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

Claire Bony,* Serge Roche,‡ Ueno Shuichi,§ Takehiko Sasaki,‖ Michael A. Crackower,‖ Josef Penninger,‖ Hiroyuki Mano,§ and Michel Pucéat‡

*The French Institute of Health and Medical Research and ‡the Center for Research of Macromolecular Biochemistry, CNRS UPR1086 Montpellier 34293, France; §Division of Functional Genomics, Jichi Medical School, Tochigi, 329-04 Japan; and ‖Amgen Institute, Ontario Cancer Institute, Department of Medical Biophysics and Immunology, Toronto, Ontario, MSG 2C1 Canada

Abstract. Purinergic stimulation of cardiomyocytes turns on a Src family tyrosine kinase–dependent pathway that stimulates PLCγ and generates IP_3, a breakdown product of phosphatidylinositol 4,5–bisphosphate (PIP2). This signaling pathway closely regulates cardiac cell autonomic activity (i.e., spontaneous cell Ca^{2+} spiking). PIP2 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 PLCγ. PI3Ks have emerged as crucial regulators of many cell functions including cell division, cell migration, cell secretion, and, via PLCγ, Ca^{2+} homeostasis. However, although PI3Kα and -β have been shown to mediate specific cell functions in nonhematopoietic cells, such a role has not been found yet for PI3Kγ.

We report that neonatal rat cardiac cells in culture express PI3Kα, -β, and -γ. The purinergic agonist predominantly activates PI3Kγ. Both wortmannin and LY294002 prevent tyrosine phosphorylation, and membrane translocation of PLCγ as well as IP_3 generation in ATP-stimulated cells. Furthermore, an anti-PI3Kγ, but not an anti-PI3Kβ, injected in the cells prevents the effect of ATP on cell Ca^{2+} spiking. A dominant negative mutant of PI3Kγ 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 PLCγ. 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-PI3Kγ antibody and a dominant negative mutant of PI3Kγ injected or transfected into cells prevent the latter event.

We conclude that PI3Kγ 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 PI3Kγ to fully activate PLCγ 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 (PIP3) after 3′ phosphorylation of phosphatidylinositol 4,5–diphosphate (PIP2). 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]P2, or PI[3,5]P2). The most studied heterodimeric class I PI3K is composed of two subgroups. PI3K of class IA is a heterodimer consisting of a regulatory subunit (p85α,β or p55) associated with a catalytic subunit (p110α, -β, or -δ) (Fruman et al., 1998). The catalytic subunit p110γ, 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

Address correspondence to Michel Pucéat, Centre de Recherches de Biochimie Macromoléculaire, CNRS UPR1086, 1919 route de Mende, 34293 Montpellier, France. Tel.: 33-4-6761-3350. Fax: 33-4-6752-1559. E-mail: puceat@crbm.cnrs-mop.fr

[1]*Abbreviations 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; PIP2, phosphatidylinositol 4,5–diphosphate; PIP3, phosphatidylinositol 3,4,5–trisphosphate.

required for PI3Kγ to be efficiently activated by βγ subunits of trimeric G proteins (Leopoldt et al., 1998). Furthermore, p101 confers substrate specificity of PI(4,5)P2 for the p110γ (Maier et al., 1999).

Whether PI3K isoforms mediate specific cell function is just an emerging issue. p110α was first shown to mediate the effects of some growth factors on DNA synthesis (Roche et al., 1994). A specific role of p110β in mitotic signals has been then uncovered in fibroblasts (Roche et al., 1998). A differential role of p110α in cell proliferation and survival and of p110β and/or p110δ 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, PI3Kα is specifically required for EGF-induced actin rearrangement. (Hill et al., 2000). Although PI3Kγ is known to link serpentine receptors to mitogen-activated protein kinase (Duckworth and Cantley, 1997; Lopez-Ilasaca et al., 1997) and c-Jun NH_2-terminal kinase (Lopez-Ilasaca 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 p110γ were generated by several groups. As expected from expression of PI3Kγ 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 PI3Kγ 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$\gamma^{-/-}$ mice.

Although PI3Ks phosphorylate PIP2, PLCs hydrolyze the phosphoinositides and produce IP_3 and diacylglycerol, the endogenous activator of protein kinase C. Activation of a plethora of cell membrane receptors produces an elevation of intracellular IP_3, a trigger and/or regulator of Ca^{2+} oscillations or Ca^{2+} waves (Berridge, 1995; Clapham, 1995). PLCs are expressed in most cell types as several isoforms including PLCβ, -δ, and -γ. PLCγ is activated by tyrosine kinase–dependent pathways (Rhee and Bae, 1997). There is compelling evidence that PLCγ 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 PLCγ and, indirectly, after activation of the tyrosine Bruton's kinase Btk (Li et al., 1997). PIP3 binding to the PH domain targets PLCγ to the membrane. Thus, PI3Ks are at the crossroad of a tyrosine kinase–mediated Ca^{2+} signaling pathway.

We recently uncovered in cardiac cells a tyrosine kinase–dependent Ca^{2+} signaling pathway. Purinergic stimulation of rat cardiomyocytes leads to PLCγ activation (Puceat and Vassort, 1996) and in turn modulates spontaneous intracellular Ca^{2+} oscillations. IP_3 generated by binding of ATP to extracellular purinergic receptors induces a mitochondrial Ca^{2+} loading, which indirectly depletes Ca^{2+} from the sarcoplasmic reticulum. This in turn slows or stops spontaneous Ca^{2+} 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 Gβγ-dependent PI3K was shown in cardiac hypertrophy (Naga Prasad et al., 2000). A gain of function of a mutant of p110α increases heart size in transgenic mice (Shioi et al., 2000).

Together, these findings prompted us to investigate the involvement of PI3Ks in the PLCγ-regulated Ca^{2+} 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$\gamma^{-/-}$ cardiomyocytes, we provide evidence for a specific role of PI3Kγ 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 PI3Kγ.

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$\gamma^{-/-}$, PI3K$\gamma^{+/-}$, 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 PI3Kγ (PI3KγK799R) and the blue or red fluorescent protein (pEBFP or pDsRed1-N1; CLONTECH Laboratories) to detect the transfected cells before Ca^{2+} measurement.

Tec Translocation

Cells were transfected with a TecGFP plasmid (Tec cDNA inserted in the EcoR1 site of pEGFP-N1; CLONTECH Laboratories) alone or with a dominant negative mutant of PI3Kγ, using effectene as described above. pEGFP-N1 was transfected as a negative control. After 36 h, cells were stimulated for 1 min by 20 μM 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 μm 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 $Na_4P_4O_7$, 1 mM EDTA, 1 mM $MgCl_2$, 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 Na_3VO_4, 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 peroxydase-conjugated antibody. The proteins were revealed using enhanced chemiluminescence.

