PLC-γ1 is required for IGF-I protection from cell death induced by loss of extracellular matrix adhesion

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Summary

Phospholipase C-γ1, a tyrosine kinase substrate, hydrolyses phosphatidylinositol 4,5-bisphosphate to produce inositol 1,4,5-trisphosphate and diacylglycerol, which act as second messenger moleculesto mobilize intracellular calcium and activate protein kinase C, respectively. We have investigated the role of phospholipase C-γ1 in anoikis, or cell death, induced by the loss of extracellular matrix adhesion. Spontaneously immortalized mouse embryonic fibroblasts nullizygous at the *Plcg1* locus (*Plcg1*$^{-/-}$), referred to as Null cells, were derived from targeted gene disruption experiments. Subsequently, phospholipase C-γ1 was re-expressed in these cells to derive Null+ cells. The Null and Null+ cells were then placed in suspension to induce cell death, which was measured directly as well as by the induction of caspase 3, as an index of programmed cell death or apoptosis. The results demonstrate that insulin-like growth factor can rescue Null+ cells but not Null cells from suspension-induced cell death. This demonstrates that phospholipase C-γ1 is required for insulin-like growth factor dependent cell survival under these conditions. Lastly, the data demonstrate that insulin-like growth factor stimulated tyrosine phosphorylation of phospholipase C-γ1 in both adherent and suspension cells.

Key words: Phospholipase C, Anoikis, Insulin-like growth factor

Introduction

Phospholipase C (PLC) isoenzymes catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI 4, 5-P_2) to inositol 1,4,5-trisphosphate and diacylglycerol, which act as intracellular second messengers to provoke the mobilization of Ca^{2+} and activation of protein kinase C, respectively (Berridge and Irvine, 1984; Nishizuka, 1986). PLC isoenzymes are organized into multiple subtypes on the basis of conservation of sequence similarities (Carpenter and Ji, 1999; Rhee, 2001). There are two isoforms of the PLC-γ subtype that mediate receptor-tyrosine-kinase-dependent PLC activity. Other PLC isoforms are not known to be downstream effectors of receptor tyrosine kinases.

PLC-γ1 and -γ2 are tyrosine kinase substrates, and tyrosine phosphorylation (Nishibe et al., 1990; Kim et al., 1991) is an essential step in their activation mechanism along with membrane translocation (Todderud et al., 1990; Falasca et al., 1998). The γ1 isoform is ubiquitously distributed in tissues and cell lines, whereas the γ2 isoform is predominantly expressed in cells of the hematopoietic lineage (Homma et al., 1989; Rhee et al., 1991). Targeted gene disruption in mice shows that *Plcg1* is essential for early embryonic development (Ji et al., 1997), whereas mice nullizygous for *Plcg2* are viable (Wang et al., 2000). Hence the two γ isoforms do not have redundant functions in the animal. A *Drosophila* PLC-γ has been identified and loss-of-function mutations lead to aberrant wing vein development (Thackeray et al., 1998).

In the absence of pharmacological inhibitors, attempts to abrogate PLC-γ1 function in growth-factor-stimulated cells have employed two distinct approaches that have yielded discordant results. In one approach, antibodies to PLC-γ1 or fragments of PLC-γ1, acting as dominant-negative molecules, have been microinjected into quiescent cells prior to treatment of the cells with platelet-derived growth factor (PDGF) or epidermal growth factor (EGF) (Roche et al., 1996; Wang et al., 1998). The conclusion drawn from these experiments is that PLC-γ1 is essential for mitogenesis induced by these growth factors. In separate experiments using different cell lines, mutagenesis of the PLC-γ1 specific association site on the PDGF β-receptor has been employed to prevent PLC-γ1 activation in PDGF-treated cells. The conclusion of these experiments is that the loss of PLC-γ1 activation has either no effect (Ronnstrand et al., 1992; Rosenkranz et al., 1999) or produces a small (30%) decrease in mitogenesis (Valius et al., 1993).

We have used mouse embryos from gene disruption experiment to establish immortalized cell lines of defined *Plcg1* genotypes (Ji et al., 1997). The *Plcg1* nullizygous cells do not mobilize Ca^{2+} in response to growth factors, whereas wild-type cells (Ji et al., 1997) or Null cells in which PLC-γ1 has been re-expressed (Ji et al., 1999) do demonstrate a Ca^{2+} response to growth factors. Comparison of these cell lines in mitogenesis assays has not shown a requirement for PLC-γ1 in the induction of DNA synthesis by EGF (Ji et al., 1998) or PDGF (Liao et al., 2001).

