

A Specific Role of Phosphatidylinositol 3-Kinase γ : A Regulation of Autonomic Ca^{2+} Oscillations in Cardiac Cells

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Abstract. Purinergic stimulation of cardiomyocytes turns on a Src family tyrosine kinase-dependent pathway that stimulates PLC γ and generates IP $_3$, a breakdown product of phosphatidylinositol 4,5-bisphosphate (PIP $_2$). This signaling pathway closely regulates cardiac cell autonomic activity (i.e., spontaneous cell Ca^{2+} spiking). PIP $_2$ is phosphorylated on 3' by phosphoinositide 3-kinases (PI3Ks) that belong to a broad family of kinase isoforms. The product of PI3K, phosphatidylinositol 3,4,5-trisphosphate, regulates activity of PLC γ . PI3Ks have emerged as crucial regulators of many cell functions including cell division, cell migration, cell secretion, and, via PLC γ , Ca^{2+} homeostasis. However, although PI3K α and β have been shown to mediate specific cell functions in nonhematopoietic cells, such a role has not been found yet for PI3K γ .

We report that neonatal rat cardiac cells in culture express PI3K α , β , and γ . The purinergic agonist predominantly activates PI3K γ . Both wortmannin and LY294002 prevent tyrosine phosphorylation, and membrane translocation of PLC γ as well as IP $_3$ generation in ATP-stimulated cells. Furthermore, an anti-PI3K γ , but not an anti-PI3K β , injected in the cells prevents the

effect of ATP on cell Ca^{2+} spiking. A dominant negative mutant of PI3K γ transfected in the cells also exerts the same action. The effect of ATP was observed on spontaneous Ca^{2+} spiking of wild-type but not of PI3K $\gamma^{-/-}$ embryonic stem cell-derived cardiomyocytes. ATP activates the Btk tyrosine kinase, Tec, and induces its association with PLC γ . A dominant negative mutant of Tec blocks the purinergic effect on cell Ca^{2+} spiking. Tec is translocated to the T-tubes upon ATP stimulation of cardiac cells. Both an anti-PI3K γ antibody and a dominant negative mutant of PI3K γ injected or transfected into cells prevent the latter event.

We conclude that PI3K γ activation is a crucial step in the purinergic regulation of cardiac cell spontaneous Ca^{2+} spiking. Our data further suggest that Tec works in concert with a Src family kinase and PI3K γ to fully activate PLC γ in ATP-stimulated cardiac cells. This cluster of kinases provides the cardiomyocyte with a tight regulation of IP $_3$ generation and thus cardiac autonomic activity.

Key words: phosphoinositide kinase • calcium • tyrosine kinase • heart • automaticity

Introduction

Activation of phosphoinositide 3-kinase (PI3K)¹ leads to cell survival and proliferation, cell motility and secretion, cytoskeletal rearrangement, cell migration, and Ca^{2+} signaling (Rameh et al., 1998; Scharenberg and Kinet, 1998;

Rameh and Cantley, 1999). These lipid/protein kinases generate phosphatidylinositol 3,4,5-trisphosphate (PIP $_3$) after 3' phosphorylation of phosphatidylinositol 4,5-diphosphate (PIP $_2$). PI3Ks belong to a broad family of enzymes, grouped in class I, II, and III based on their sequence substrate preference (PI, PIP, PI[3,4]P $_2$, or PI[3,5]P $_2$). The most studied heterodimeric class I PI3K is composed of two subgroups. PI3K of class IA is a heterodimer consisting of a regulatory subunit (p85 α , β or p55) associated with a catalytic subunit (p110 α , β , or δ) (Fruman et al., 1998). The catalytic subunit p110 γ , which belongs to class IB, does not bind to p85 but to a p101 adapter (Stoyanov et al., 1995; Kurosu et al., 1997). p101 is

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¹Abbreviations used in this paper: CCD, charge-coupled device; ES, embryonic stem; GFP, green fluorescent protein; GST, glutathione S-transferase; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PIP $_2$, phosphatidylinositol 4,5-diphosphate; PIP $_3$, phosphatidylinositol 3,4,5-trisphosphate.

required for PI3K γ to be efficiently activated by $\beta\gamma$ subunits of trimeric G proteins (Leopoldt et al., 1998). Furthermore, p101 confers substrate specificity of PI(4,5)P₂ for the p110 γ (Maier et al., 1999).

Whether PI3K isoforms mediate specific cell function is just an emerging issue. p110 α was first shown to mediate the effects of some growth factors on DNA synthesis (Roche et al., 1994). A specific role of p110 β in mitotic signals has been then uncovered in fibroblasts (Roche et al., 1998). A differential role of p110 α in cell proliferation and survival and of p110 β and/or p110 δ in migration has been then found in macrophages (Vanhaesebroeck et al., 1999) and in human colon carcinoma cells (Benistant et al., 2000). In breast cancer cells, PI3K α is specifically required for EGF-induced actin rearrangement. (Hill et al., 2000). Although PI3K γ is known to link serpentine receptors to mitogen-activated protein kinase (Duckworth and Cantley, 1997; Lopez-Illasaca et al., 1997) and c-Jun NH₂-terminal kinase (Lopez-Illasaca et al., 1998), two major cell signaling pathways a specific cell function has not yet been assigned to this isoform. Recently, gene targeted mice lacking the p110 γ were generated by several groups. As expected from expression of PI3K γ in hematopoietic cells, the mice featured defects in thymocyte development, T cell activation and functions of neutrophils (Hirsch et al., 2000; Sasaki et al., 2000a). In addition to blood cells, Northern blot analysis revealed that mRNAs of PI3K γ were abundant in heart, skeletal muscle, liver, and pancreas (Stoyanov et al., 1995). However, defects in functions of these tissues were not sought in PI3K γ ^{-/-} mice.

Although PI3Ks phosphorylate PIP₂, PLCs hydrolyze the phosphoinositides and produce IP₃ and diacylglycerol, the endogenous activator of protein kinase C. Activation of a plethora of cell membrane receptors produces an elevation of intracellular IP₃, a trigger and/or regulator of Ca²⁺ oscillations or Ca²⁺ waves (Berridge, 1995; Clapham, 1995). PLCs are expressed in most cell types as several isoforms including PLC β , δ , and γ . PLC γ is activated by tyrosine kinase-dependent pathways (Rhee and Bae, 1997). There is compelling evidence that PLC γ activity is also increased by PIP₃ both directly through binding to the pleckstrin homology (PH) (Falasca et al., 1998) and the SH2 domains (Rameh et al., 1998) of PLC γ and, indirectly, after activation of the tyrosine Bruton's kinase Btk (Li et al., 1997). PIP₃ binding to the PH domain targets PLC γ to the membrane. Thus, PI3Ks are at the crossroad of a tyrosine kinase-mediated Ca²⁺ signaling pathway.

We recently uncovered in cardiac cells a tyrosine kinase-dependent Ca²⁺ signaling pathway. Purinergic stimulation of rat cardiomyocytes leads to PLC γ activation (Puceat and Vassort, 1996) and in turn modulates spontaneous intracellular Ca²⁺ oscillations. IP₃ generated by binding of ATP to extracellular purinergic receptors induces a mitochondrial Ca²⁺ loading, which indirectly depletes Ca²⁺ from the sarcoplasmic reticulum. This in turn slows or stops spontaneous Ca²⁺ oscillations in neonatal rat cardiomyocytes (Jaconi et al., 2000). In cardiac cells, little is known about expression, function, and signaling roads of PI3Ks. To our knowledge, two studies reported an involvement of p85 in leukemia inhibitory factor and angiotensin signaling pathways in cardiomyocytes (Rabkin et al., 1997; Oh et al., 1998). More recently, activation of a G $\beta\gamma$ -depen-

dent PI3K was shown in cardiac hypertrophy (Naga Prasad et al., 2000). A gain of function of a mutant of p110 α increases heart size in transgenic mice (Shioi et al., 2000).