IP_3 Measurements

Intracellular IP_3 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, $MnCl_2$ 10 mM, 1 mM DTT, 5 μCi [γ-^{32}P]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-PLCγ 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-PI3Kα and -β have been previously characterized (Roche et al., 1998). The polyclonal anti-PI3Kγ antibody used in microinjection and immunoprecipitation experiments was raised against the amino acids 742–756 of p110γ within the Gβγ and p101 binding domains (Stoyanov et al., 1995). The monoclonal anti-PI3Kγ antibody, used in Western blot, binds to the NH_2-terminal stretch (amino acids 87–302 including the predicted PH domain) of p110γ (Leopoldt et al., 1998). The blocking activity of the polyclonal anti-PI3Kγ antibody was tested in vitro. A recombinant p110γ was expressed as a glutathione *S*-transferase (GST)–p110γ fusion protein in Sf9 cells infected with a baculovirus and purified (Rubio et al., 1999). Activity was then measured in vitro as described above. p110γ was decreased by 40% in two experiments in which the specific polyclonal anti-p110γ antibody was added to the kinase assay. Anti-p110α or -p110β did not affect p110γ activity (see Fig. 5 A, inset).

Microspectrofluorimetry and Imaging of Cell Ca^{2+}

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 NaH_2PO_4, 4.4 mM $NaHCO_3$, 1.7 mM $MgCl_2$, 1.8 mM $CaCl_2$. 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 ($F - F_o$) and divided by the first one to take into account fluo3 inhomogeneity within the cell. To calculate the frequency of Ca^{2+} 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 PLCγ

First, we tested the effects of PI3K inhibitors on purinergic activation of PLCγ. PLCγ 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 PLCγ, 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 βγ-regulated PI3K (0.5 μM) (Stephens et al., 1994) or LY 294002 (25 μM), the purinergic effect on PLCγ membrane translocation was fully abolished (Fig. 1 A). PI3K inhibitors used under the same experimental conditions as the ones described above, prevented PLCγ 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 PLCγ 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 PLCγ phosphorylation and membrane translocation (data not shown).

We then addressed whether PLCγ activity was also inhibited by a PI3K inhibitor. Neonatal rat cardiomyocytes stimulated with ATP for 1 min featured a twofold increase in intracellular IP_3 content. Cell incubation with LY294002 fully abolished this rise and even decreased basal content of IP_3 (Fig. 1 C). This finding is consistent with inhibition of both phosphorylation and membrane translocation of PLCγ by PI3K inhibitors.

PI3K Activity Underlies the IP_3-mediated Purinergic Regulation of Cardiac Automatic Activity

IP_3, generated by activation of purinergic P2Y receptors, is a crucial regulator of rhythmic Ca^{2+} spiking in neonatal rat cardiomyocytes plated at high density. After an intracellular Ca^{2+} release, IP_3 slows or stops cell spontaneous Ca^{2+} oscillations (Jaconi et al., 2000). We tested the effect of PI3K inhibitors on purinergic effect in spontaneous Ca^{2+} spiking cells. Extracellular ATP, acting through P2Y receptors slowed the rate of Ca^{2+} spiking of fluo3-loaded cardiomyocytes after an intracellular Ca^{2+} release, revealed by a transient rise in diastolic Ca^{2+} (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

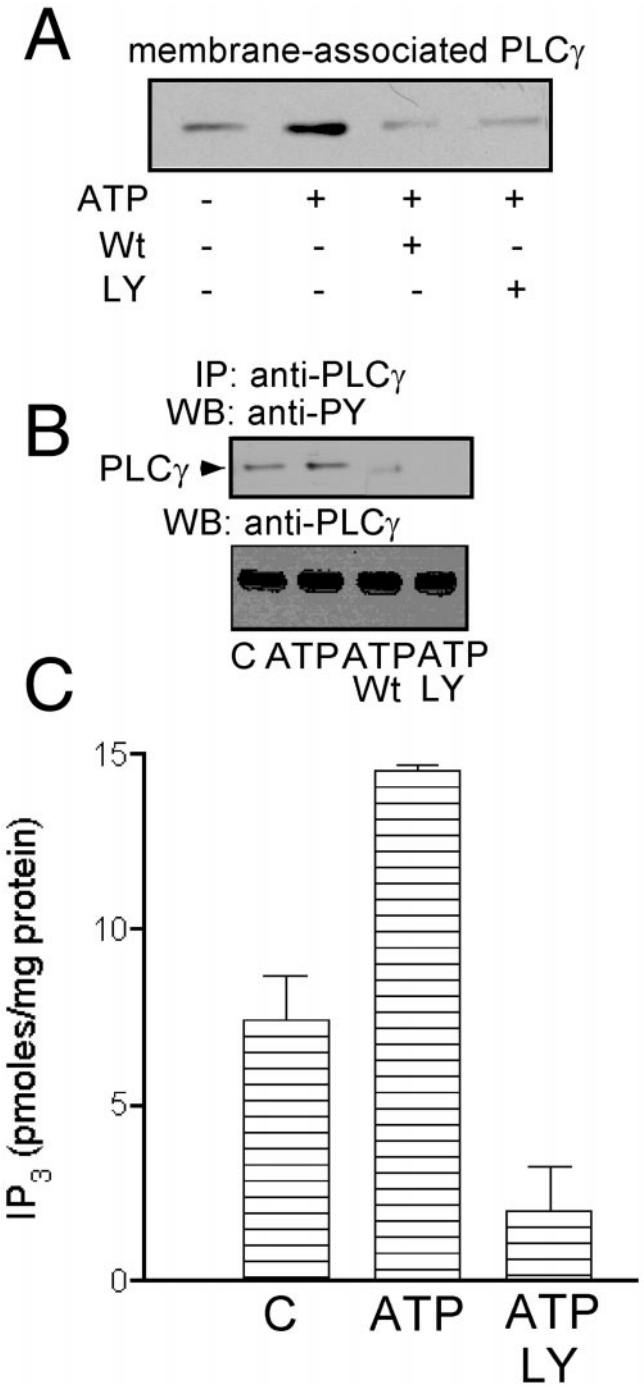

Figure 1. PI3K is required for ATP-induced IP_3 generation. Cardiomyocytes were stimulated with 20 μM ATP in the absence or presence of wortmannin (Wt) or LY294002 (LY). After stimulation, cells were subfractionated in cytosolic and membrane fractions (A). Membrane proteins were subjected to Western blot analysis using an anti-PLCγ antibody. (B) PLCγ was immunoprecipitated, and the immunocomplex was analyzed by Western blot using an anti-phosphotyrosine or an anti-PLCγ antibody. (C) The amount of IP_3 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.

