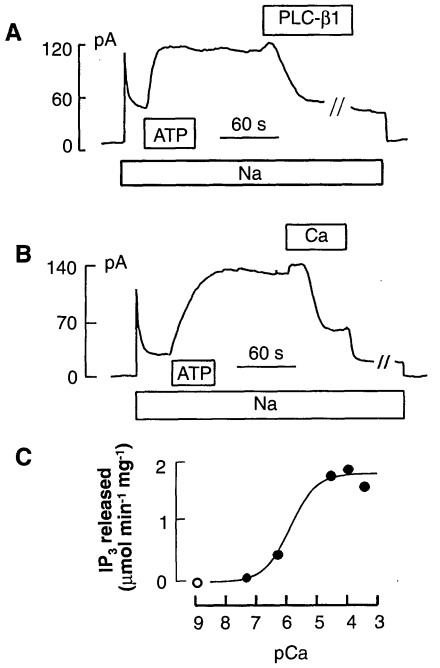

Fig. 2. Reversal of the stimulatory effect of ATP on cardiac Na^+,Ca^{2+} exchange current by PLC and Ca^{2+}. (**A**) ATP (2 mM) was applied for 45 s, and PLC-β1 was applied 80 s after ATP removal. (**B**) Cytoplasmic free Ca^{2+} was increased from 0.5 to 20 μM 80 s after ATP removal. Cytoplasmic Ca^{2+} activates the exchanger through a regulatory site (14), and the ATP effect increases the apparent affinity for Ca^{2+} (4). Thus, exchange current increased only slightly when the concentration of Ca^{2+} was increased to 20 μM, because the exchanger was already fully activated; in contrast, exchange current decreased substantially if the concentration of free Ca^{2+} was decreased after the ATP effect reversed. (**C**) Ca^{2+} dependence of PLC activity in cardiac membranes prepared from myocytes. The same solutions and conditions were used for PLC assays and patch experiments. The open circle gives PLC activity in the absence of added calcium. Closed circles give PLC activity with calcium at calculated pCa (−log[Ca^{2+}]) values.

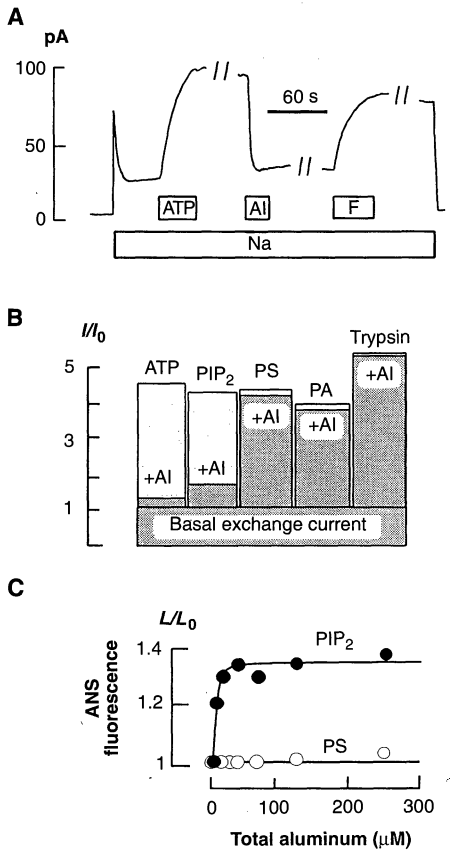

Fig. 3. Reversal of the stimulatory effect of ATP on Na^+,Ca^{2+} exchange current by high-affinity aluminum binding to PIP_2. (**A**) ATP was applied for 40 s, and aluminum (Al) (50 μM) was applied 2 min after ATP removal. Current returned to the basal level within 15 s and was stable after removal of aluminum. Up to 2 min later, fluoride (F) (0.5 mM), which had no effect in patches not exposed to aluminum, reactivated the current. (**B**) Effect of aluminum (50 μM) after stimulation of exchange current by ATP (2 mM), PIP_2 vesicles (50 μM), PA vesicles (0.2 mM), PS vesicles (0.2 mM), or trypsin treatment (0.3 mg ml^{-1} for 1 min). The plotted values (I/I_o) give exchange current magnitudes (I) divided by the current magnitude before stimulation (I_o in each patch. (**C**) Effect of aluminum on ANS fluorescence in the presence of 10 mM EGTA and PIP_2:PC (1:4) or PS:PC (1:4) vesicles. The plotted values (L/L_o) give fluorescence light intensities (L) divided by the intensity without aluminum (L_o). Fluoride (20 mM), but not EDTA (2 mM), reversed the increase of fluorescence caused by 120 μM aluminum.

the negative charges of PIP_2 were neutralized (Fig. 3C). The response saturated at <65 μM aluminum with 90 μM PIP_2 in the assay, indicating that about one aluminum ion was bound for every two molecules of PIP_2.