Together, these findings prompted us to investigate the involvement of PI3Ks in the PLC γ -regulated Ca²⁺ signaling pathway in cardiomyocytes. Herein, using a pharmacological approach and intracellular microinjection of specific and blocking anti-PI3Ks antibodies, as well as embryonic stem (ES)-derived PI3K γ ^{-/-} cardiomyocytes, we provide evidence for a specific role of PI3K γ in cardiac cell autonomic activity. Also, we report that Tec, a Btk family tyrosine kinase, acts in concert with a Src family kinase to support the cardiac function of PI3K γ .

Materials and Methods

Cell Isolation and Culture

Cardiomyocytes were isolated from 2–3-d-old neonatal rats according to Puceat et al. (1994) and kept in culture for 5 d.

PI3K γ ^{-/-}, PI3K γ ^{+/-}, and wild-type ES cells were cultured as previously described (Meyer et al., 2000; Sasaki et al., 2000b). Differentiation into cardiac cells was performed within embryoid bodies as previously described (Meyer et al., 2000).

Cell Transfection

Cells were transfected using fugene (Boehringer) or effectene (QIAGEN) according to the manufacturer's instructions using a fugene/DNA ratio (vol/wt) of 3:1 and a effectene/DNA ratio of 5:1, respectively. Cells were cotransfected with a dominant negative mutant of Tec (kinase-dead mutant; Mano et al., 1995) or a dominant negative mutant of PI3K γ (PI3K γ K799R) and the blue or red fluorescent protein (pEBFP or pDsRed1-N1; CLONTECH Laboratories) to detect the transfected cells before Ca²⁺ measurement.

Tec Translocation

Cells were transfected with a TecGFP plasmid (Tec cDNA inserted in the EcoRI site of pEGFP-N1; CLONTECH Laboratories) alone or with a dominant negative mutant of PI3K γ , using effectene as described above. pEGFP-N1 was transfected as a negative control. After 36 h, cells were stimulated for 1 min by 20 μ M ATP, washed with cold PBS, and then fixed for 10 min with 3% paraformaldehyde. After washing and mounting of coverslips in mowiol, green fluorescent protein (GFP) fluorescence was detected with a Micromax 1300YHS charge-coupled device (CCD) camera mounted on a Leica microscope.

Cell Immunostaining and Image Analysis

Cardiomyocytes were fixed with 3% paraformaldehyde and permeabilized with 0.5% Triton X-100. Immunostaining was performed as previously described using the anti-Tec antibody and a secondary TRITC-conjugated anti-rabbit Ig antibody (Puceat et al., 1995). The images were acquired with a Micromax 1300YHS CCD camera. Objectives were set on a piezo-electric device. The images were acquired in the z direction using a step of the piezo-electric system of 0.2 μ m and stored as single TIFF images or as a volume file ("stack" of z-sections images) using the Metamorph software (Universal Imaging Corp.). To improve the resolution and the signal/noise ratio of the volume data, digital deconvolution was applied to stacks of images. The images were restored using the Huygens software (Huygens v2.2.1; Scientific Volume Imaging) and visualized using Imaris (Bitplane). All calculations were performed using an Octane workstation (Silicon Graphics).

Cell Fractionation

Cardiomyocytes were washed in ice-cooled PBS for centrifugation at 1,000 g for 4 min at 4°C. The pellet was thoroughly resuspended in hypotonic lysis buffer, containing 10 mM Tris, 10 mM Na₄P₄O₇, 1 mM EDTA, 1 mM MgCl₂, pH 8, 10 mM NaF supplemented with 0.1 mM PMSF, and homogenized. After centrifugation, the pellet was resuspended with NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0) supple-

mented with 1% Nonidet-P40, 50 mM NaF, 1 mM Na₃VO₄, 0.1 mM PMSF, and 10 µg/ml leupeptin and kept on ice for 15 min. Myofilaments were removed by centrifugation at 12,000 g for 20 min at 4°C, and the resulting supernatant (crude membrane fraction) was mixed with 4× Laemmli buffer and was boiled 1 min before Western analysis.

Immunoprecipitation of Proteins and Western Blotting

Whole cell lysates were subjected to immunoprecipitation as described previously (Puceat and Vassort, 1996). The samples were run in 7.5% SDS-PAGE and electrophoretically transferred to nitrocellulose filter. The blots were treated as described previously (Puceat et al., 1995) and probed with the antibody recognizing the protein of interest and a secondary peroxidase-conjugated antibody. The proteins were revealed using enhanced chemiluminescence.

IP₃ Measurements

Intracellular IP₃ was measured with a radiobinding assay (NEN Life Science Products) as described previously (Puceat and Vassort, 1996)

Measurement of Tyrosine Kinase Activities

Kinase activities were measured as described previously (Puceat et al., 1998b). In brief, cardiac cell lysates prepared from control or ATP-stimulated cells were subjected to immunoprecipitation using an anti-Tec antibody. The autophosphorylation assay was carried out in Hepes 50 mM, MnCl₂ 10 mM, 1 mM DTT, 5 µCi [γ -³²P]ATP, and 10 µM ATP for 15 min at 30°C. The kinase reactions were stopped by adding Laemmli buffer and heating at 100°C for 1 min. The complex was run in SDS-PAGE. After staining and destaining, the gel was dried and exposed to autoradiography films. Tec autophosphorylation was quantified using SCION IMAGE software. In some experiments, the Tec immunocomplexes were split in two fractions. Half of the immunocomplex was used for kinase activity and half was subjected to Western blotting. The blot was probed with an anti-PLC γ or anti-PY antibody.

PI3K Activity

PI3K were immunoprecipitated from control or ATP-stimulated cells using specific antibodies. PI3K activity was measured using PI as a substrate (Roche et al., 1998). Anti-PI3K α and - β have been previously characterized (Roche et al., 1998). The polyclonal anti-PI3K γ antibody used in microinjection and immunoprecipitation experiments was raised against the amino acids 742–756 of p110 γ within the G $\beta\gamma$ and p101 binding domains (Stoyanov et al., 1995). The monoclonal anti-PI3K γ antibody, used in Western blot, binds to the NH₂-terminal stretch (amino acids 87–302 including the predicted PH domain) of p110 γ (Leopoldt et al., 1998). The blocking activity of the polyclonal anti-PI3K γ antibody was tested *in vitro*. A recombinant p110 γ was expressed as a glutathione *S*-transferase (GST)–p110 γ fusion protein in Sf9 cells infected with a baculovirus and purified (Rubio et al., 1999). Activity was then measured *in vitro* as described above. p110 γ was decreased by 40% in two experiments in which the specific polyclonal anti-p110 γ antibody was added to the kinase assay. Anti-p110 α or -p110 β did not affect p110 γ activity (see Fig. 5 A, inset).

Microspectrofluorimetry and Imaging of Cell Ca²⁺

Cells or embryoid bodies at days 9 and 10 were loaded with 3 or 10 µM fluo3/AM, respectively, for 20 min and then transferred to the stage of an epifluorescence microscope and superfused with a medium containing 20 mM Hepes, 117 mM NaCl, 5.7 mM KCl, 1.2 mM NaH₂PO₄, 4.4 mM NaHCO₃, 1.7 mM MgCl₂, 1.8 mM CaCl₂. The field was illuminated at 485 ± 22 nm with a Xenon lamp. Fluo3 emission fluorescence was recorded through a dichroic mirror (cutoff 510 nm) and a long pass emission filter (cutoff 520 nm) as described previously (Jaconi et al., 2000). Expression of the blue or red fluorescent protein was detected using a 360- or 515-nm excitation filter and a dichroic mirror, respectively (cutoff 405 or 590 nm). The fluorescence or images were recorded at 530 nm or with a 590-nm-long pass filter, respectively, using a photomultiplier tube coupled to a diaphot (Nikon) microscope or a CCD camera (Hamamatsu) and digitized on line by a computer (Metamorph software; Universal Imaging Corp.). To plot the line scan graphs, the first image was subtracted from the other ones (F – F₀) and divided by the first one to take into account fluo3 inhomogeneity within the cell. To calculate the frequency of Ca²⁺ spikes in embryoid bodies, a region of interest was selected within a beating area,

and the average intensity of pixels was plotted as a function of time (Metamorph software). The experiments were performed at 35°C ± 2°C.