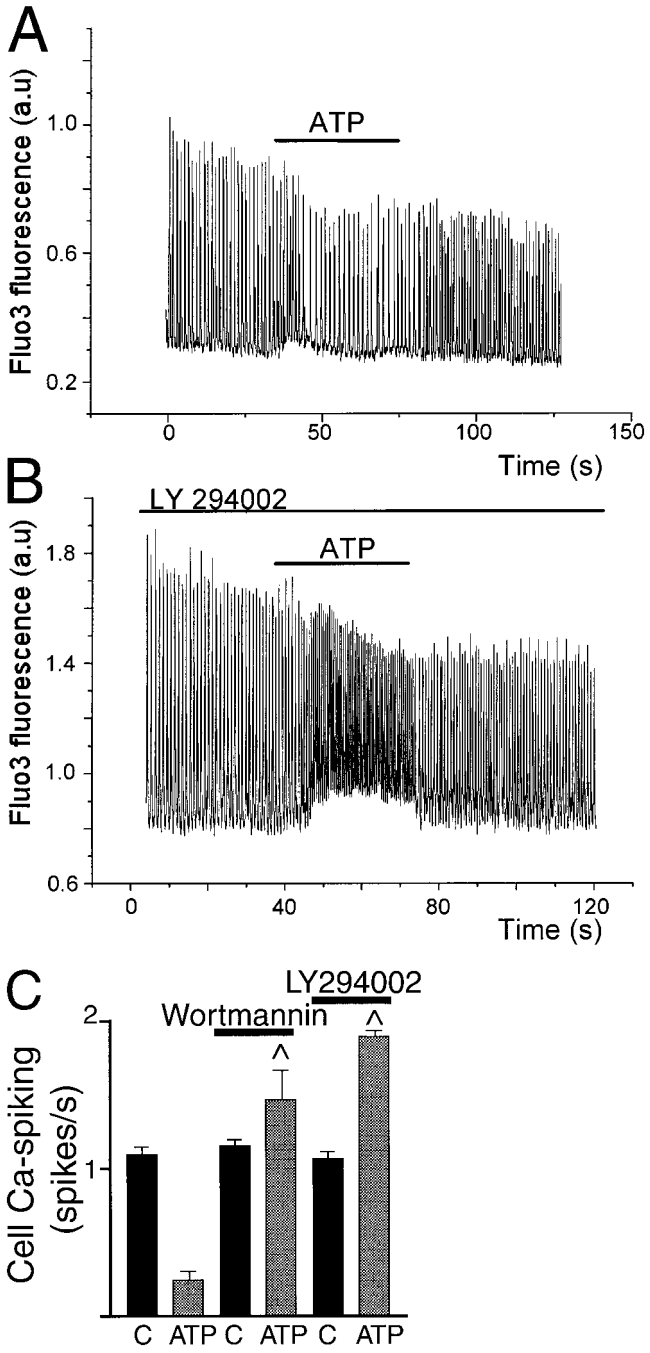

Figure 2. PI3K is required for ATP-induced slowing of cell Ca^{2+} spiking rate. A fluo3-loaded cell was superfused in the absence (A) or presence (B) of LY294002 with 20 μM ATP. Fluo3 fluorescence was recorded every 30 ms with a photomultiplier. The figure is representative of 10 similar experiments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

(Fig. 2, B and C). Acceleration of the rate and sustained increase in diastolic Ca^{2+} in the presence of ATP can be attributed to a transient membrane depolarization after activation of the ATP-gated ion channel through P2X receptors, an effect that is unmasked when IP_3 generation by P2Y receptors is blocked (Jaconi et al., 2000). Wortmannin and LY294002 added alone did not affect the rate of Ca^{2+} oscillations (Fig. 2 C).

Tec Is Involved in the PLCγ Signaling Pathway

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

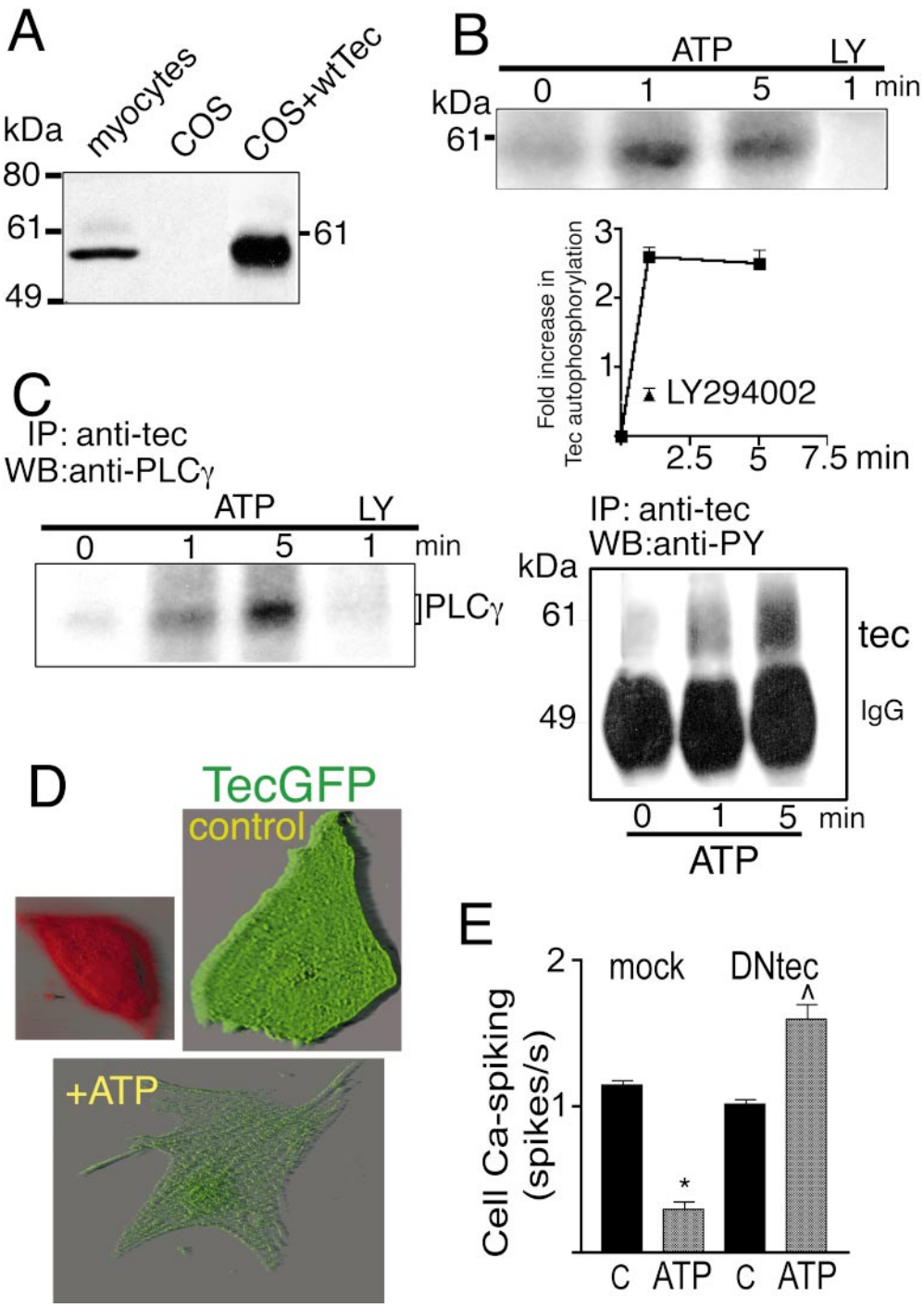

Figure 3. Tec is involved in ATP-induced slowing of cell Ca^{2+} spiking rate. (A) Whole cell lysate proteins from cardiac cells, mock Cos, or Cos cells transfected with wild-type Tec (from left to right lane), were subjected to Western blotting using an anti-Tec antibody. (B) After ATP stimulation, Tec was immunoprecipitated. The immunocomplex was used for the autophosphorylation assay using [γ-^{32}P]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-PLCγ antibody to detect the presence of PLCγ. 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 PLCγ-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