The control of fibroblastic cell proliferation involves not only signaling from receptor tyrosine kinases but also signaling from adhesion receptors (Schwartz, 1997; Giancotti and Ruoslahti, 1999; Aplin et al., 1999). Integrin activation is known to increase intracellular Ca^{2+}, although it remains unclear whether this involves PLC-γ or another mechanism (Clark and Brugge, 1995; Sjaastad and Nelson, 1996). It is

possible, therefore, that adhesion-dependent signals are able to substitute for the loss of PLC-γ1 from growth factor receptor signaling pathways. Therefore, we have attempted to determine whether PLC-γ1 has a role in the survival of cells placed in suspension and in the absence of adhesion-dependent signaling.

Materials and Methods

Materials

Poly (2-hydroxyl methacrylate) (Poly-HEMA), Protein A Sepharose, aprotinin, leupeptin, pepstatin and phenylmethylsulfonyl fluoride, hydrogen peroxide and reagents for enhanced chemiluminescence (ECL) were obtained from Sigma. Recombinant insulin-like growth factor (IGF-I) was purchased from Gibco. Phosphotyrosine antibodies were purchased from Zymed Laboratories Inc., IGF-I receptor antibodies were from Transduction Laboratories, and anti-PLC-γ1 was reported elsewhere (Arteaga et al., 1991). Immobilin-P membranes were from MCI. The following buffers were used: cell lysis buffer (10 mM Tris-HCl, 10 mM $NaH_2PO_4/NaHPO_4$, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaPPi), protease assay buffer (20 mM HEPES, pH 7.5, 10% glycerol, 2 mM DTT), TGH buffer (1% Triton X-100, 10% glycerol, 50 mM HEPES, pH 7.2).

Cell line

Spontaneously immortalized *Plcg1*$^{-/-}$ mouse embryonic fibroblasts, referred to as Null cells, were prepared from E9.5 mouse embryos with targeted disruption of the *Plcg1* gene (Ji et al., 1997). The rat PLC-γ1 full-length cDNA (a generous gift from Sue Goo Rhee, National Institutes of Health) was stably expressed in the *Plcg1*$^{-/-}$ cells by retroviral infection to generate PLC-γ1 re-expressing cells referred to as Null+ cells (Ji et al., 1999). Null+ cells have been derived from two independent Null cell lines referred to as targeting vector (TV)-I and TV-II. Unless otherwise stated, TV-I Null and Null+ cells are employed in the experiments reported in this manuscript. All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Suspension culture

Regular cell culture dishes were coated with a film of poly-HEMA following the protocol reported by Folkman and Moscona (Folkman and Moscona, 1978). Briefly, a solution of 120 mg/ml of polyHEMA in 95% ethanol was mixed overnight, centrifuged at 800 ***g*** to remove undissolved particles and diluted 1:10 in 95% ethanol. The resulting solution was then placed in the culture dishes. The dishes were left to dry at room temperature overnight. Before use, the coated dishes were washed twice with phosphate buffered saline (PBS).

Anoikis protocol

Near-confluent cells on regular plastic tissue culture dishes were trypsinized to produce a single cell suspension. Trypsin was inactivated by the addition of either soybean trypsin inhibitor or 10% FBS. Approximately 2.5×10^5 cells were placed in each well of a six-well dish precoated with poly-HEMA to prevent cell attachment. At the indicated times, cells were collected, washed with PBS and resuspended by trypsinization. Viable cells were counted in a hemocytometer using the trypan blue exclusion procedure.

Caspase 3 activity assay

Caspase 3 activity present in cells under different culture conditions was measured using a fluorometric assay (Pharmingen Inc.) according to the manufacturer's protocol. In brief, cells were harvested, washed with PBS and lysed by incubating with chilled cell lysis buffer for 15 minutes at 4°C. The supernatant was collected by centrifugation at 14,000 ***g*** for 15 minutes, and the protein concentration was measured by the Bradford method using bovine serum albumin as the standard. Cell extracts (30 μg protein) were incubated for 1 hour at 37°C in a protease assay buffer containing the caspase-3-specific substrate N-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin). After 1 hour of incubation, the release of the fluorogenic compound AMC from the substrate was measured in a spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength at 440 nm. The fluorescence unit (2.5×10^6) for the emission at 440 nm for PLC-γ1 Null cell in serum-free condition was considered as 100%.

