Purinergic stimulation of rat cardiomyocytes induces tyrosine phosphorylation and membrane association of phospholipase Cγ: a major mechanism for InsP_3 generation

Michel PUCEAT* and Guy VASSORT
INSERM U-390, Laboratoire de Physiopathologie Cardiovasculaire, C.H.U. Arnaud de Villeneuve, 34295 Montpellier Cedex, France

Phospholipase Cγ (PLCγ) expression and activation by a purinergic agonist were investigated in adult rat cardiomyocytes. PLCγ is expressed in isolated cardiomyocytes. Stimulation of cells with extracellular ATP induces a rapid increase in membrane-associated PLCγ immunoreactivity most probably due to redistribution of the lipase from the cytosol to the membrane. The purine triggers a significant phosphorylation on tyrosine residues of a cytosolic pool of PLCγ with a time course that correlates with that of translocation. Extracellular ATP also increases intracellular Ins(1,4,5)P_3 content. All these events (translocation and phosphorylation of PLCγ, InsP_3 formation) are blocked by genistein, a tyrosine kinase inhibitor. The purinergic effect on both PLCγ translocation and phosphorylation are Ca-sensitive. We thus propose that the purinergic stimulation activates a non-receptor tyrosine kinase that phosphorylates PLCγ in the presence of an increased Ca level and induces PLCγ redistribution to the membrane. There, PLCγ becomes activated leading to the hydrolysis of phosphatidylinositol diphosphate and in turn Ins(1,4,5)P_3 formation. This cascade of events may play a significant role in the induction of arrhythmogenesis by purinergic agonists.

INTRODUCTION

Phospholipase C (PLC) activation by extracellular hormones, neuromediators or growth factors results in the breakdown of inositol-containing lipids. This hydrolytic process results in the generation of InsP_3 and diacylglycerol, the endogeneous activator of protein kinase C. The PLCs comprise a family of several isoforms including PLCs β, γ and δ which are encoded by different genes [1,2]. PLCβ activity is activated by binding of agonists to seven-transmembrane-domain receptors that are coupled to a Gq protein [1,2]. Whether PLCδ is activated by extracellular stimuli is still unknown. PLCγ is a major substrate for tyrosine kinase. Its phosphorylation on tyrosine residues is a key step in its activation [3]. Thus, for many years, the activity of this isoform was believed to be regulated by binding of agonists to receptors containing intracellular tyrosine kinase domains such as growth factor receptors [4]. More recently, carbachol and angiotensin, agonists that bind to seven-transmembrane-domain G-protein-coupled receptors, were shown to activate PLCγ through tyrosine phosphorylation [5–7]. A cytosolic tyrosine kinase of the c-*src* family was suggested to be involved in this activation [6].

Most purinergic receptors that have been cloned so far, namely the P_1 (adenosine-sensitive) and P_2 (ATP-sensitive) receptor subtypes (except P_{2x}), belong to the superfamily of G-protein-coupled seven-transmembrane-domain receptors [8]. The signal transduction pathways underlying the purinergic stimulation have been extensively studied in various cell types [9]. The purine generates the formation of InsP_3 in most tissues so far explored. In cardiac cells that are likely to express both P_{2x} and P_{2y}, extracellular ATP considered as a P_2-purinergic agonist also increases intracellular InsP_3 [10,11] and activates protein kinase C [12]. A better knowledge of the phosphoinositide turnover pathway is necessary to understand the various pathophysiological actions of extracellular ATP. Indeed, the purine released by both sympathetic and parasympathetic nerve terminals under physiological situations, as well as by cardiomyocytes and various vascular cell types in cardiac ischaemia, increases contractility [10] and transiently slows down cardiac rhythm driven by the sinus pacemaker. At higher concentrations, the purine triggers a sinus tachychardia and can be an arrhythmic agent under pathological situations [13]. PLC activation and in turn InsP_3 formation is likely to play a significant role in this later effect [14] by modulating the calcium-induced calcium release [15] or inducing Ca oscillations [16]. It had been assumed that ATP was a ligand for G-protein-coupled receptor-activated PLCβ. However, we previously found that extracellular ATP stimulates the Cl/HCO_3 exchanger, an intracellular-pH-regulatory mechanism, following activation of a tyrosine kinase [17]. Moreover, PLCγ has been suggested to be the major PLC isoform in the heart [18]. Altogether, these data prompted us to investigate whether, and if so how, ATP activates the tyrosine kinase-regulated PLCγ.