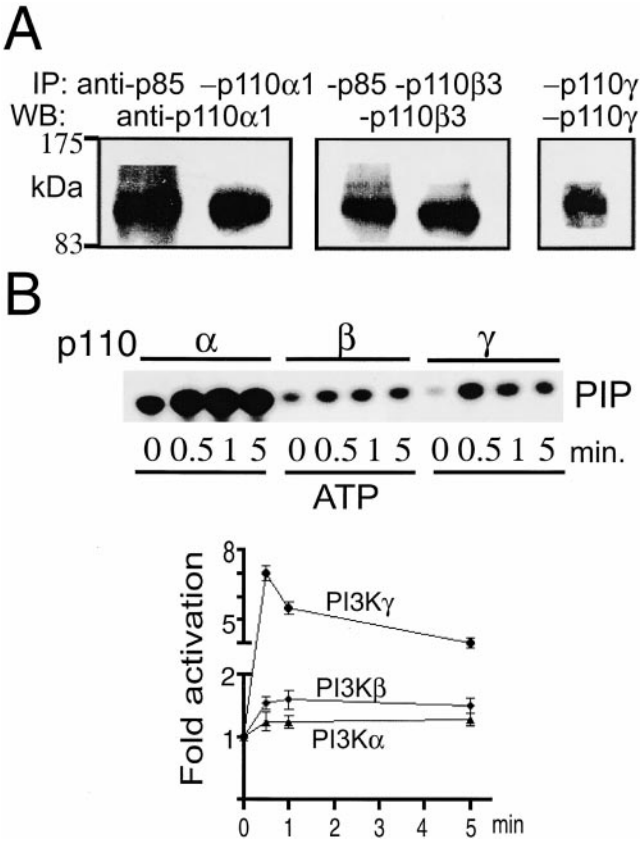

Figure 4. ATP specifically activates PI3Kγ. (A) PI3K subunits p85, p110α, -β and -γ were immunoprecipitated from a cardiac whole cell lysate and subjected to Western blot analysis using either an anti-p110 α1, β3, or γ antibody. The polyclonal anti-p110γ antibody was used for the immunoprecipitation and the monoclonal anti-p110γ antibody was used for Western blot. (B) Cells were stimulated for 0, 0.5, 1, or 5 min with 20 μM ATP, and PI3 kinase activity was assayed after PI3K immunoprecipitation. The experiment was repeated at least three times on three separate cultures. Means ± 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 ≥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 PLCγ (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 PLCγ 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 plasmid 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 PLCγ function and thus Ca^{2+} signaling, we tested the effect of expression of a dominant negative mutant of Tec lacking the kinase domain (Yamashita et al., 1998) on IP_3-regulated cell automatic Ca^{2+} spiking. In cells expressing the dominant negative mutant of Tec, ATP had no effect or increased the rate of Ca^{2+} spiking, whereas it stopped the spontaneous Ca^{2+} spikes in mock cells, a hallmark of IP_3 action (Fig. 3 E).

The Purinergic Agonist Specifically Activates p110 PI3Kγ

Next, we investigated expression of PI3Ks isoforms in neonatal rat cardiac cells. Using specific antibodies directed against the adapter p85 or the catalytic subunits p110α, -β, or -γ, the proteins were immunoprecipitated from a cardiac cell lysate and then immunoblotted with an anti-p110 antibody. p110α, -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 p110γ activity as revealed by Phosphorous-32 labeling of PIP. Maximal activation was reached within 0.5 min. p110β activity was slightly but more sustainely increased in ATP-stimulated cells. Basal activity of PI3Kα was high but barely affected by purinergic stimulation of cardiomyocytes (Fig. 4 B).

p110γ but Not p110β Is Required for IP_3-mediated Purinergic Effect on Cell Ca^{2+} Spiking and for Tec Membrane Translocation

PI3K inhibitors prevented ATP from slowing the rate of cell Ca^{2+} spiking. Both p110β and p110γ 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 Ca^{2+} spiking cardiomyocytes, and the effect of ATP was further tested. In cells injected with an anti-p110β antibody, ATP induced a transient release in intracellular Ca^{2+} and then slowed and stopped spontaneous cell Ca^{2+} 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-p110γ antibody (n = 11 cells), ATP did not induce an intracellular Ca^{2+} release and transiently accelerated spontaneous Ca^{2+} spiking (Fig. 5 A). Expression of a dominant negative mutant of PI3Kγ (K399R) that lacks lipid kinase activity (Stoyanova et al., 1997) in the cells also significantly prevented the slowing effect of ATP on cell Ca^{2+} spiking (Fig. 5 A).

To further test the involvement of PI3Kγ in the ATP effect on cell Ca^{2+} spiking, we used PI3K$\gamma^{-/-}$ 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 IP_3 receptors (Kolossov 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 Ca^{2+} (Viatchenko-Karpinski et al., 1999). In fluo3-loaded wild-type or PI3K$\gamma^{+/-}$ embryoid bodies, ATP significantly slowed down the spontaneous Ca^{2+} 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-PI3Kβ 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-PI3Kγ 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 μM LY294002 (data not shown). In cells transfected with a dominant negative mutant of PI3Kγ (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 PI3Kγ and that activity of the latter, together with the tyrosine kinase Tec, is required to mediate the purinergic effect on autonomic Ca^{2+} 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 μM, respectively) that inhibit the G$\beta\gamma$-regulated PI3K (Stephens et al., 1994) prevent both phosphorylation and translocation of PLCγ to the membrane of cardiac cells. Definite evidence in favor of a requirement of PI3K activity for PLCγ activation is brought by the IP_3 measurement assay. Indeed, PI3K inhibitors fully blocked ATP-induced IP_3 generation. Wortmannin also inhibits PI4K, a kinase required for the synthesis of PIP2. A limitation of this substrate may decrease IP_3 formation. However, LY294002 also prevents IP_3 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 PLCγ-dependent IP_3 pathway in purine-stimulated cells.

Previously, we showed that tyrosine phosphorylation is a key step for purinergic activation of PLCγ 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 PLCγ (Falasca et al., 1998). We found that LY294002 not only inhibits ATP-induced IP_3 production but also decreases the basal level (Fig. 1 C). Also, we observed such an effect of the drug on IP_3 generation in wortmannin-treated cells and on PLCγ 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 IP_3 in these cells (Fig. 1 C). In agreement with our findings on IP_3 generation, PI3Ks inhibitors prevent the effect of ATP on autonomic Ca^{2+} spiking, an effect mediated by IP_3 (Jaconi et al., 2000). Our present data demonstrate that activation of PI3K, together with PLCγ, is required for ATP-induced IP_3 formation and, in turn, regulation of Ca^{2+} spiking in primary culture of cardiomyocytes. This is in line with observations reported in the HepG2 cells in which PI3K mediates PDGF-induced intracellular Ca^{2+} release (Rameh et al., 1998).

Extracellular ATP predominantly activates PI3Kγ and, to some but much less extent, PI3Kβ but only barely affects PI3Kα 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 PI3Kγ was not only much stronger but also more transient than the one of PI3Kα and -β. This suggests that PI3Kβ and -α may be involved in more time lasting cell events such as cell survival or cell hypertrophy (Shioi et al., 2000), whereas PI3Kγ mediates short-term regulation of cardiac autonomic activity.