Tests for regulation of cardiac Na^+ channels and Na^+,K^+ pumps by PIP_2 were negative (*25*), but tests for K_{ATP} potassium channels were positive (*26*). Potassium current was recorded as inward current at 0 mV (extracellular potassium, 140 mM; cytoplasmic potassium, 20 mM; free cytoplasmic Ca^{2+}, 0.5 μM) (Fig. 4). Application of 2 mM ATP immediately suppressed a small inward current. Then the current increased for about 1 min in the continued presence of ATP (*27*). The large magnitude of the stimulatory effect was evident only after removal of ATP, which relieved its immediate inhibitory effect. Potassium current was usually stable after ATP removal in the presence of 0.5 μM or less free Ca^{2+}, and it was completely blocked when a cytoplasmic solution containing 120 mM cesium was applied.

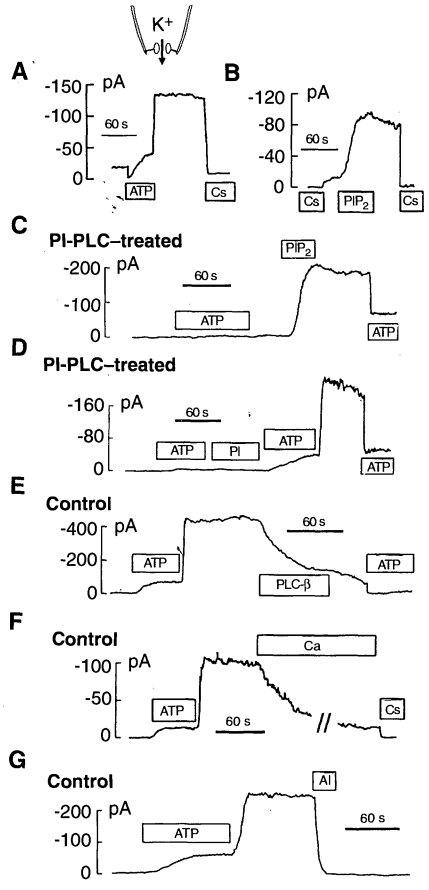

Fig. 4. Stimulation of inward K_{ATP} current by ATP and PIP_2. (**A**) Effect of ATP. (**B**) Effect of PIP_2 (50 μM PIP_2), applied for 60 s. A cytoplasmic solution with 120 mM cesium completely blocked the potassium current. (**C**) Effects of ATP and PIP_2 on a patch pretreated with PI-PLC for 4 min. A nearly complete lack of potassium current was typical after 4 min for both control and treated patches. Application of 50 μM PIP_2 for 40 s activated a large current that was inhibited for the most part by 4 mM ATP. (**D**) Restoration of the stimulatory effect of ATP by PI on a PI-PLC–treated patch. ATP (2 mM) was applied for 1 min with no effect. Then PI (0.2 mM) was applied for 1 min with no effect. Thereafter, ATP (2 mM) activated a large K_{ATP} current. (**E**) Reversal of the stimulatory effect of ATP by application of PLC-β1. ATP (2 mM) was applied for 1 min and removed. After 80 s, PLC-β1 (0.2 mg ml^{-1}) was added, and the current decreased by about 80%. (**F**) Reversal of the stimulatory effect of ATP by high concentrations of cytoplasmic free Ca^{2+}. ATP (2 mM) was applied for 1 min and removed. After 80 s, free Ca^{2+} was increased from 0.5 to 10 μM, and the ATP effect reversed over 2 min. (**G**) Reversal of the stimulatory effect of ATP by aluminum (50 μM). ATP (2 mM) was applied for 90 s and removed. Current declined to baseline within 20 s on application of aluminum.

The stimulatory effect of ATP on potassium current was similar to the ATP effect on Na^+,Ca^{2+} exchange current. The time courses and dependence on ATP concentrations were similar; the stimulated current was potently blocked by pentalysine (5 μM), which binds to negatively charged lipids (*3*); hydrolyzable ATP was required; several protein kinase inhibitors had no effect; and potassium currents were highly activated by 50 μM PIP_2 in the absence of ATP (Fig. 4B).