In this study, we show that in isolated rat cardiac ventricular myocytes, extracellular ATP induces the phosphorylation on tyrosine residues of PLCγ. This leads to the translocation of PLCγ from the cytosolic to the membrane fraction. Both events depend upon extracellular calcium and result in the formation of intracellular Ins(1,4,5)P_3.

EXPERIMENTAL

Materials

Collagenase was purchased from Boehringer. Polyclonal anti-PLCγ and monoclonal anti-PLCγ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and UBI (Lake Placid, NY, U.S.A.), respectively. Anti-phosphotyrosine antibody was purchased from Transduction Laboratories (Lexing-

Abbreviations used: PLC, phospholipase C; BAPTA/AM, 1,2-bis-(2-aminophenoxy)ethane-*NNN′N′*-tetra-acetic acid acetoxymethyl ester; PNP, phenylnitrophosphate; ECL, enhanced chemiluminescence; NP40, Nonidet P40; Ca_i, intracellular Ca; EGF, epidermal growth factor.

* To whom correspondence should be addressed.

ton, KY, U.S.A.). Enhanced chemiluminescence (ECL) detection reagent was provided by NEN Dupont de Nemours. Genistein, herbimycin A and all other chemicals were obtained from Sigma (La Verpillère, France).

Preparation of a cardiac homogenate from rat hearts

Homogenates from whole hearts were prepared as previously described [19].

Isolation of cardiomyocytes

Cardiomyocytes were isolated from hearts of 200–250 g male Wistar rats as previously described [19]. Briefly, the hearts were first perfused for 5 min at 35 °C with a nominally Ca-free Hepes-buffered solution containing (in mM): NaCl 117, KCl 5.7, $NaHCO_3$ 4.4, KH_2PO_4 1.5, $MgCl_2$ 1.7, Hepes 21, glucose 11, taurine 20, and then for 55 min with the same solution containing 1.2 mg/ml collagenase (Type A, Boehringer) and 20 μM Ca. The heart was removed from the perfusion set-up and gently dissociated through the bore of a large-tip pipette. After filtration, the cells were allowed to decant and the pellet was resuspended in collagenase-free Hepes solution. The cells were incubated for 15 min at 37 °C. Meanwhile Ca concentration was increased stepwise up to 0.3 mM. Cells were then washed and resuspended in Hepes solution containing 1 mM Ca and 0.25 % BSA. This method routinely obtained $(6–10) \times 10^6$ rod-shaped cells/heart. Dissociations giving a low yield of viable cells were discarded.

Cell fractionation

Cells were stimulated in Hepes-buffered solution containing 0.25 % BSA and 1 mM Ca, or in nominally Ca-free solution with 50 μM ATP for 0.5, 1, 5 or 15 min at 37 °C and pelleted by centrifugation. The pellet was resuspended in glycerophosphate–sucrose buffer B [12] containing 1 mM Na_3VO_4, 50 mM NaF, 10 mM phenylnitrophosphate (PNP), 100 μM PMSF and 1 μg/ml leupeptin. After homogenization with a glass–glass homogenizer, the homogenate was spun down for 30 min at 12000 ***g***. The supernatant was saved as the cytosolic fraction. The pellet was resuspended in buffer A [12] containing 1 % Triton X-100, 1 mM Na_3VO_4, 50 mM NaF, 10 mM PNP, 100 μM PMSF and 1 μg/ml leupeptin. After 30 min of incubation on ice, the extract was spun down for 30 min at 12000 ***g***. The supernatant was saved as the crude membrane fraction. The lack of lactate dehydrogenase activity (measured by an enzymic spectrofluorimetric assay) and of anti-($\alpha\beta$)tubulin immunoreactivity in the membrane fraction excludes contamination of this fraction by cytosolic or cytoskeleton proteins, respectively. Laemmli buffer was added to both the cytosolic and membrane fractions and the samples were boiled for 3 min. In some experiments, the pellet of myofibrils containing both cytoskeleton and myofilaments was saved and the proteins were submitted to Western blotting.