However, we also found that microinjection of an anti-PI3Kγ antibody into cardiomyocytes prevents the slowing effect of ATP on autonomic cell Ca^{2+} oscillations. In these microinjected cells, ATP increases the rate in cells in which the IP_3-dependent pathway was disrupted, as previously observed (Jaconi et al., 2000) (Fig. 2 B). Such an in vivo blocking effect of the anti-PI3Kγ 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 p110γ activity in in vitro assay. Finally, the antibody recognizes the region of PI3Kγ 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-PI3Kβ antibody, ATP still slows the rate of Ca^{2+} spiking after triggering an intracellular Ca^{2+}release, a hallmark of IP_3 action. This further demonstrates that PI3Kγ is mandatory for ATP-induced PLCγ 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-

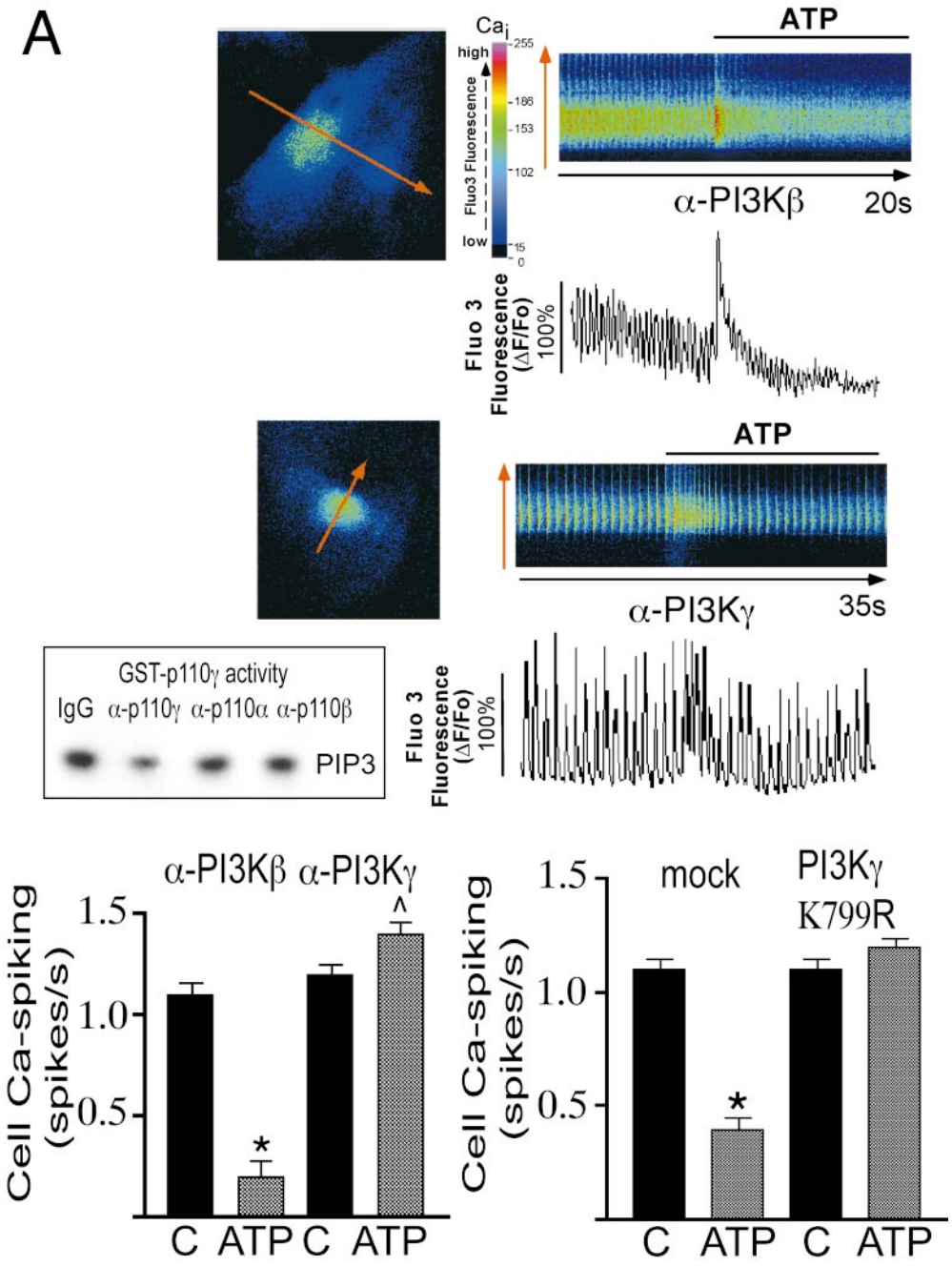

Figure 5. PI3Kγ mediates both ATP-induced slowing of cell Ca^{2+} spiking and Tec translocation to the cell membrane. (A) Cardiomyocytes were microinjected with a specific anti PI3Kβ or -γ polyclonal antibody together with fluo3. Cell Ca^{2+} spiking was monitored with a CCD camera. After background 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_o). 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 PI3Kγ, and the effects of ATP on cell Ca^{2+} spiking was measured 24 h later on 10 different cells from two separate cultures (right). A recombinant p110γ was expressed as a GST-p110γ fusion (inset). PIP3K activity was then measured in vitro in the presence of rabbit IgG, the specific polyclonal anti-p110γ, or the anti-p110α or -p110β antibodies. (B) Beating embryoid bodies (days 9 and 10) generated from PI3Kγ$^{-/-}$, PI3Kγ$^{+/-}$, or wild-type (WT) ES cells were loaded with fluo3, and the effect of ATP was tested on spontaneous Ca^{2+} spiking of cardiomyocytes. The results are expressed as means ± SEM from 15 PI3Kγ$^{-/-}$, 6 PI3Kγ$^{+/-}$, and 6 wild-type embryoid bodies. (C) A specific anti-PI3Kβ (left) or γ antibody (middle) was injected, or a dominant negative mutant (K399R) of PI3Kγ 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 PI3Kγ, 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.

fere 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 PI3Kγ, expressed in neonatal cardiomyocytes, blocks the effect of ATP on both Ca^{2+} spiking and Tec membrane association. In spontaneously beating cardiomyocytes generated from PI3Kγ$^{-/-}$ ES cells, ATP did not affect the frequency of Ca^{2+} spiking whereas it significantly decreased it in embryoid bodies generated from PI3Kγ$^{+/-}$ or wild-type ES cells, as observed in neonatal cardiomyocytes. Together, our findings demonstrate that PI3Kγ mediates purinergic activation of PLCγ, subsequent IP_3 formation, and modulation of spontaneous cell Ca^{2+} spiking in cardiac cells. To our knowledge, ATP is the first agonist, binding to a