Microinjection of Neonatal Rat Cardiomyocytes

Microinjection of neonatal rat cardiac cells was performed according to Shubeita et al. (1992). The pipette concentration of antibodies was 1 mg/ml in 150 mM KCl, 0.025 mM EGTA, 1 mM Pipes, pH 7.2. The polyclonal anti-PI3Ks antibodies (Stoyanov et al., 1995; Roche et al., 1998) were affinity purified. In the experiments of microinjection of TecGFP transfected cells, cells were coinjected with PI3K antibodies and rhodamine-conjugated dextran to identify microinjected cells.

Results

A PI3K Activity Is Required in ATP-induced Activation of PLC γ

First, we tested the effects of PI3K inhibitors on purinergic activation of PLC γ . PLC γ is phosphorylated and then translocated to the plasma membrane after ATP stimulation of adult rat cardiomyocytes (Puceat and Vassort, 1996). Short purinergic stimulation of neonatal rat cardiomyocytes plated at high density increased membrane-associated immunoreactivity of PLC γ , as previously reported in adult rat ventricular myocytes (Puceat and Vassort, 1996). In cardiomyocytes pretreated with either wortmannin, at a concentration required to inhibit the $\beta\gamma$ -regulated PI3K (0.5 µM) (Stephens et al., 1994) or LY 294002 (25 µM), the purinergic effect on PLC γ membrane translocation was fully abolished (Fig. 1 A). PI3K inhibitors used under the same experimental conditions as the ones described above, prevented PLC γ phosphorylation, as detected after immunoprecipitation of the lipase and Western blotting with an antiphosphotyrosine antibody (Fig. 1 B). We consistently observed that the phosphorylation level of PLC γ was below the control value in wortmannin- or LY294002-treated cells. Lower concentrations of wortmannin (0.1 µM) or LY294002 (10 µM) also partially decreased ATP-induced PLC γ phosphorylation and membrane translocation (data not shown).

We then addressed whether PLC γ activity was also inhibited by a PI3K inhibitor. Neonatal rat cardiomyocytes stimulated with ATP for 1 min featured a twofold increase in intracellular IP₃ content. Cell incubation with LY294002 fully abolished this rise and even decreased basal content of IP₃ (Fig. 1 C). This finding is consistent with inhibition of both phosphorylation and membrane translocation of PLC γ by PI3K inhibitors.

PI3K Activity Underlies the IP₃-mediated Purinergic Regulation of Cardiac Automatic Activity

IP₃, generated by activation of purinergic P2Y receptors, is a crucial regulator of rhythmic Ca²⁺ spiking in neonatal rat cardiomyocytes plated at high density. After an intracellular Ca²⁺ release, IP₃ slows or stops cell spontaneous Ca²⁺ oscillations (Jaconi et al., 2000). We tested the effect of PI3K inhibitors on purinergic effect in spontaneous Ca²⁺ spiking cells. Extracellular ATP, acting through P2Y receptors slowed the rate of Ca²⁺ spiking of fluo3-loaded cardiomyocytes after an intracellular Ca²⁺ release, revealed by a transient rise in diastolic Ca²⁺ (Fig. 2 A), as described previously (Jaconi et al., 2000). In contrast, the purinergic agonist had no effect or accelerated this rate in cardiomyocytes treated with wortmannin or LY294002

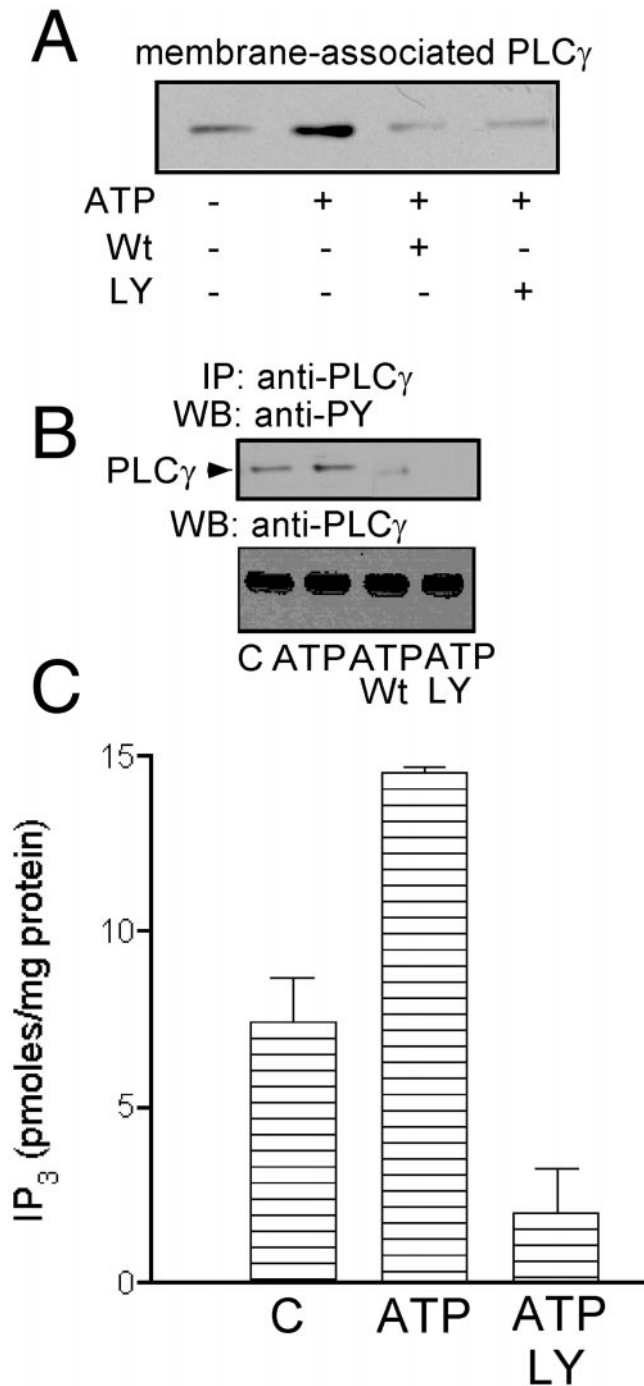


Figure 1. PI3K is required for ATP-induced IP₃ generation. Cardiomyocytes were stimulated with 20 μ M ATP in the absence or presence of wortmannin (Wt) or LY294002 (LY). After stimulation, cells were subfractionated in cytosolic and membrane fractions (A). Membrane proteins were subjected to Western blot analysis using an anti-PLC γ antibody. (B) PLC γ was immunoprecipitated, and the immunocomplex was analyzed by Western blot using an anti-phosphotyrosine or an anti-PLC γ antibody. (C) The amount of IP₃ was assayed in a protein-free cell extract. The figure is representative of at least three experiments performed on three separate cell cultures. WB, Western blotting.