Immunoprecipitation of tyrosine-phosphorylated proteins

Cells were stimulated with the agonist as described above. The cells were quickly pelleted by centrifugation and the pellet was resuspended in immunoprecipitation buffer (NET buffer) containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 1 % Nonidet P40 (NP40), 1 mM Na_3VO_4, 50 mM NaF, 10 mM PNP, 0.1 mM PMSF and 1 μM leupeptin adjusted to pH 8.8. Cells were lysed for 20 min on ice and the lysate was spun down for 30 min at 12000 ***g***. The protein amount was estimated in the supernatant using Bradford reagent and adjusted to the same value for every sample. SDS (2 %) was added to the samples that were boiled for 3 min. SDS concentration was decreased to 0.5 % by dilution with the NET buffer. The samples were precleared for 30 min at 4 °C with Protein A–Sepharose and 8 μg aliquots of anti-phosphotyrosine antibody were added to the tubes. The samples were incubated with gentle shaking at 4 °C for 3.5 h. During the last hour, Protein A–Sepharose was added to the tubes. The Protein A–Sepharose beads were spun down for 4 min at 2000 ***g***, washed twice with NET buffer containing 1 % NP40 and once with NET buffer. Laemmli buffer was added to the samples that were boiled for 3 min. After centrifugation, the supernatant was loaded on the electrophoresis gel.

Western blotting of proteins

Proteins from cytosolic and membrane fractions and immunoprecipitates were loaded on 6 % or 7.5 % acrylamide gels and run according to the method of Laemmli [20]. The proteins were transferred to nitrocellulose membrane according to the method of Towbin et al. [21]. The blots were probed overnight at 4 °C with anti-PLCγ antibody and revealed using a secondary peroxidase-conjugated antibody and ECL detection according to the manufacturer's instructions. The blots were quantified by a digital imaging system (Scion Image).

Blot stripping

The blots were stripped for 30 min at 50 °C in a Tris buffer containing 62.5 mM Tris, 2 % SDS and 10 mM mercaptoethanol adjusted to pH 6.7.

Measurement of intracellular Ins(1,4,5)P_3

Cardiomyocytes (5×10^5 cells/0.5 ml) were preincubated for 10 min with 10 mM LiCl and were stimulated at 37 °C for 15 s, 30 s, 1 and 5 min with 50 μM ATP. The incubations were stopped by rapid centrifugation (5 s) and addition of 0.2 ml perchloric acid (1 M) to the cell pellet. The samples were incubated for 10 min on ice and the protein precipitate was pelleted by centrifugation. The supernatant was neutralized with 10 M KOH. Ins(1,4,5)P_3 content was estimated in the supernatant by a binding protein assay kit (NEN) according to the manufacturer's instructions.

Measurement of intracellular ATP

Proteins from control and genistein-treated cells were precipitated by perchloric acid. After centrifugation, the supernatant was neutralized with 5 M K_2CO_3. The perchlorate precipitate was pelleted and ATP was measured in the supernatant by a luciferin–luciferase chemiluminescent assay (using a Boehringer kit) according to the manufacturer's instructions.

RESULTS

Expression and intracellular distribution of PLCγ in cardiomyocytes

Western blot analysis was used to investigate the expression of PLCγ in the heart. Blots probed with a monoclonal anti-PLCγ antibody revealed the presence in a whole-rat-heart homogenate of a significant immunoreactivity of PLCγ that migrated with an apparent molecular mass of 145 kDa (Figure 1A). In this preparation, the antibody also reacted with a 130 kDa protein. Similar results were obtained with a polyclonal anti-PLCγ antibody. In isolated ventricular cardiomyocytes only the 145 kDa band was observed, indicating that PLCγ is specifically

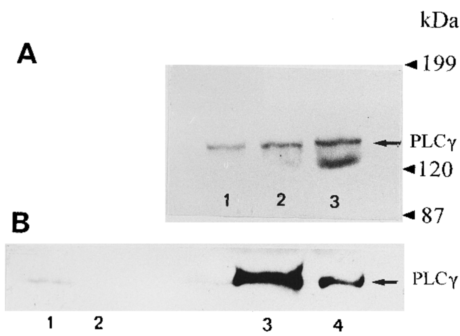

Figure 1 Expression of PLCγ in rat heart and isolated cardiomyocytes

(**A**) A cardiac homogenate was prepared from rat hearts and 50 μg, 100 μg and 200 μg aliquots of protein (lanes 1, 2 and 3, respectively) were run on SDS/PAGE. Proteins were electrophoretically transferred to a nitrocellulose membrane and the blot was probed with a monoclonal anti-PLCγ antibody (1/3000) and then with a peroxidase-conjugated anti-(mouse IgG) (1/3000). (**B**) Isolated cardiomyocytes were fractionated in cytosolic and membrane fractions. Aliquots (150 and 50 μg) of membrane (lanes 1 and 2) and cytosolic (lanes 3 and 4) proteins were run on SDS/PAGE. The proteins were transferred to a nitrocellulose membrane and the blot probed with a monoclonal anti-PLCγ antibody. Positions of prestained molecular mass markers are shown on the right. The proteins were revealed using ECL. The autoradiography films were exposed for a few minutes.