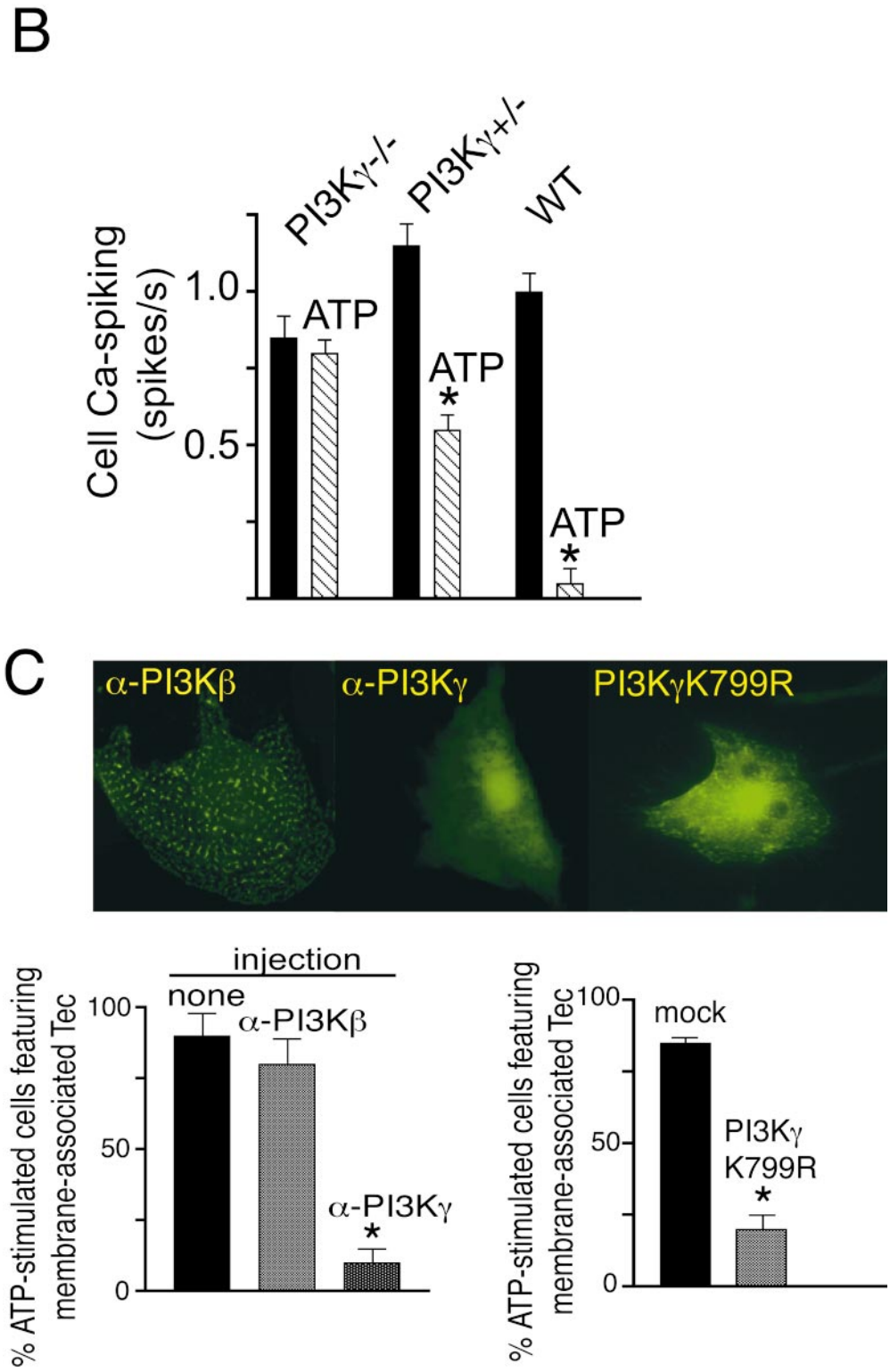

Figure 5 (continued)

serpentine receptor, to activate a specific isoform of PI3K and the first agonist to activate PI3Kγ in excitable cells.

Previously, we reported that Src family kinases mediate the effects of ATP on both PLCγ activity (Puceat and Vassort, 1996) and Ca^{2+} 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 PLCγ in ATP-stimulated cardiomyocytes (Fig. 3) and is a key element in the regulation of cell Ca^{2+} spiking by

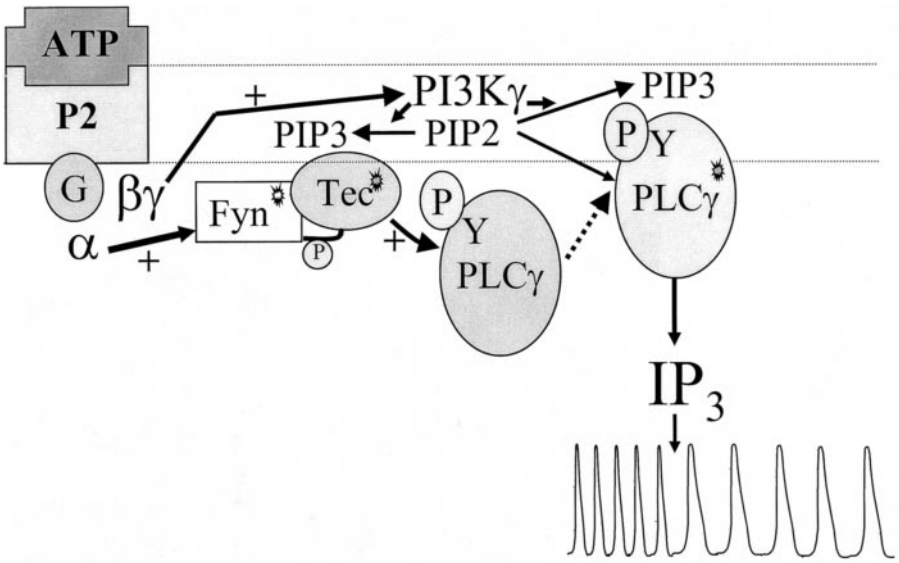

Figure 6. Proposed signaling pathway of the purinergic receptor: regulation of cardiac autonomic activity. Activation of the G protein–coupled P2 purinergic receptor leads to dissociation of α and βγ subunits. α activates Fyn, whereas βγ stimulates PI3Kγ. Tec is then transphosphorylated by Fyn. This leads to phosphorylation and membrane translocation of PLCγ. PIP3, the product of PI3K, facilitates the anchor of both Tec and PLCγ. 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 PI3Kγ activation, as revealed by the inhibitory effect of intracellular microinjection of a specific and blocking anti-PI3Kγ antibody (Fig. 5), PIP3 is likely to anchor at the cell membrane, not only PLCγ 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 PLCγ 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 PI3Kγ and PLCγ 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 βγ subunits. α_s is likely to directly activate Fyn and Src (Ma et al., 2000), whereas βγ activates PI3Kγ. In cardiac cells, this regulatory nexus between Fyn, Tec, and PI3Kγ 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.

We thank Dr. G. Vassort for his support at the beginning of this study; Drs. Wetzker and Rubio (Fredrich-Schillem University, Jena, Germany) for the gift of anti-PI3Kγ antibodies and GST-p110γ baculovirus; Dr. Wymann (Institute of Biochemistry, Foribourg, Switzerland) for the gift of the plasmid encoding a dominant negative form (K799R) of PI3K; Valérie Simon for the purification of GST-p110γ; and Dr. Travo for skilful advice in the acquisition and analysis of images at the imaging core facility (Centre de Recherches de Biochimie Macromoléculaire, Montpelier, France).