(Fig. 2, B and C). Acceleration of the rate and sustained increase in diastolic Ca²⁺ in the presence of ATP can be attributed to a transient membrane depolarization after activation of the ATP-gated ion channel through P2X re-

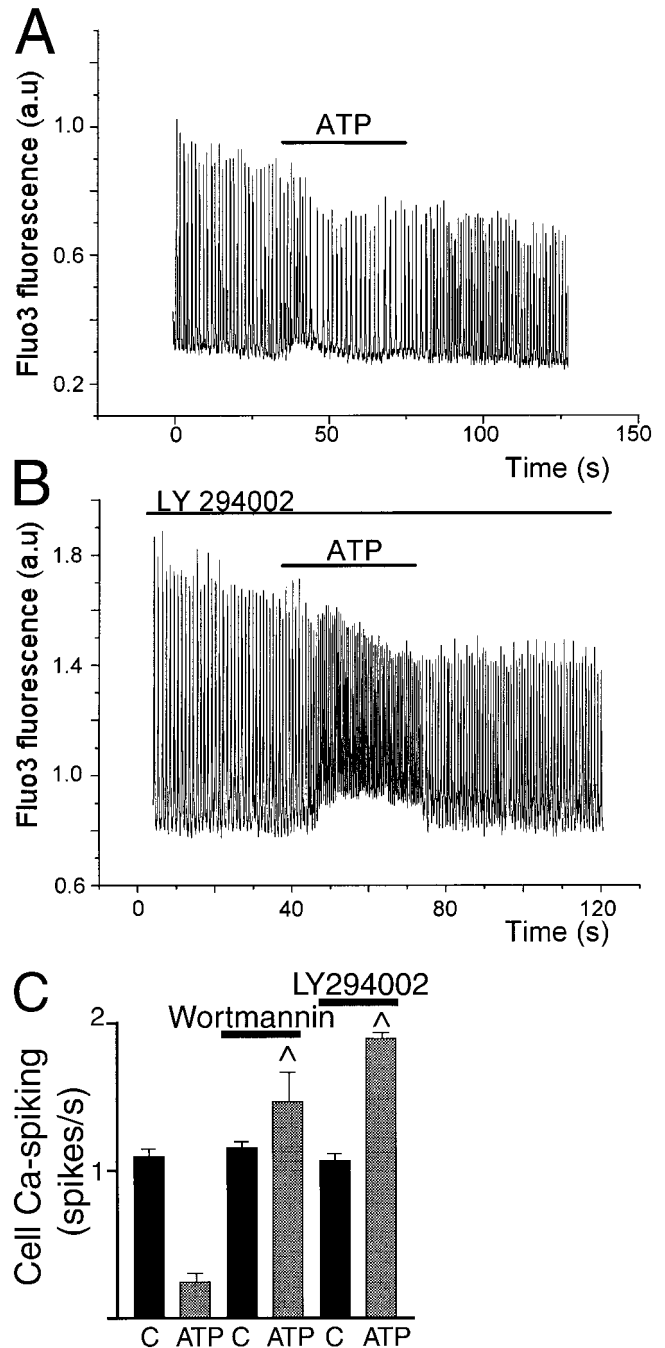


Figure 2. PI3K is required for ATP-induced slowing of cell Ca²⁺ spiking rate. A fluo3-loaded cell was superfused in the absence (A) or presence (B) of LY294002 with 20 μ M ATP. Fluo3 fluorescence was recorded every 30 ms with a photomultiplier. The figure is representative of 10 similar experiments performed using two different cell cultures. Data are compared in the bar graph shown in (C). \wedge Significantly increased. ($p \leq 0.01$). ATP, ATP-stimulated cells; C, control.

ceptors, an effect that is unmasked when IP₃ generation by P2Y receptors is blocked (Jaconi et al., 2000). Wortmannin and LY294002 added alone did not affect the rate of Ca²⁺ oscillations (Fig. 2 C).

Tec Is Involved in the PLC γ Signaling Pathway

We showed that Fyn associates with PLC γ in ATP-stimulated cells (Puecat and Vassort, 1996). In hematopoietic

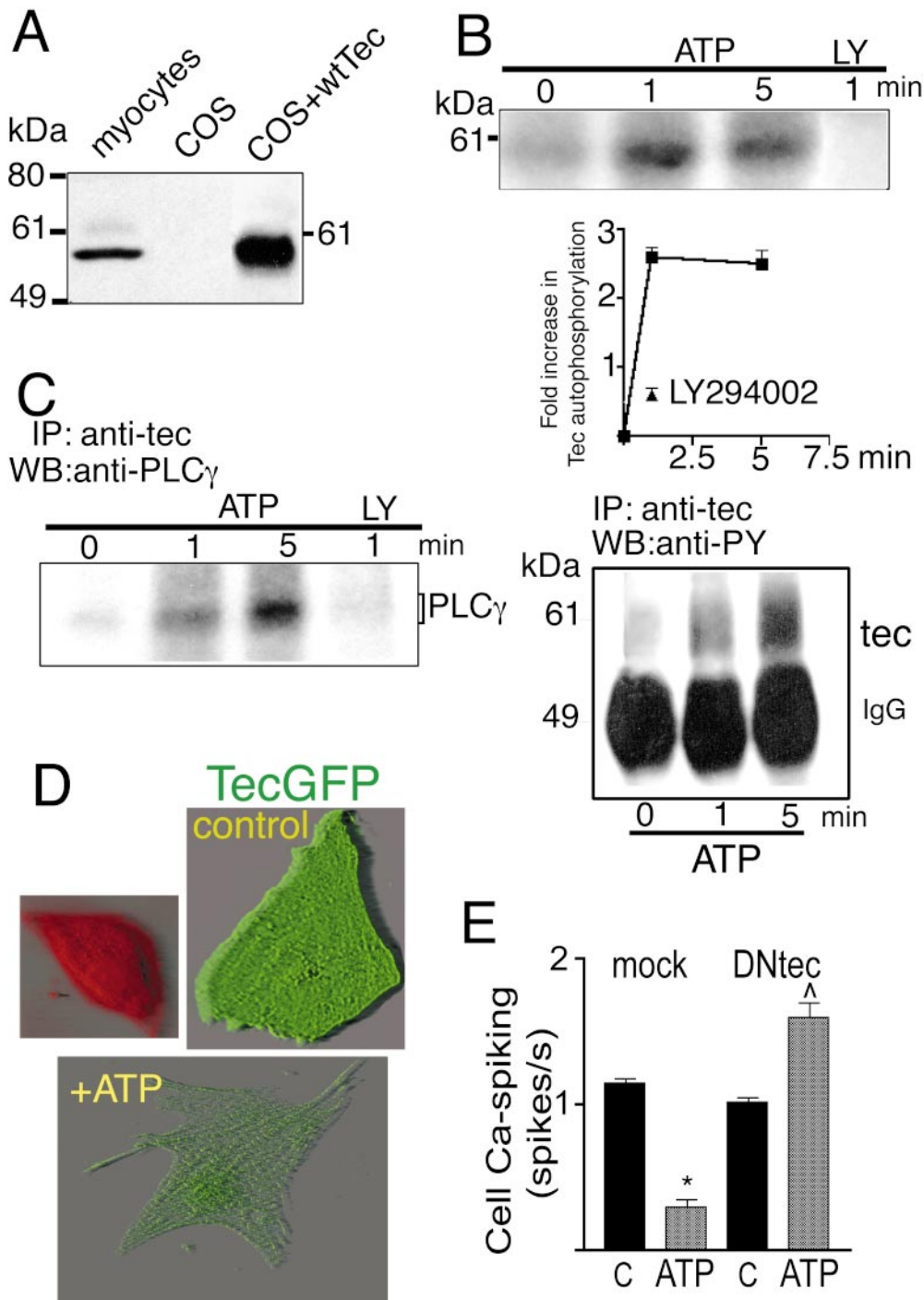


Figure 3. Tec is involved in ATP-induced slowing of cell Ca^{2+} spiking rate. (A) Whole cell lysate proteins from cardiac cells, mock Cos, or Cos cells transfected with wild-type Tec (from left to right lane), were subjected to Western blotting using an anti-Tec antibody. (B) After ATP stimulation, Tec was immunoprecipitated. The immunocomplex was used for the autophosphorylation assay using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of MnCl_2 and was analyzed by autoradiography. Cells were pretreated for 10 min with LY294002 before ATP stimulation (top). Data from three experiments are gathered in the line graph (middle). After Tec immunoprecipitation, a Western blot antiphosphotyrosine was performed to confirm the identity of the Tec amino acid phosphorylated after purinergic stimulation of cells (bottom). (C) After immunoprecipitation of Tec, the immunocomplex was subjected to Western blotting using an anti-PLC γ antibody to detect the presence of PLC γ . Similar experiments were repeated three times. (D) A TecGFP plasmid was transfected into cardiomyocytes. 36 h later, transfected cells were stimulated (ATP) or not (control) with 20 μM ATP. The inset shows the labeling of a cardiomyocyte by the anti-Tec antibody and a secondary TRITC-conjugated antibody. Specificity of the labeling was confirmed by a staining of cardiomyocytes overexpressing a wild-type Tec. The images were obtained after digital deconvolution (Huygens software) and visualized