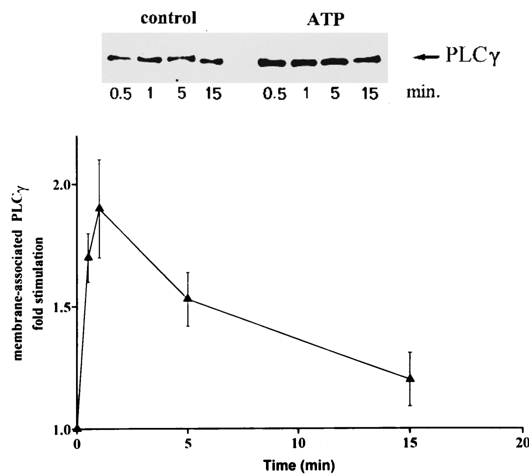

Figure 2 Extracellular ATP increases membrane PLCγ immunoreactivity

Upper panel: cardiomyocytes were stimulated for 0.5 to 15 min with 50 μM ATP and were fractionated into cytosol and membrane fractions. Aliquots (100 μg) of membrane proteins were run on SDS/PAGE and transferred to a nitrocellulose membrane. The blot was probed with a polyclonal anti-PLCγ antibody (1/2000) and then with a peroxidase-conjugated anti-(rabbit IgG) antibody (1/3000). The proteins were revealed by ECL. The autoradiography films were scanned using an imaging system. Lower panel: the graph represents the time course of ATP-induced increase in membrane PLCγ immunoreactivity obtained from 4 to 9 experiments. Results are expressed as means $\pm$ S.E.M.

expressed in the muscle cell type. To look at the intracellular localization of PLCγ, rat isolated ventricular cells were fractionated into cytosolic and membrane fractions. As illustrated in Figure 1(B), 80–90 % of 145 kDa PLCγ immunoreactivity was found in the cytosol and only 10–20 % was associated with the crude membrane fraction under resting conditions. A weak immunoreactivity was observed in the myofibrils (results not shown).

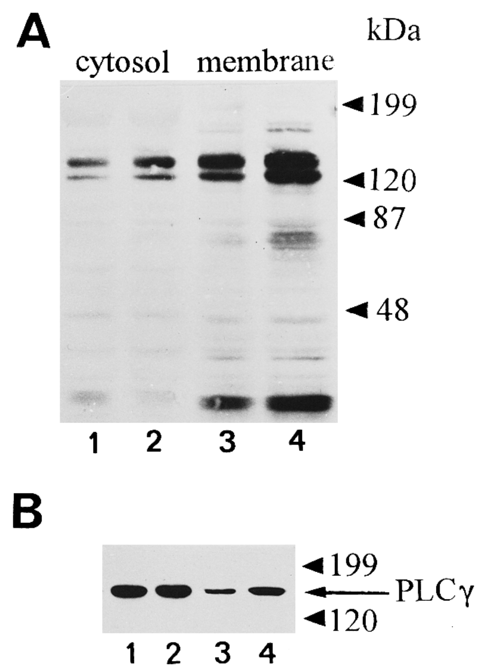

Figure 3 Extracellular ATP induces tyrosine phosphorylation of proteins including PLCγ

Cardiomyocytes were stimulated for 1 min with 50 μM ATP and were then fractionated into cytosol and membrane fractions. 70 μg of cytosolic and 200 μg of membrane proteins were subjected to Western blotting. (**A**) The blot was probed first with an anti-phosphotyrosine antibody (1/2000) and a secondary peroxidase-conjugated anti-(mouse IgG) antibody. (**B**) The blot was stripped and then reprobed with a polyclonal anti-PLCγ antibody and a secondary peroxidase-conjugated anti-(rabbit IgG). Lanes 1 and 3: proteins from control cells; lanes 2 and 4: proteins from ATP-stimulated cells. The proteins were revealed by ECL and autoradiography. Scanning of the blots indicates a 2-fold increase in membrane PLCγ immunoreactivity and a 2- and 3-fold increase in 145 kDa protein phosphorylation in membrane and cytosol, respectively. Similar results were obtained in two other experiments.

Purinergic stimulation increases membrane-associated PLCγ immunoreactivity

Purinergic stimulation of isolated cardiomyocytes using 50 μM ATP increased by 2-fold membrane-associated PLCγ immunoreactivity. This event occurred as early as 30 s and maximal effect was reached at 1 min. Membrane-associated PLCγ immunoreactivity then decreased slowly to its basal value within 15 min (Figure 2). No clear decrease in cytosolic PLCγ immunoreactivity could be detected (results not shown). The faint myofibrillar PLCγ immunoreactivity was not changed following purinergic stimulation (results not shown).