M. Pucéat and S. Roche are supported by Institut National de la Santé et de la Recherche Médicale. Part of this work was supported by Association pour la Recherche contre le Cancer.

Submitted: 5 April 2000
Revised: 27 October 2000
Accepted: 21 December 2000

References

August, A., A. Sadra, B. Dupont, and H. Hanafusa. 1997. Src-induced activation of inducible T cell kinase (ITK) requires phosphatidylinositol 3-kinase activity and the Pleckstrin homology domain of inducible T cell kinase. *Proc. Natl. Acad. Sci. USA*. 94:11227–11232.

Benistant, C., H. Chapuis, and S. Roche. 2000. A specific function for phosphatidylinositol 3-kinase α (p85-p110α) in cell survival and for phosphatidylinositol 3-kinase β (p85-p110β) in de novo DNA synthesis of human colon carcinoma cells. *Oncogene*. 19:5083–5093.

Berridge, M.J. 1995. Inositol trisphosphate and calcium signaling. *Ann. NY Acad. Sci.* 766:31–43.

Clapham, D.E. 1995. Calcium signaling. *Cell*. 80:259–268.

Downing, G.J., S. Kim, S. Nakanishi, K.J. Catt, and T. Balla. 1996. Characterization of a soluble adrenal phosphatidylinositol 4-kinase reveals wortmannin sensitivity of type III phosphatidylinositol kinases. *Biochemistry*. 35: 3587–3594.

Duckworth, B.C., and L.C. Cantley. 1997. Conditional inhibition of the mitogen-activated protein kinase cascade by wortmannin. Dependence on signal strength. *J. Biol. Chem*. 272:27665–27670.

Falasca, M., S.K. Logan, V.P. Lehto, G. Baccante, M.A. Lemmon, and J. Schlessinger. 1998. Activation of phospholipase C gamma by PI 3-kinase-induced PH domain-mediated membrane targeting. *EMBO (Eur. Mol. Biol. Organ.) J*. 17:414–422.

Fruman, D.A., R.E. Meyers, and L.C. Cantley. 1998. Phosphoinositide kinases. *Annu. Rev. Biochem*. 67:481–507.

Hill, K., S. Welti, J. Yu, J.T. Murray, S.C. Yip, J.S. Condeelis, J.E. Segall, and J.M. Backer. 2000. Specific requirement for the p85-p110α phosphatidylinositol 3-kinase during epidermal growth factor-stimulated actin nucleation in breast cancer cells. *J. Biol. Chem*. 275:3741–3744.

Hirsch, E., V.L. Katanaev, C. Garlanda, O. Azzolino, L. Pirola, L. Silengo, S. Sozzani, A. Mantovani, F. Altruda, and M.P. Wymann. 2000. Central role for G protein-coupled phosphoinositide 3-kinase γ in inflammation. *Science*. 287:1049–1053.

Jaconi, M., C. Bony, S. Richards, A. Terzic, S. Arnaudeau, G. Vassort, and M. Pucéat. 2000. Inositol 1,4,5-trisphosphate directs Ca^{2+} flow between mitochondria and the endoplasmic/sarcoplamic reticulum: a role in regulating autonomic cardiac Ca^{2+} spiking. *Mol. Biol. Cell.* 11:1845–1858.

Kolossov, E., B.K. Fleischmann, Q. Liu, W. Bloch, S. Viatchenko-Karpinski, O. Manzke, G.J. Ji, H. Bohlen, K. Addicks, and J. Hescheler. 1998. Functional characteristics of ES cell–derived cardiac precursor cells identified by tissue-specific expression of the green fluorescent protein. 1998. *J. Cell Biol*. 143: 2045–2056.

Kurosu, H., T. Maehama, T. Okada, T. Yamamoto, S. Hoshino, Y. Fukui, M. Ui, O. Hazeki, and T. Katada. 1997. Heterodimeric phosphoinositide 3-kinase consisting of p85 and p110β is synergistically activated by the βγ subunits of G proteins and phosphotyrosyl peptide. *J. Biol. Chem*. 272: 24252–24256.

Leopoldt, D., T. Hanck, T. Exner, U. Maier, R. Wetzker, and B. Nurnberg. 1998. Gβγ stimulates phosphoinositide 3-kinase-γ by direct interaction with two domains of the catalytic p110 subunit. *J. Biol. Chem*. 273:7024–7029.

Li, Z., M.I. Wahl, A. Eguinoa, L.R. Stephens, P.T. Hawkins, and O.N. Witte. 1997. Phosphatidylinositol 3-kinase-gamma activates Bruton's tyrosine ki-

nase in concert with Src family kinases. *Proc. Natl. Acad. Sci. USA*. 94: 13820–13825.

Lopez-Ilasaca, M., P. Crespo, P.G. Pellici, J.S. Gutkind, and R. Wetzker. 1997. Linkage of G protein–coupled receptors to the MAPK signaling pathway through PI 3-kinase γ. *Science*. 275:394–397.

Lopez-Ilasaca, M., J.S. Gutkind, and R. Wetzker. 1998. Phosphoinositide 3-kinase gamma is a mediator of Gbetagamma-dependent Jun kinase activation. *J. Biol. Chem*. 273:2505–2508.

Ma, Y.C., J. Huang, S. Ali, W. Lowry, and X.Y. Huang. 2000. Src tyrosine kinase is a novel direct effector of G proteins. *Cell*. 102:635–646.

Mahajan, S., J. Fargnoli, A.L. Burkhardt, S.A. Kut, S.J. Saouaf, and J.B. Bolen. 1995. Src family protein tyrosine kinases induce autoactivation of Bruton's tyrosine kinase. *Mol. Cell. Biol*. 15:5304–5311.

Maier, U., A. Babich, and B. Nurnburg. 1999. Roles of Non-catalytic subunits in Gβγ-induced activation of class i phosphoinositide 3-kinase isoforms β and γ. *J. Biol. Chem*. 274:29311–29317.

Mano, H., Y. Yamashita, K. Sato, Y. Yazaki, and H. Hirai. 1995. Tec protein-tyrosine kinase is involved in interleukin-3 signaling pathway. *Blood*. 85: 343–350.

Meng, F., and C.A. Lowell. 1998. A beta 1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. *EMBO (Eur. Mol. Biol. Organ.) J*. 17:4391–4403.

Meyer, N., M. Jaconi, A. Ladopoulou, P. Fort, and M. Puceat. 2000. A fluorescent reporter gene as a marker for ventricular specification in ES-derived cardiac cells. *FEBS Lett*. 478:151–158.

Naga Prasad, S.V., G. Esposito, L. Mao, W.J. Koch, and H.A. Rockman. 2000. Gβγ-dependent phosphoinositide 3-kinase activation in hearts with in vivo pressure overload hypertrophy. *J. Biol. Chem*. 275:4693–4698.

Oh, H., Y. Fujio, K. Kunisada, H. Hirota, H. Matsui, T. Kishimoto, and K. Yamauchi-Takihara. 1998. Activation of phosphatidylinositol 3-kinase through glycoprotein 130 induces protein kinase B and p70 S6 kinase phosphorylation in cardiac myocytes. *J. Biol. Chem*. 273:9703–9710.