using a shadow projection (Imaris software). In this series of experiments $85 \pm 5\%$ of cells featured a membrane staining of TecGFP (E), mock cells or cells transfected with a dominant negative (DN) mutant of Tec were loaded with fluo3 and superfused with ATP. Fluo3 fluorescence was recorded in a region of interest including the whole cell with a CCD camera. Two to three images/s were captured by a CCD camera. A similar result was obtained in 12 cells isolated from two separate cultures as shown in the bar graph. *Significantly decreased; ^significantly increased ($p \leq 0.01$). wt, Wild-type; ATP, ATP-stimulated cells; C, control.

cells, Btk family of tyrosine kinase was reported to be activated by PI3K in concert with Src family kinases (Li et al., 1997). PIP3, a product of PI3K, and Btk have been involved in PLC γ -mediated Ca^{2+} signaling (Scharenberg and Kinet, 1998). Therefore, we first sought for expression of tyrosine kinases of the Btk family in cardiac cells. Btk has a restricted expression in hematopoietic cells and was not found in cardiac myocytes (data not shown). A specific

anti-Tec antibody (Mano et al., 1995) detected in a cardiac cell lysate a 58-kD protein and one upper molecular mass variant (62 kD) of Tec, a member of the Btk family (Fig. 3 A), was previously found in T cells (Yang et al., 1999). To further characterize the Tec immunoreactive bands found in cardiac cell lysates, Cos cells, which lack Tec, were transfected with a plasmid-encoding wild-type Tec. Western blot analysis of a lysate from mock and transfected

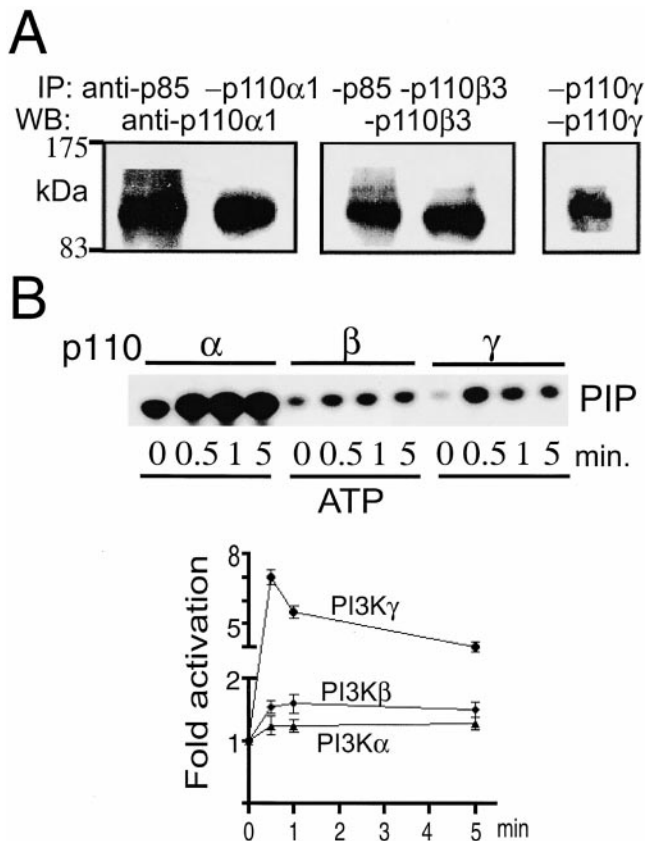


Figure 4. ATP specifically activates PI3K γ . (A) PI3K subunits p85, p110 α , - β and - γ were immunoprecipitated from a cardiac whole cell lysate and subjected to Western blot analysis using either an anti-p110 α 1, β 3, or γ antibody. The polyclonal anti-p110 γ antibody was used for the immunoprecipitation and the monoclonal anti-p110 γ antibody was used for Western blot. (B) Cells were stimulated for 0, 0.5, 1, or 5 min with 20 μ M ATP, and PI3 kinase activity was assayed after PI3K immunoprecipitation. The experiment was repeated at least three times on three separate cultures. Means \pm SEM of fold activation of PI3Ks are plotted as a function of the duration of purinergic stimulation of cells after scanning the autoradiographic films exposed to TLC plates (bottom).

cells revealed that wild-type Tec encodes a protein migrating with an apparent molecular weight of 58–60 kD, similar to the main Tec immunoreactive protein found in cardiac cells. Using both autophosphorylation of the kinase and antiphosphotyrosine Western blotting after Tec immunoprecipitation as an index of activation, we found that purinergic stimulation of cardiomyocytes induced within 1 min and for \geq 5 min, a two- to threefold increase in Tec autophosphorylation as shown by a labeled band migrating at 60 kD (Fig. 3 B). This activation was accompanied with an association of the kinase with PLC γ (Fig. 3 C), which suggested that Tec may be translocated to the cell membrane upon cell stimulation with ATP. Tyrosine phosphorylation of Tec was detected in the Tec immunocomplex in a Western blot using an antiphosphotyrosine antibody (Fig. 3 B). Both Tec autophosphorylation and association with PLC γ were inhibited by cell treatment with the PI3K inhibitor LY2940002. (Fig. 3, B and C). To test whether Tec may be translocated to the membrane upon ATP cell stimulation, cardiomyocytes were transfected with a plas-

mid encoding a GFP-tagged Tec. TecGFP was homogeneously distributed throughout the cytosol and in the nucleus under resting conditions as the endogenous Tec was revealed by the anti-Tec antibody (Fig. 3 D, inset). Short ATP stimulation (1 min) of cells induced a translocation of TecGFP to the T-tubes (i.e., plasma membrane invaginations) of cardiac cells, as revealed by the regular rib-like GFP staining (Fig. 3 D). To investigate whether Tec may, like Btk, regulate PLC γ function and thus Ca²⁺ signaling, we tested the effect of expression of a dominant negative mutant of Tec lacking the kinase domain (Yamashita et al., 1998) on IP₃-regulated cell automatic Ca²⁺ spiking. In cells expressing the dominant negative mutant of Tec, ATP had no effect or increased the rate of Ca²⁺ spiking, whereas it stopped the spontaneous Ca²⁺ spikes in mock cells, a hallmark of IP₃ action (Fig. 3 E).