Purinergic stimulation of cardiomyocytes induces phosphorylation of PLCγ on tyrosine residues

We next carried out experiments to look for the signal that triggers PLCγ translocation. Because phosphorylation of PLCγ is required for its activation [2], we tested the possibility that phosphorylation of the lipase in the cytosol triggers its redistribution to the membrane where it could be activated. Cardiomyocytes were stimulated for 1 min (peak of translocation) by extracellular ATP and were then fractionated into cytosolic and membrane fractions. Proteins from both fractions were run in electrophoresis and submitted to Western blotting using an anti-phosphotyrosine antibody. The blot shown in Figure 3(A) revealed a 145 kDa protein that was phosphorylated

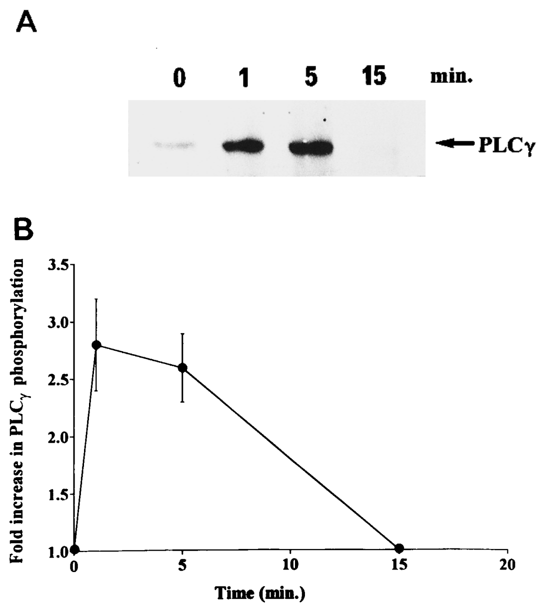

Figure 4 Extracellular ATP induces PLCγ phosphorylation

Cardiomyocytes were stimulated for 1, 5 or 15 min with 50 μM ATP. The proteins phosphorylated on tyrosine residues were immunoprecipitated from a whole-cell lysate using an anti-phosphotyrosine antibody. They were then subjected to Western blotting and the blot was probed with a polyclonal anti-PLCγ antibody. PLCγ was revealed by a secondary peroxidase-conjugated anti-(rabbit IgG) antibody and ECL detection (**A**). PLCγ is indicated by an arrow. (**B**) Graph summarizing the results. Data are expressed as means ± S.E.M. from four experiments in which autoradiograms were scanned using an imaging system.

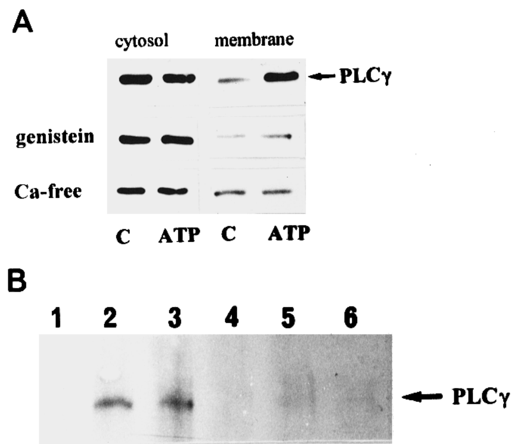

Figure 5 Genistein and incubation of cardiomyocytes in Ca-free buffer prevents ATP-induced increase in membrane-associated PLCγ immunoreactivity and PLCγ phosphorylation

(**A**) Cardiomyocytes were incubated in the absence (top blot), in the presence of 50 μM genistein for 30 min at 37 °C in a Ca-containing buffer (middle blot), or in a Ca-free buffer (bottom blot). Cells were then stimulated for 1 min with 50 μM ATP and fractionated into cytosolic and membrane fractions. 40 μg and 100 μg of cytosolic and membrane proteins, respectively, were submitted to Western blotting. (**B**) Cardiomyocytes were not stimulated (lane 1) or were stimulated with 50 μM ATP for 1, 5 or 15 min (lanes 2, 3 and 4, respectively) in a Ca-containing buffer. Cells were incubated in the presence of 50 μM genistein for 30 min before stimulation with ATP for 5 min (lane 5). Cells were stimulated for 5 min with ATP in Ca-free buffer (lane 6). Protein phosphorylated on tyrosine residues were immunoprecipitated from a whole-cell lysate and run on SDS/PAGE. The proteins were transferred to a nitrocellulose membrane. The blots were probed with a polyclonal anti-PLCγ antibody. PLCγ was detected by a secondary peroxidase-conjugated anti-(rabbit IgG) antibody and ECL reagent. Similar results were obtained in two other experiments.