Treatment of patches with PI-PLC reduced the stimulatory effect of ATP by 97%, measured as the difference between current magnitudes before and after ATP ($P < 0.001$; six treated patches, eight control patches) (Fig. 4C). PIP_2 (50 μM) strongly activated the potassium current after depletion of PI in the membrane. When 4 mM ATP was then applied, the current was largely inhibited (*28*). An ATP effect was restored in PI-PLC–treated patches by application of PI vesicles (Fig. 4D). The stimulatory effect of ATP was reversed by PLC-β1 (Fig. 4E), and PLC-β1 had no effect in the absence of cytoplasmic Ca^{2+} (*12*). A high concentration of free Ca^{2+} (10 μM) reversed the ATP effect over 3 min (Fig. 4F). The stimulatory effect of ATP was also reversed in seconds by aluminum (50 μM) with 10 mM EGTA. Thus, all criteria implicating PIP_2 in the stimulatory effect of ATP on the Na^+,Ca^{2+} exchange current also apply to the K_{ATP} potassium current.

Our results demonstrate that PIP_2, generated by endogenous lipid kinases in membrane patches, potently activates the cardiac Na^+,Ca^{2+} exchanger and K_{ATP} channels. Possible targets of PIP_2 include membrane-associated cytoskeletal elements (*29*), which may interact with calcium (*30*) and K_{ATP} (*31*) channels. Global changes of actin polymerization probably do not occur in giant patches, because cytochalesin D and other agents that disrupt the cytoskeleton do not affect the ATP responses (*32*). The plasmalemmmal Ca^{2+} pump (*33*) and phospholipase D (*34*) may be directly regulated by PIP_2, and the cardiac Na^+,Ca^{2+} exchanger has a positively charged, cytoplasmic regulatory domain (*35*), which is a potential PIP_2 binding site (*36*). Possible mediators of the PIP_2 effects on K_{ATP} channels include G proteins (*37*) and sulfonylurea receptors (*38*). In the heart, the regulation via cyclic adenosine monophosphate of Ca^{2+} channels and sarcoplasmic reticulum Ca^{2+} pumps optimizes the frequency, speed, and force of contraction (*39*). Regulation PIP_2 of Na^+,Ca^{2+} exchange and potassium channels that do not participate in pacemaking might modulate only the force of contraction. The PIP_2 sensitivity of Na^+,Ca^{2+} exchange and K_{ATP} channels may be an important linkage of PI signaling pathways to surface membrane ion transporters and channels.

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9. Giant patches were formed and excised from guinea pig myocytes as described (*4, 8*). All recordings were at 37°C with a holding potential of 0 mV. For the Na^+,Ca^{2+} exchange current, the extracellular (pipette) solution contained: 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 100 mM *N*-methyl-D-glucamine (NMG)–Cl, 20 mM CsCl, 20 mM tetraethylammonium (TEA)–MES, 20 mM NaCl, 10 mM Hepes (pH 7.0 with NMG), and 0.2 mM ouabain. The cytoplasmic solution contained 20 mM CsCl, 20 mM TEA, 10 mM EGTA, 0.5 mM $MgCl_2$, 6 mM $CaCl_2$ for 0.5 μM free calcium, 20 mM Hepes (pH 7 with NMG), and either 90 mM NaCl or 90 mM CsCl. For the potassium current, the extracellular solution contained 120 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA, 10 mM Hepes (pH 7.0 with KOH), and 0.2 mM ouabain. The cytoplasmic solution contained 120 mM NMG, 20 mM KCl, 10 mM EGTA, 0.5 mM $MgCl_2$, 20 mM Hepes, 6 mM $CaCl_2$ for 0.5 μM free calcium, and NMG to set the pH to 7.0. ATP was added as Mg-ATP. PIP_2 was from Calbiochem, and all other phospholipids were from Avanti Polar Lipids (Alabaster, AL). PI-PLC (from *Bacillus cereus*) was from Calbiochem.
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11. Exchange current was recorded in 22 excised patch-