The Purinergic Agonist Specifically Activates p110 PI3K γ

Next, we investigated expression of PI3Ks isoforms in neonatal rat cardiac cells. Using specific antibodies directed against the adapter p85 or the catalytic subunits p110 α , - β , or - γ , the proteins were immunoprecipitated from a cardiac cell lysate and then immunoblotted with an anti-p110 antibody. p110 α , - β , and - γ are all expressed in cardiac cells (Fig. 4 A). We further addressed whether all PI3K isoforms were activated by purinergic stimulation of cardiac cells. Cells were stimulated for 0.5, 1, and 5 min, and the catalytic p110 subunit was immunoprecipitated from cell lysates. PI3K activities, assayed in the immunocomplexes, revealed that ATP mainly and rapidly increased p110 γ activity as revealed by Phosphorous-32 labeling of PIP. Maximal activation was reached within 0.5 min. p110 β activity was slightly but more sustainably increased in ATP-stimulated cells. Basal activity of PI3K α was high but barely affected by purinergic stimulation of cardiomyocytes (Fig. 4 B).

p110 γ but Not p110 β Is Required for IP₃-mediated Purinergic Effect on Cell Ca²⁺ Spiking and for Tec Membrane Translocation

PI3K inhibitors prevented ATP from slowing the rate of cell Ca²⁺ spiking. Both p110 β and p110 γ activities were significantly activated by ATP. We thus designed microinjection experiments to identify the PI3K isoform that underlies this effect. To reach such an aim, antibodies specific of PI3K isoforms were injected into rhythmic Ca²⁺ spiking cardiomyocytes, and the effect of ATP was further tested. In cells injected with an anti-p110 β antibody, ATP induced a transient release in intracellular Ca²⁺ and then slowed and stopped spontaneous cell Ca²⁺ spiking ($n = 9$ cells), as previously found in noninjected cells (Fig. 2) (Jaconi et al., 2000). In contrast, in cells injected with a specific and blocking (Fig. 5 A, inset) anti-p110 γ antibody ($n = 11$ cells), ATP did not induce an intracellular Ca²⁺ release and transiently accelerated spontaneous Ca²⁺ spiking (Fig. 5 A). Expression of a dominant negative mutant of PI3K γ (K399R) that lacks lipid kinase activity (Stoyanova et al., 1997) in the cells also significantly prevented the slowing effect of ATP on cell Ca²⁺ spiking (Fig. 5 A).

To further test the involvement of PI3K γ in the ATP effect on cell Ca²⁺ spiking, we used PI3K γ ^{-/-} ES cells to gen-

erate cardiomyocytes within embryoid bodies (Meyer et al., 2000). At day 9 of differentiation, 80% of PI3K $\gamma^{-/-}$, PI3K $\gamma^{+/-}$, or wild-type embryoid bodies ($n = 25$) featured several spontaneously beating areas, showing that ES cells differentiated into contractile cardiomyocytes (Meyer et al., 2000; Sasaki et al., 2000b). At this stage of differentiation (day 9), functional type I IP₃ receptors (Kolossoff et al., 1998) are already strongly expressed in cardiomyocytes and distributed mainly around the nucleus and in a network spreading from the nuclear area (data not shown), as found in neonatal myocytes (Jaconi et al., 2000). In contrast, ryanodine receptors are still poorly expressed in ES-derived cardiomyocytes (Meyer et al., 2000), a situation also similar to neonatal cardiomyocytes. Furthermore, spontaneous beating activity of embryoid bodies (i.e., automaticity) is closely dependent on intracellular Ca²⁺ (Viatchenko-Karpinski et al., 1999). In fluo3-loaded wild-type or PI3K $\gamma^{+/-}$ embryoid bodies, ATP significantly slowed down the spontaneous Ca²⁺ spiking of cardiomyocytes. This effect was not observed in PI3K $\gamma^{-/-}$ embryoid bodies (Fig. 5 B).

Also, we looked at the effects of anti-PI3K antibodies on TecGFP membrane translocation. In cells transfected with TecGFP and microinjected with an anti-PI3K β antibody, ATP still triggered a membrane translocation of Tec, as revealed by the regular labeling of T-tubes. By contrast, in cells microinjected with an anti-PI3K γ antibody, Tec remained in the cytosol after a 1-min purinergic stimulation (Fig. 5 C), as also seen in cells pretreated for 10 min with 20 μ M LY294002 (data not shown). In cells transfected with a dominant negative mutant of PI3K γ (K399R), ATP did not trigger Tec translocation to the membrane (Fig. 5 C).

Discussion

We found that cardiac cells express several PI3Ks of class I including PI3K γ and that activity of the latter, together with the tyrosine kinase Tec, is required to mediate the purinergic effect on autonomic Ca²⁺ spiking of neonatal rat cardiomyocytes.

Both inhibitors of PI3Ks, wortmannin and LY294002, used for a short time (10 min) at concentrations (0.1 or 0.5 and 20 μ M, respectively) that inhibit the G $\beta\gamma$ -regulated PI3K (Stephens et al., 1994) prevent both phosphorylation and translocation of PLC γ to the membrane of cardiac cells. Definite evidence in favor of a requirement of PI3K activity for PLC γ activation is brought by the IP₃ measurement assay. Indeed, PI3K inhibitors fully blocked ATP-induced IP₃ generation. Wortmannin also inhibits PI4K, a kinase required for the synthesis of PIP₂. A limitation of this substrate may decrease IP₃ formation. However, LY294002 also prevents IP₃ formation at a concentration far below the one that inhibits PI4K (Downing et al., 1996). Thus, we propose that PI3K is mandatory to turn on the PLC γ -dependent IP₃ pathway in purine-stimulated cells.

Previously, we showed that tyrosine phosphorylation is a key step for purinergic activation of PLC γ in cardiac cells (Puceat and Vassort, 1996). Our current findings indicate that PIP₃, the product of PI3K, is also necessary to activate the phospholipase. PIP₃ is likely to provide a membrane anchoring site for the PH domain of PLC γ (Falasca

et al., 1998). We found that LY294002 not only inhibits ATP-induced IP₃ production but also decreases the basal level (Fig. 1 C). Also, we observed such an effect of the drug on IP₃ generation in wortmannin-treated cells and on PLC γ membrane translocation and phosphorylation in both LY294002- and wortmannin-treated cells. This suggests that cardiac cells feature a tonic activation of PI3K as previously found in Cos cells (Wennstrom and Downward, 1999). This may be in part responsible for a high phosphoinositide turnover and in turn for the high masses of endogenous IP₃ in these cells (Fig. 1 C). In agreement with our findings on IP₃ generation, PI3Ks inhibitors prevent the effect of ATP on autonomic Ca²⁺ spiking, an effect mediated by IP₃ (Jaconi et al., 2000). Our present data demonstrate that activation of PI3K, together with PLC γ , is required for ATP-induced IP₃ formation and, in turn, regulation of Ca²⁺ spiking in primary culture of cardiomyocytes. This is in line with observations reported in the HepG2 cells in which PI3K mediates PDGF-induced intracellular Ca²⁺ release (Rameh et al., 1998).

Extracellular ATP predominantly activates PI3K γ and, to some but much less extent, PI3K β but only barely affects PI3K α whose basal activity is already high in cardiac cells (Fig. 3). The relative extent of purinergic activation of PI3K isoforms may be taken with caution. In vitro PI3Ks activity using a phosphoinositol as a substrate may be different from the in vivo situation in which membrane phospholipids are more complex. Substrate specificity of PI3K isoforms may also differ in vivo compared with the in vitro assays. It is interesting to note that purinergic activation of PI3K γ was not only much stronger but also more transient than the one of PI3K α and β . This suggests that PI3K β and α may be involved in more time lasting cell events such as cell survival or cell hypertrophy (Shioi et al., 2000), whereas PI3K γ mediates short-term regulation of cardiac autonomic activity.