on tyrosine residues following purinergic stimulation. The phosphorylation was detected both in cytosolic and in membrane fractions. Some other proteins migrating with apparent molecular masses of 125 and 25 kDa in both cytosolic and membrane fractions and 180 and 80 kDa in membrane fractions were phosphorylated following purinergic stimulation. To check whether the 145 kDa phosphoprotein was PLCγ, the blot was stripped and reprobed with an anti-PLCγ antibody. The latter reacted with the same 145 kDa band (Figure 3B). The increase in membrane-associated PLCγ immunoreactivity suggested a redistribution of the enzyme from the cytosol to the membrane. An increase in phosphotyrosine immunoreactivity of PLCγ in the membrane fraction could only be due to an increase in the amount of the enzyme but not to a rise in the number of PO_4 residues incorporated in the protein. As an alternative approach to more specifically investigate and to better characterize PLCγ phosphorylation, the cardiomyocytes were stimulated for 1, 5 and 15 min with ATP and the phosphoproteins were immunoprecipitated from a whole-cell lysate using an anti-phosphotyrosine antibody. The proteins were run in electrophoresis and were submitted to Western blotting. The blots were probed with an anti-PLCγ antibody. Such experiments revealed that PLCγ was already phosphorylated after 1 min of purinergic stimulation (Figure 4A). The protein phosphorylation was slowly decreased within 15 min (Figures 4A and 4B). A parallel pattern of increase in PLCγ membrane association is shown in Figure 2.

Phosphorylation of PLCγ is necessary for its redistribution to the membrane

The observation that the time course of PLCγ phosphorylation closely correlates to the time course of its redistribution to the membrane suggests that phosphorylation and redistribution to the membrane of PLCγ are two interdependent events. To determine whether the phosphorylation was necessary for the intracellular redistribution of PLCγ, cardiomyocytes were treated for 30 min in the presence of a concentration of 50 μM of the specific tyrosine-kinase inhibitor, genistein, before purinergic stimulation. Such a treatment fully prevented both the ATP-induced increase in membrane-associated PLCγ immunoreactivity (Figure 5A) and the phosphorylation as assessed by immunoprecipitation (Figure 5B). Because genistein was reported to exert some side-effect on the metabolic state of cells [22], we considered the possibility that the tyrosine kinase inhibitor prevents the phosphorylation of PLC only by decreasing the intracellular ATP concentration. However, the assay of intracellular ATP content in the cardiomyocytes by chemiluminescence revealed no significant difference between control and genistein-treated cells (15.1 ± 1.1 in control versus 15.6 ± 2.6 nmol/mg of protein in genistein-treated cells, $n = 9$). Thus, under our experimental conditions, genistein did not affect the energetic state of cardiac cells. The target of the inhibitory effect of genistein is thus likely to be a tyrosine kinase.

Extracellular Ca is required for both phosphorylation and redistribution to the membrane of PLCγ

Extracellular ATP triggers an increase in intracellular calcium (Ca_i) following membrane depolarization. This rise is Ca_i mainly depends upon extracellular Ca following the opening of the voltage-dependent Ca-channels [23]. This prompted us to test the role of Ca_i in both phosphorylation and increase in membrane-immunoreactivity of PLCγ. Two protocols were used to prevent the increase in Ca_i triggered by extracellular ATP. Cells were either loaded with 1,2-bis-(2-aminophenoxy)ethane-*NNN′N′*tetra-acetic acid acetoxymethyl ester (BAPTA-AM) [23]

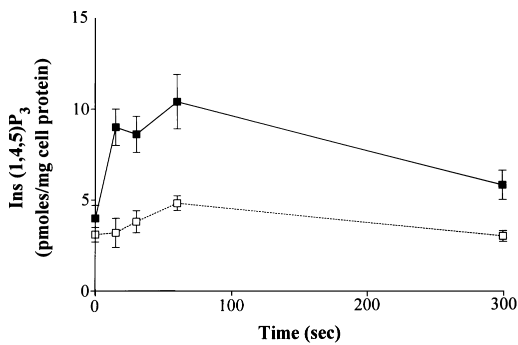

Figure 6 Genistein treatment of cardiomyocytes prevent ATP-induced increased in Ins(1,4,5)P_3 formation