Immunoprecipitation and western blotting

Cells were placed in 100 mm tissue culture dishes and incubated at 37°C for 48 hours in medium containing 10% FBS. At a confluence level of 50-70%, the cells were serum-starved by incubating overnight in medium containing 0.5% FBS. After trypsinization, the cells were placed in suspension culture in poly-HEMA-coated dishes. IGF-I (100 ng/ml) was added as indicated, and the cells were incubated at 37°C for 15 minutes. The cells were then collected, washed with PBS and lysed by incubating with TGH lysis buffer for 20 minutes at 4°C. Insoluble materials were then removed by centrifugation (14,000 ***g*** for 10 minutes) at 4°C. Protein concentrations were assayed by the method of Bradford. To immunoprecipitate the IGF-I receptor or PLC-γ1, the primary antibodies were incubated with 1 mg of cell lysate overnight at 4°C. This was followed by a 1 hour incubation with Protein A Sepharose. Immune complexes were subsequently washed three times with TGH lysis buffer, resuspended in 1× Laemmli buffer and boiled for 5 minutes. The samples were then electrophoresed on an 8% SDS-polyacrylamide gel and subsequently transferred to nitrocellulose membranes for western blotting. Blotting was performed as described elsewhere (Ji et al., 1999). Bound antibody was detected by enhanced ECL.

Results

Influence of PLC-γ1 on cell survival during anoikis

To prevent cell death, mammalian cells require survival signals generated from adhesion to the extracellular matrix (ECM) (Frisch and Francis, 1994; Meredith et al., 1993) and/or from growth factors (Aplin et al., 1999; Giancotti and Ruoslahti, 1999; Schwartz, 1997). Generally, epithelial cells require survival signals from both growth factors and from ECM attachment, whereas fibroblast survival depends on signals either from growth factors or from ECM adhesion (Meredith and Schwartz, 1997). We have previously generated spontaneously immortalized mouse embryonic fibroblasts, nullizygous at the *Plcg1* locus (*Plcg1*$^{-/-}$) (Ji et al., 1997), which are referred to herein as Null cells. Subsequently PLC-γ1 was re-expressed by retroviral infection in the Null cells to derive Null+ cells (Ji et al., 1999).

To explore the role of PLC-γ1 in cell survival signaling, we compared the sensitivity to cell death of *Plcg1* Null and Null+ cells in the absence of cell adhesion. Trypsinized cells were plated in tissue culture dishes or in dishes pre-coated with poly-HEMA to prevent cell attachment. Cell viability was determined in these adherent and suspension cultures in the presence or absence of 10% FBS as a source of growth-factor-dependent survival signals. Trypan blue staining was employed to calculate the percentage of non-viable cells 20 hours after plating. The data in Fig. 1A show that, when Null and Null+

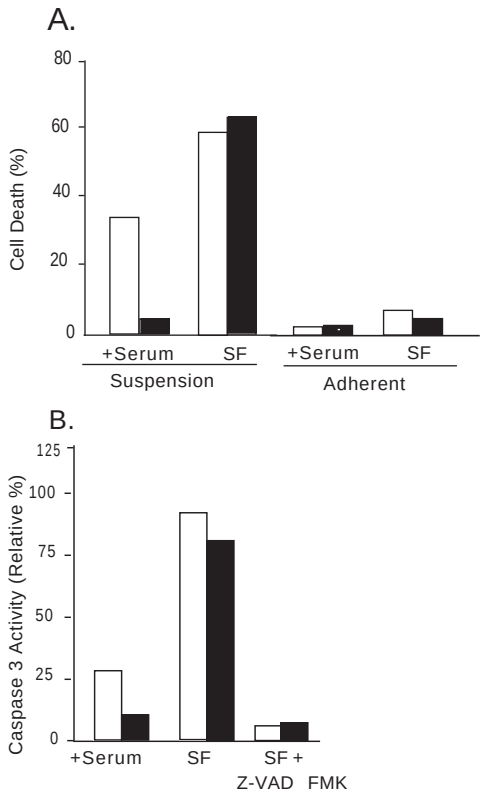

Fig. 1. The influence of PLC-γ1 on cell survival. (A) Null and Null+ cells were plated on either regular tissue culture plastic dishes to adhere or on tissue culture dishes coated with poly-HEMA for suspension culture. 10% FBS was added as indicated (+Serum) and cell viability was measured 20 hours later by calculating the ratio of trypan-blue-positive cells to live cells. The open and closed bars represent Null and Null+ cells, respectively. Each bar indicates the average of triplicate assays. SF indicates serum-free media. (B) Null and Null+ cells were seeded on poly-HEMA coated dishes. 10% FBS or a broad spectrum caspase inhibitor Z-VAD-FMK (20 μM) were added as indicated. Cells were collected 20 hours later, washed with PBS and cell lysates were prepared using cell lysis buffer. Caspase 3 activity present in the cell lysates was determined as described in the Materials and Methods. Open and closed bars indicate Null and Null+ cells, respectively.