es, 7 of which disrupted before completion of the experiment. Results from the nine stable control patches and six stable PI-PLC–treated patches were compared by Student's t tests. The average peak current after 4 min of recording was 67 ± 7 pA (SEM) in control patches and 51 ± 9 pA in PI-PLC treated patches. The difference was not significant ($P > 0.1$). During application of ATP, exchange current increased by 4.7 ± 0.9 times in control patches to 88 ± 14 pA and by 0.19 ± 0.05 times in treated patches to 14 ± 2 pA. The differences were highly significant ($P < 0.001$).
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15. With continuous application of 10 μM free Ca^{2+}, ATP effects were small because the exchanger was more highly activated by Ca^{2+} (*14*). Reversal took place with a time constant of about 30 s, and guanosine 5′-*O*-(3′-triotriphosphate) (0.2 mM) did not affect this time course. With >20 μM free Ca^{2+}, the stimulatory effects of ATP were small and transitory, and subsequent effects of ATP with 0.5 μM free Ca^{2+} were small or absent. ATP effects were restored by PI.
16. Cardiac membranes were prepared by homogenization on ice of 2 g of isolated myocytes in 1 ml of solution containing 20 mM KCl, 10 mM Hepes (pH 7.0), 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μM leupeptin. Nuclei and cell debris were removed by centrifugation at 400*g* for 5 min at 4°C. The membrane fraction was recovered after centrifugation at 150,000*g* for 30 min at 4°C and resuspended at 8 milligrams of protein per milliliter in a solution containing 20 mM KCl, 20 mM Hepes (pH 7), and 1 mM EGTA. The PLC assay was done as described (*13*), except that sonicated vesicles containing 250 μM phosphatidylethanolamine and 50 μM (inositol-2-3H)-PIP_2were used, and the assay solution contained 20 mM Hepes (pH 7), 100 mM NaCl, 40 mM KCl, 10 mM EGTA, 0.5 mM $MgCl_2$, 20 mM CsCl, 20 mM TEA-Cl, and the indicated concentrations of free Ca^{2+}.
17. Fast reversal was blocked by EDTA (0.5 mM), fluoride (0.2 mM), 5 mM phosphate, and 5′-adenylimidodiphosphate (2 mM), which all bind polyvalent cations (*18, 19*).
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21. With 50 μM PIP_2 and 10 mM EGTA in the assay buffer, aluminum (50 μM) inhibited PLC-β1 and cardiac membrane PLC activities by 99% with 0.5 μM free Ca^{2+} and by 60% with 20 μM free Ca^{2+}. Aluminum (50 μM) had no effect on Ca^{2+}-activated PI hydrolysis in our assays, but inhibition has been reported for higher free aluminum concentrations, probably by aluminum binding to PI (*20*). As was consistent with a PI-aluminum interaction in cardiac patches, high concentrations of aluminum (100 to 500 μM with 10 mM EGTA) slowed development of the ATP effect and decreased its magnitude (*12*).
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28. In giant patches, ATP inhibited the potassium current with an inhibition constant (K_i) of 0.5 to 2 mM, which is a lower affinity than in most recordings in small patches. Usually, a component of current clearly could not be inhibited by ATP. Because current-voltage relations showed inward rectification, it is likely that inwardly rectifying potassium channels other than K_{ATP} channels are also activated by PIP_2.
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Coupling of the RAS-MAPK Pathway to Gene Activation by RSK2, a Growth Factor–Regulated CREB Kinase

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A signaling pathway has been elucidated whereby growth factors activate the transcription factor cyclic adenosine monophosphate response element–binding protein (CREB), a critical regulator of immediate early gene transcription. Growth factor–stimulated CREB phosphorylation at serine-133 is mediated by the RAS–mitogen-activated protein kinase (MAPK) pathway. MAPK activates CREB kinase, which in turn phosphorylates and activates CREB. Purification, sequencing, and biochemical characterization of CREB kinase revealed that it is identical to a member of the $pp90^{RSK}$ family, RSK2. RSK2 was shown to mediate growth factor induction of CREB serine-133 phosphorylation both in vitro and in vivo. These findings identify a cellular function for RSK2 and define a mechanism whereby growth factor signals mediated by RAS and MAPK are transmitted to the nucleus to activate gene expression.

Growth factors transmit signals from the plasma membrane to the nucleus to activate programs of immediate early gene (IEG) transcription that are critical for cell proliferation and differentiation (*1*). Growth factor binding to receptor tyrosine kinases produces stimulation of a RAS-dependent kinase cascade that includes the sequential phosphorylation and activation of RAF, MEK (MAPK or extracellular signal–regulated kinase kinase), mitogen-activated protein kinase (MAPK), and ribosomal protein S6 kinase ($pp90^{rsk}$ or RSK) (*2*). Once activated, MAPK translocates to the nucleus where it phosphorylates and activates the transcription factor ELK-1. ELK-1 binds,

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