Control (■) or genistein-treated cells (□) were stimulated for 15 s, 30 s, 1 min and 5 min with 50 μM ATP. The incubation was stopped by rapid centrifugation and addition of perchloric acid (1 M) to the cell pellet. Ins(1,4,5)P_3 content was measured in neutralized extract using a binding-protein assay. Results are expressed as pmol of Ins(1,4,5)P_3/mg of cell protein and are means $\pm$ S.E.M. of five experiments performed in duplicate.

to buffer Ca_i, or incubated in nominally Ca-free buffer. Under both experimental conditions, the purinergic agonist lost the ability to both increase membrane PLCγ immunoreactivity and to phosphorylate PLCγ (Figure 5).

ATP activates PLCγ

We next designed experiments to test the origin of Ins(1,4,5)P_3 formation induced by purinergic stimulation. If PLCγ is responsible for this effect, prevention of phosphorylation and redistribution of PLCγ to the membrane by genistein should block its activity and thus InsP_3 generation. Basal intracellular Ins(1,4,5)P_3 content of isolated cardiomyocytes was 4.0 $\pm$ 0.7 pmol/mg of protein. This value was not significantly affected following genistein treatment of cells. ATP (50 μM) triggered a 2-fold increase in intracellular Ins(1,4,5)P_3 content. This effect occurred within 1 min; then, InsP_3 content decreased towards the basal value within 5 min. Pretreatment of cells for 30 min in the presence of 50 μM genistein fully prevented ATP-induced Ins(1,4,5)P_3 generation (Figure 6). Treatment of cardimyocytes for 30 min with 2 μg/ml herbimycin A, another tyrosine kinase inhibitor, also inhibited purinergic stimulation of Ins(1,4,5)P_3 formation (results not shown).

DISCUSSION

Our results document that PLCγ is expressed in rat cardiomyocytes and mainly localized in the cytosolic compartment. We also report for the first time that neurohormonal stimulation of cardiac cells activates PLCγ following tyrosine phosphorylation. Two different approaches were used to investigate the activation of PLCγ. First, extracellular ATP induces a rapid increase in membrane-associated PLCγ immunoreactivity. The failure to observe a concomitant decrease in cytosolic PLCγ immunoreactivity is most probably due to the fact that a rise in the small amount of membrane-associated PLCγ is more easily detectable than a loss in the large amount of cytosolic PLCγ, as already noticed for protein kinase C in cardiomyocytes [12]. This finding thus suggests that PLCγ is translocated from the cytosol to the membrane following purinergic stimulation. It should also be noted that, in contrast to epidermal growth factor (EGF) [24], ATP does not affect PLCγ binding to the cytoskeleton. Because PLCγ is primarily regulated by tyrosine phosphorylation, PLCγ translocation to the membrane further points to a purinergic activation of the tyrosine kinase-dependent pathway in adult rat cardiomyocytes. Indeed, extracellular ATP induces phosphorylation of PLCγ on tyrosine residues. Using a cell fractionation protocol, we observed that the phosphorylation is likely to occur in the cytosolic compartment. Although we found an increase in the phosphotyrosine immunoreactivity in the membrane fraction of ATP-stimulated cells, this observation does not allow us to conclude about a rise in the phosphorylation of the protein since its amount was also increased to the same extent in this compartment following the translocation process. After immunoprecipitation of tyrosine-phosphorylated proteins from a whole-cell lysate, we found an increase in phosphorylation of PLCγ after purinergic stimulation. Because the cytosolic PLCγ pool is much larger than the membrane pool, this result also indicates that phosphorylation of PLCγ occurs in the cytosolic compartment.

In the A431 cell line, ATP failed to phosphorylate PLCγ [25]. Cardiomyocytes are thus the first cell type that displays a tyrosine kinase-dependent activation of PLCγ by a purinergic agonist. We can speculate that extracellular ATP, that most probably binds to a G-protein-coupled seven-transmembrane-domain receptor, turns on the activity of a cytosolic tyrosine kinase in cardiac cells. Therefore, it should be pointed out that tyrosine kinase can regulate short-term events in cardiomyocytes, a cell type that lost the ability to divide. In this regard, we could detect a significant immunoreactivity of pp60$^{c\text{-}src}$ and fyn in adult rat cardiomyocytes. Using a dot-blot kinase-assay, we also measured a tyrosine kinase activity in the cytosolic compartment but not in the crude membrane fraction of adult rat cardiomyocytes (results not shown).