However, we also found that microinjection of an anti-PI3K γ antibody into cardiomyocytes prevents the slowing effect of ATP on autonomic cell Ca²⁺ oscillations. In these microinjected cells, ATP increases the rate in cells in which the IP₃-dependent pathway was disrupted, as previously observed (Jaconi et al., 2000) (Fig. 2 B). Such an in vivo blocking effect of the anti-PI3K γ antibody could be expected. First, the specificity of the antibody has been recently confirmed by the absence of any cross-reactivity with other PI3Ks in PI3K $\gamma^{-/-}$ mice (Sasaki et al., 2000b). Second, this antibody partially blocks p110 γ activity in in vitro assay. Finally, the antibody recognizes the region of PI3K γ that binds both p101 and G $\beta\gamma$ (Leopoldt et al., 1998), which most likely accounts for the neutralizing in vivo effect. In contrast, in cardiomyocytes injected with an anti-PI3K β antibody, ATP still slows the rate of Ca²⁺ spiking after triggering an intracellular Ca²⁺ release, a hallmark of IP₃ action. This further demonstrates that PI3K γ is mandatory for ATP-induced PLC γ activation. In this study, we used a powerful approach of microinjection of PI3K antibodies, whose isoform specificity has been previously demonstrated (Stoyanov et al., 1995; Roche et al., 1998). This approach, already successful in cardiac cells (Puceat et al., 1998b), was first chosen herein because it is more reliable and more specific than the use of kinase dead mutants of p110PI3Ks. The latter may partially inter-

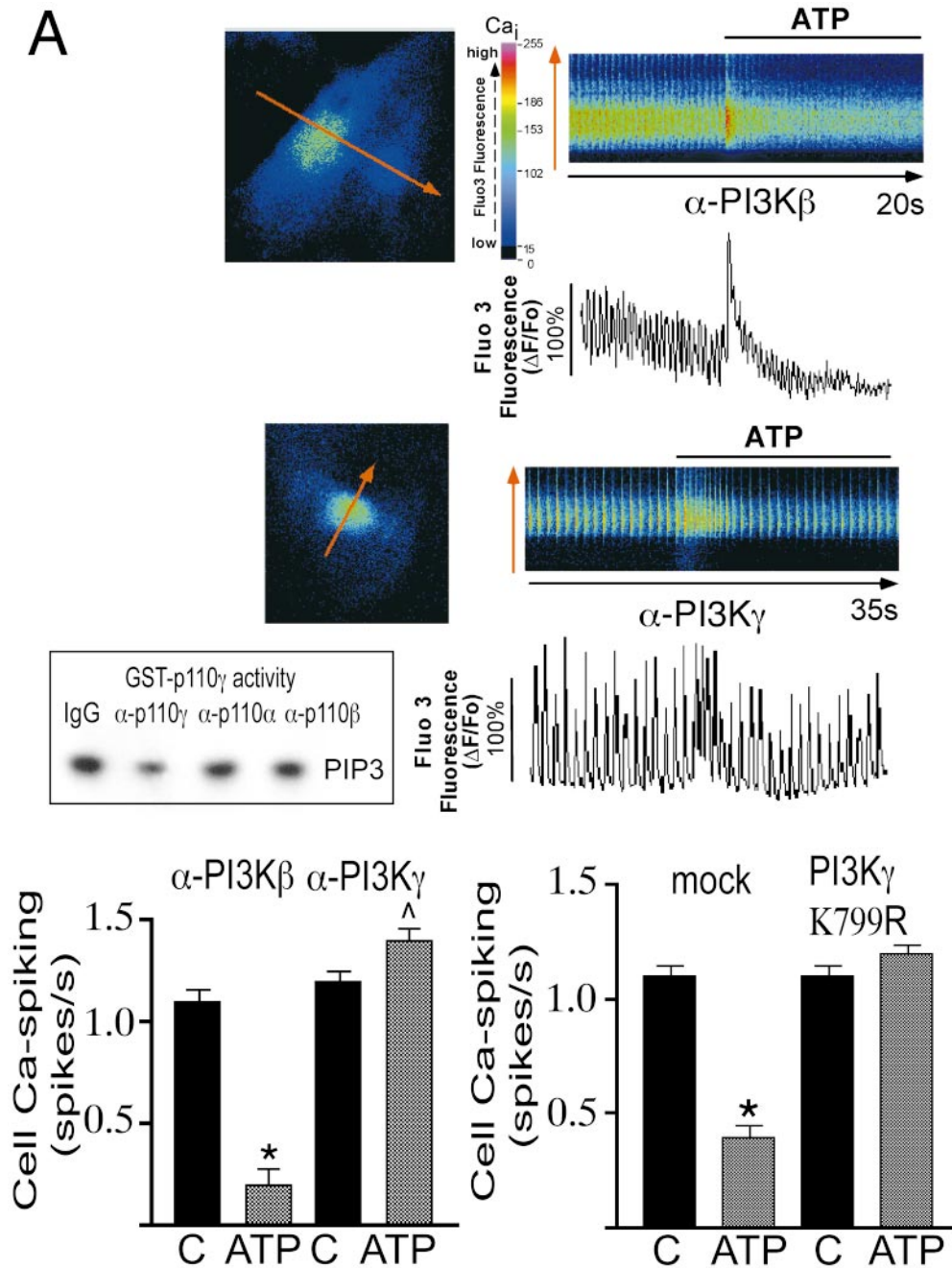


Figure 5. PI3K γ mediates both ATP-induced slowing of cell Ca²⁺ spiking and Tec translocation to the cell membrane. (A) Cardiomyocytes were microinjected with a specific anti PI3K β or γ polyclonal antibody together with fluo3. Cell Ca²⁺ spiking was monitored with a CCD camera. After background subtraction, a line was set offline along a cell (arrow). Fluorescence was recorded along the line as a function of time. The line scan images of adjacent lines were reconstructed using ANALYZE software (Mayo Foundation). The graphs below the images represent the change in fluorescence (ΔF) along the time after subtraction of the first image (F_0). Similar results were obtained on at least 10 cells from two separate cell cultures as shown in the bar graph on the left. Cells were also transfected with a dominant negative mutant (K399R) of PI3K γ , and the effects of ATP on cell Ca²⁺ spiking was measured 24 h later on 10 different cells from two separate cultures (right). A recombinant p110 γ was expressed as a GST-p110 γ fusion (inset). PIP3K activity was then measured in vitro in the presence of rabbit IgG, the specific polyclonal anti-p110 γ , or the anti-p110 α or -p110 β antibodies. (B) Beating embryoid bodies (days 9 and 10) generated from PI3K γ ^{-/-}, PI3K γ ^{+/-}, or wild-type (WT) ES cells were loaded with fluo3, and the effect of ATP was tested on spontaneous Ca²⁺ spiking of cardiomyocytes. The results

are expressed as means \pm SEM from 15 PI3K γ ^{-/-}, 6 PI3K γ ^{+/-}, and 6 wild-type embryoid bodies. (C) A specific anti-PI3K β (left) or γ antibody (middle) was injected, or a dominant negative mutant (K399R) of PI3K γ was transfected into cells (right). Cardiomyocytes transfected with a TecGFP plasmid were microinjected. 1 h later, cells were stimulated for 1 min with ATP and fixed before image acquisition. Similar results were obtained in 15 cells from three separate cell cultures, as shown in the bar graph on left. Cells were also transfected with a dominant negative mutant (K399R) of PI3K γ , and the effects of ATP on Tec membrane translocation was tested on eight transfected or mock cells (right). *Significantly decreased; [^]significantly increased ($p \leq 0.01$). ATP, ATP-stimulated cells; C, control.

interfere with other isoforms with their regulatory subunits, as recently demonstrated (Vanhaesebroeck et al., 1999). However, we confirmed the findings obtained with the antibody using two other experimental approaches. A dominant negative mutant of PI3K γ , expressed in neonatal cardiomyocytes, blocks the effect of ATP on both Ca²⁺ spiking and Tec membrane association. In spontaneously beating cardiomyocytes generated from PI3K γ ^{-/-}

ES cells, ATP did not affect the frequency of Ca²⁺ spiking whereas it significantly decreased it in embryoid bodies generated from PI3K γ ^{+/-} or wild-type ES cells, as observed in neonatal cardiomyocytes. Together, our findings demonstrate that PI3K γ mediates purinergic activation of PLC γ , subsequent IP₃ formation, and modulation of spontaneous cell Ca²⁺ spiking in cardiac cells. To our knowledge, ATP is the first agonist, binding to a