Puceat, M., and G. Vassort. 1996. Purinergic stimulation of rat cardiomyocytes induces tyrosine phosphorylation and membrane association of phospholipase Cγ: a major mechanism for $InsP_3$ generation. *Biochem. J*. 318:723–728.

Puceat, M., R. Hilal-Dandan, B. Strulovici, L.L. Brunton, and J.H. Brown. 1994. Differential regulation of protein kinase C isoforms in isolated neonatal and adult rat cardiomyocytes. *J. Biol. Chem*. 269:16938–16944.

Puceat, M., I. Korichneva, R. Cassoly, and G. Vassort. 1995. Identification of band 3-like proteins and Cl-/HCO3- exchange in isolated cardiomyocytes. *J. Biol. Chem*. 270:1315–1322.

Puceat, M., C. Bony, M. Jaconi, and G. Vassort. 1998a. Specific activation of adenylyl cyclase V by a purinergic agonist. *FEBS Lett*. 431:189–194.

Puceat, M., S. Roche, and G. Vassort. 1998b. Src family tyrosine kinase regulates intracellular pH in cardiomyocytes. *J. Cell Biol*. 141:1637–1646.

Rabkin, S.W., V. Goutsouliak, and J.Y. Kong. 1997. Angiotensin II induces activation of phosphatidylinositol 3-kinase in cardiomyocytes. *J. Hypertens*. 15: 891–899.

Rameh, L.E., and L.C. Cantley. 1999. The role of phosphoinositide 3-kinase lipid products in cell function. *J. Biol. Chem*. 274:8347–8350.

Rameh, L.E., S.G. Rhee, K. Spokes, A. Kazlauskas, L.C. Cantley, and L.G. Cantley. 1998. Phosphoinositide 3-kinase regulates phospholipase Cγ-mediated calcium signaling. *J. Biol. Chem*. 273:23750–23757.

Rawlings, D.J., A.M. Scharenberg, H. Park, M.I. Wahl, S. Lin, R.M. Kato, A.C. Fluckiger, O.N. Witte, and J.P. Kinet. 1996. Activation of BTK by a phosphorylation mechanism initiated by Src family kinases. *Science*. 271:822–825.

Rhee, S.G., and Y.S. Bae. 1997. Regulation of phosphoinositide-specific phospholipase C isozymes. *J. Biol. Chem*. 272:15045–15048.

Roche, S., M. Koegl, and S.A. Courtneidge. 1994. The phosphatidylinositol 3-kinase α is required for DNA synthesis induced by some, but not all, growth factors. *Proc. Natl. Acad. Sci. USA*. 91:9185–9189.

Roche, S., J. Downward, P. Raynal, and S.A. Courtneidge. 1998. A function for phosphatidylinositol 3-kinase γ (p85α-p110β) in fibroblasts during mitogenesis: requirement for insulin- and lysophosphatidic acid-mediated signal transduction. *Mol. Cell. Biol*. 18:7119–7129.

Rubio, I., U. Wittig, C. Meyer, R. Heinze, D. Kadereit, H. Waldmann, J. Downward, and R. Wetzker. 1999. Farnesylation of Ras is important for the interaction with phosphoinositide 3-kinase gamma. *Eur. J. Biochem*. 266:70–82.

Sasaki, T., J. Irie-Sasaki, R.G. Jones, A.J. Oliveira-dos-Santos, W.L. Stanford, B. Bolon, A. Wakeham, A. Itie, D. Bouchard, I. Kozieradzki, et al. 2000a. Function of PI3Kγ in thymocyte development, T cell activation, and neutrophil migration. *Science*. 287:1040–1046.

Sasaki, T., J. Irie-Sasaki, Y. Horie, K. Bachmaier, J.E. Fata, M. Li, A. Suzuki, D. Bouchard, A. Ho, M. Redston, et al. 2000b. Colorectal carcinomas in mice lacking the catalytic subunit of PI(3)Kγ. *Nature*. 406:897–902.

Scharenberg, A.M., and J.P. Kinet. 1998. PtdIns-3,4,5-P3: a regulatory nexus between tyrosine kinases and sustained calcium signals. *Cell*. 94:5–8.

Shioi, T., P.M. Kang, P.S. Douglas, J. Hampe, C.M. Yballe, J. Lawitts, L.C. Cantley, and S. Izumo. 2000. The conserved phosphoinositide 3-kinase pathway determines heart size in mice. *EMBO (Eur. Mol. Biol. Organ.) J*. 19: 2537–2548.

Shubeita, H.E., J. Thorburn, and K.R. Chien. 1992. Microinjection of antibodies and expression vectors into living myocardial cells. Development of a novel approach to identify candidate genes that regulate cardiac growth and hypertrophy. *Circulation*. 85:2236–2246.

Stephens, L., A. Smrcka, F.T. Cooke, T.R. Jackson, P.C. Sternweis, and P.T. Hawkins. 1994. A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein βγ subunits. *Cell*. 77:83–93.

Stoyanov, B., S. Volinia, T. Hanck, I. Rubio, M. Loubtchenkov, D. Malek, S. Stoyanova, B. Vanhaesebroeck, R. Dhand, B. Nurnberg, et al. 1995. Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science*. 269:690–693.

Stoyanova, S., G. Bulgarelli-Leva, C. Kirsch, T. Hanck, R. Klinger, R. Wetzker, and M.P. Wymann. 1997. Lipid kinase and protein kinase activities of G-protein-coupled phosphoinositide 3-kinase gamma: structure-activity analysis and interactions with wortmannin. *Biochem. J*. 324:489–495.

Vanhaesebroeck, B., G.E. Jones, W.E. Allen, D. Zicha, R. Hooshmand-Rad, C. Sawyer, C. Wells, M.D. Waterfield, and A.J. Ridley. 1999. Distinct PI(3)Ks mediate mitogenic signalling and cell migration in macrophages. *Nat. Cell Biol*. 1:69–71.

Varnai, P., K.I. Rother, and T. Balla. 1999. Phosphatidylinositol 3-kinase-dependent membrane association of the Bruton's tyrosine kinase pleckstrin homology domain visualized in single living cells. *J. Biol. Chem*. 274:10983–10989.

Viatchenko-Karpinski, S., B.K. Fleischmann, Q. Liu, H. Sauer, O. Gryshchenko, G.J. Ji, and J. Hescheler. 1999. Intracellular Ca^{2+} oscillations drive spontaneous contractions in cardiomyocytes during early development. *Proc. Natl. Acad. Sci. USA*. 96:8259–8264.

Wennstrom, S., and J. Downward. 1999. Role of phosphoinositide 3-kinase in activation of ras and mitogen-activated protein kinase by epidermal growth factor. *Mol. Cell. Biol*. 19:4279–4288.

Yamashita, Y., S. Watanabe, A. Miyazato, K. Ohya, U. Ikeda, K. Shimada, N. Komatsu, K. Hatake, Y. Miura, K. Ozawa, and H. Mano. 1998. Tec and Jak2 kinases cooperate to mediate cytokine-driven activation of c-fos transcription. *Blood*. 91:1496–1507.

Yang, W.C., M. Ghiotto, B. Barbarat, and D. Olive. 1999. The role of Tec protein-tyrosine kinase in T cell signaling. *J. Biol. Chem*. 274:607–617.