As an index for PLCγ activity, we measured InsP_3 generation following purinergic stimulation. As expected from previous studies [10,11] the purinergic agonist triggers a fast increase in intracellular Ins(1,4,5)P_3 content. The increase in the content of the specific Ins(1,4,5)P_3 isomer is probably limited by its metabolism. Although inositol phosphatase activity was blocked by LiCl, InsP_3 kinase [26] most probably participates in the degradation of Ins(1,4,5)P_3 following phosphorylation to Ins(1,3,4,5)P_4 and dephosphorylation to Ins(1,3,4)P_3.

Altogether, our results raised the interesting question of how these events (i.e. translocation of PLCγ to the membrane, phosphorylation of PLCγ, and InsP_3 generation) are related. The tyrosine kinase inhibitor genistein prevents both phosphorylation and translocation of PLCγ to the membrane following purinergic stimulation of cardiomyocytes. These results indicate that phosphorylation is necessary for translocation of PLCγ to the membrane. This finding is in line with the observation that in fibroblasts genistein can induce translocation of PLCγ from the membrane to the cytosol [27]. Phosphorylation is thus likely to be the first event to occur. Besides EGF receptor kinase, genistein preferentially inhibits cytosolic tyrosine kinase [28], which further suggests that ATP acts through the activation of a non-receptor tyrosine kinase and rules out the idea that the purine could exert its phosphorylating effect on PLCγ following the inhibition of a tyrosine phosphatase. Both genistein and herbimycin also block ATP-induced InsP_3 formation. Because each of these tyrosine kinase inhibitors has different mechanisms of action (i.e. competition with kinase ATP-binding site [28] versus protein thiol group interaction with herbimycin [29]), this finding further shows that tyrosine phosphorylation is required for PLCγ activation.

Prevention of ATP-induced Ca_i-increase by loading the cells with BAPTA or by bathing them in nominally Ca-free buffer

inhibits both phosphorylation and translocation of PLCγ from the cytosolic fraction to the membrane fraction. The rise in Ca_i turns out to be the key step in the activation of PLCγ by extracellular ATP. It should be pointed out that Ca could also play a direct role in PLCγ activation as expected from the presence of a C-2 Ca-binding domain on PLCγ [30] and with the close Ca-dependence of the enzymic activity [31]. Alternatively, Ca could facilitate the interaction between PLCγ and the tyrosine kinase as shown for other src substrates [32]. Anyhow, the above findings allow us to propose the following cascade: ATP $\rightarrow$ tyrosine kinase $\rightarrow$ Ca-regulated cytosolic PLCγ phosphorylation $\rightarrow$ PLCγ translocation to the membrane $\rightarrow$ InsP_3 generation.

We thus report for the first time on the same cell type the three events, namely, phosphorylation, translocation and activation of PLCγ, that occur following stimulation of a seven-trans-membrane-domain receptor and that lead to phosphoinositides breakdown and in turn InsP_3 formation. Our result may be of pathophysiological significance. We previously reported that the purinergic agonist ATP can trigger cardiac arrhythmias by depolarizing the cardiomyocytes and in turn triggering Ca_i-rise [13,23]. This cascade of events includes activation of a tyrosine kinase that phosphorylates the Cl/HCO_3 exchanger protein leading to an intracellular acidosis [17]. PLCγ-mediated InsP_3 generation may add further to this phenomenon since InsP_3 is able to enhance Ca-induced Ca release from the sarcoplasmic reticulum [15] or to induce Ca oscillations [16]. Moreover, Du et al. [33] recently observed that gentamicin, a potent inhibitor of Ins(1,4,5)P_3 release, prevents arrhythmias *in vivo*. Therefore, the next question to address is to identify the tyrosine kinase(s) activated by extracellular ATP and the pathway that links the purinergic receptor to the cytosolic tyrosine kinase. Among other candidates, an 'src-like' tyrosine kinase could be the one that is stimulated by ATP in cardiac cells. The data obtained from such an investigation added to our present findings should help in the development of a pharmacological strategy designed to prevent deleterious purinergic modification of cardiac rhythm.

Note added in proof (received 5 July 1996)

While this paper was being proofed out, we observed a co-immunoprecipitation of PLCγ with a tyrosine kinase using an anti-cst1 antibody that recognizes both $pp^{60\text{-}cbrc}$ and fyn.

We are thankful to Dr. J.H. Brown for critical reading of the manuscript and to M. Rongier for technical assistance.

Received 12 March 1996/3 May 1996; accepted 14 May 1996

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