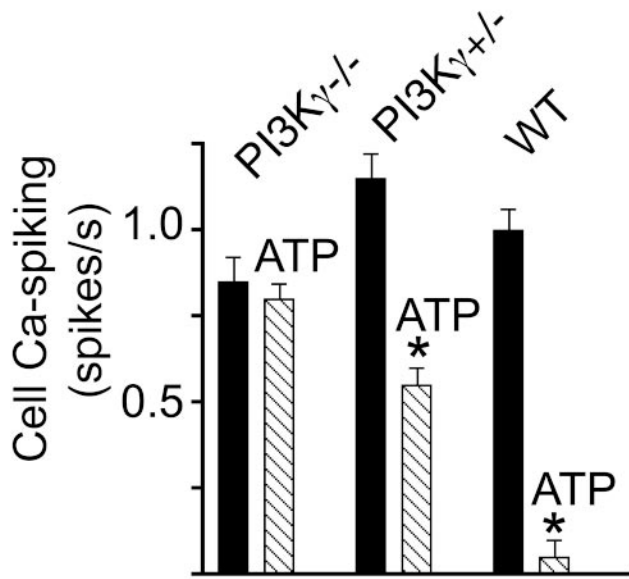
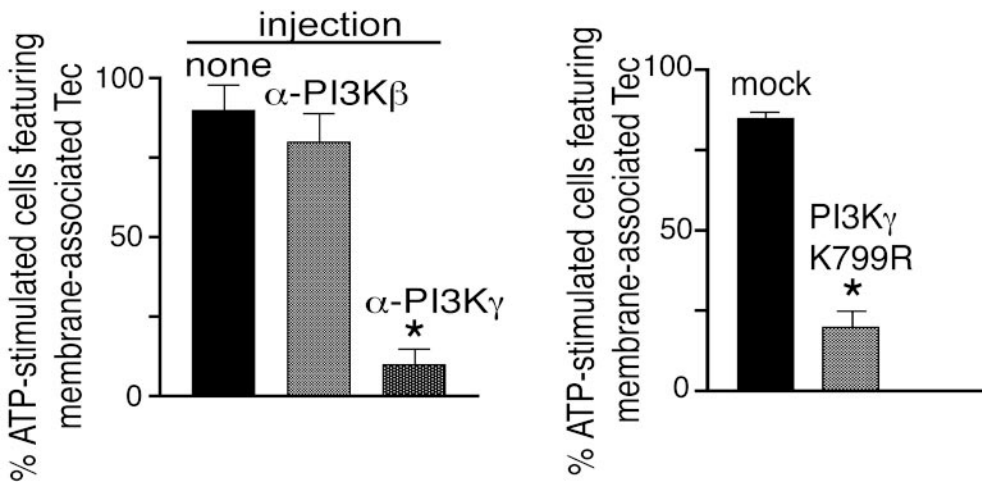
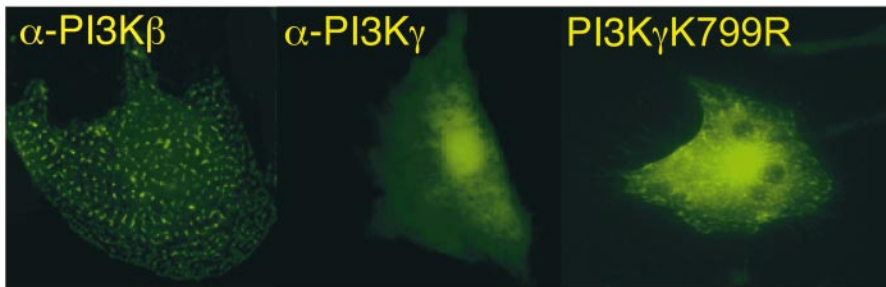
B**C**

Figure 5 (continued)

serpentine receptor, to activate a specific isoform of PI3K and the first agonist to activate PI3K γ in excitable cells.

Previously, we reported that Src family kinases mediate the effects of ATP on both PLC γ activity (Puceat and Vassort, 1996) and Ca²⁺ signaling (Jaconi et al., 2000). Herein,

we show that, in response to extracellular ATP, Tec, the tyrosine kinase of the Btk family, is phosphorylated and translocated into the T-tubes, plasma membrane invagination in cardiomyocytes (Figs. 3 and 5). Tec also associates with PLC γ in ATP-stimulated cardiomyocytes (Fig. 3) and is a key element in the regulation of cell Ca²⁺ spiking by

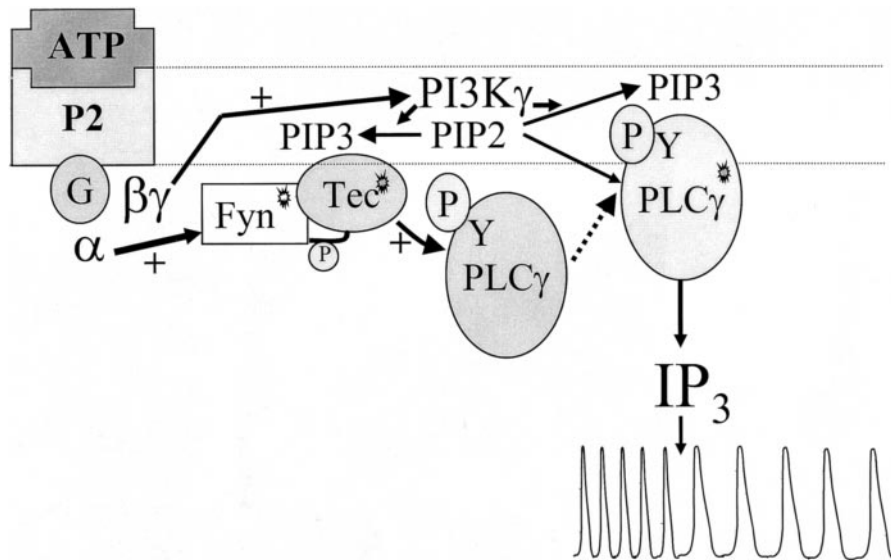


Figure 6. Proposed signaling pathway of the purinergic receptor: regulation of cardiac autonomic activity. Activation of the G protein-coupled P2 purinergic receptor leads to dissociation of α and $\beta\gamma$ subunits. α activates Fyn, whereas $\beta\gamma$ stimulates PI3K γ . Tec is then transphosphorylated by Fyn. This leads to phosphorylation and membrane translocation of PLC γ . PIP3, the product of PI3K, facilitates the anchor of both Tec and PLC γ . The latter is fully activated, generating IP $_3$, which regulates the autonomic activity of the cardiomyocyte.

ATP (Fig. 3). These data further suggest that Tec is likely to be transphosphorylated by Src family kinases, activated as demonstrated for other members of the Btk family (Mahajan et al., 1995; Rawlings et al., 1996). Since Tec membrane translocation depends on PI3K γ activation, as revealed by the inhibitory effect of intracellular microinjection of a specific and blocking anti-PI3K γ antibody (Fig. 5), PIP3 is likely to anchor at the cell membrane, not only PLC γ but also the tyrosine kinase Tec, through its PH domain (August et al., 1997; Varnai et al., 1999). These data also argue in favor of ATP-induced PLC γ activation, mediated by Src/Tec tyrosine kinases, together with PI3K (August et al., 1997).

Together, our findings obtained in excitable cells support the idea that a cluster of kinases comprising Btk and Src family kinases as well as PI3K γ and PLC γ constitute a regulatory network of intracellular Ca $^{2+}$ homeostasis (Scharenberg and Kinet, 1998). Such a multistep process in which two families of tyrosine kinases are implicated has been previously reported for T cell activation (August et al., 1997) and for cell spreading (Meng and Lowell, 1998). It is likely that the purinergic receptor is coupled to a Gs protein, as previously found (Puceat et al., 1998a). Activation of this receptor by ATP leads to dissociation of α_s and $\beta\gamma$ subunits. α_s is likely to directly activate Fyn and Src (Ma et al., 2000), whereas $\beta\gamma$ activates PI3K γ . In cardiac cells, this regulatory nexus between Fyn, Tec, and PI3K γ constitutes an amplification cascade to ensure a fast but transient accumulation of IP $_3$ (Fig. 6). It also provides the heart with a fine regulation of a critical cell function, namely cell autonomic activity, by factors that activate a tyrosine kinase-dependent pathway.

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