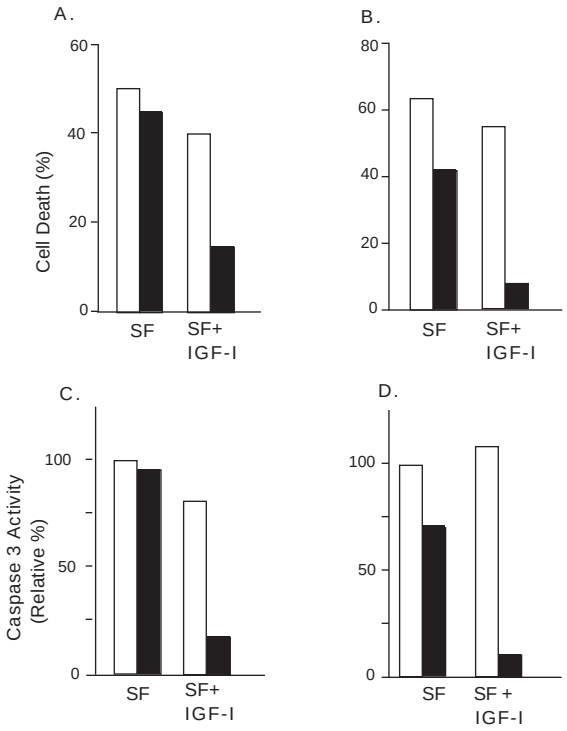

Fig. 2. Capacity of IGF-I to protect Null and Null+ cells from loss of extracellular-matrix-induced cell death. Both TVI (A,C) and TVII (B,D) Null and Null+ cells were placed in suspension by seeding on poly-HEMA coated dishes. IGF-I (100 ng/ml) was added to the medium as indicated, and the cells were harvested 20 hours later. Cell viability was determined using trypan blue staining (A,B) or caspase 3 activity (C,D). Open and closed bars represent results for Null and Null+ cells, respectively.

cells are placed in suspension under serum-free conditions, cell death occurs at nearly equivalent levels (approximately 60%) in both cell types. In the presence of serum, however, cell death was considerably higher in the Null cells (35%) compared with the Null+ cells (5%). When the Null and Null+ cells are allowed to adhere to the culture dish surface in the presence or absence of serum for the same amount of time, the data show that no significant cell death is induced in either Null and Null+ cells.

These results indicate that Null+ cells demonstrate the expected characteristics of fibroblasts for cell survival in suspension. Namely, that the presence of serum growth factors prevent cell death. However, Null cells in suspension are significantly less protected from cell death by serum growth factors. Similar results were obtained when cell death was assayed by propidium iodide exclusion.

Cell death measured under these conditions for Null and Null+ cells can be considered to reflect programmed cell death because of the DNA fragmentation or laddering (data not shown) and the induction of caspase 3 as shown in Fig. 1B. In this experiment, caspase 3 was induced to about the same level in Null and Null+ cells placed in suspension under serum-free conditions. However, in suspension cultures to which serum was added to promote survival, caspase induction in Null cells was three-fold greater than in Null+ cells. Therefore, the presence of PLC-γ1 is likely to be necessary molecule for serum-mediated survival of cells deprived of matrix attachment. The addition of the caspase inhibitor Z-VAD-FMK confirms that the proteolytic activity measured in this assay is caused by caspase activity.

Influence of IGF-I on the survival of Null and Null+ cells

IGF-I is a growth factor component of serum and is considered to be a major inhibitor of apoptosis in serum (Baserga et al., 1997). Therefore, we examined the influence of IGF-I on the survival of Null and Null+ cells placed in suspension under serum-free conditions. In addition, we tested a second independent pair of Null and Null+ cells, referred to as TV-II, for their capacity to survive under suspension conditions. In Fig. 2A,B, the results of cell viability assays (trypan blue staining) are reported, and the induction of caspase 3 activity is shown in Fig. 2C,D. Both assays show that IGF-I protects Null+ cells much more effectively than Null cells from cell

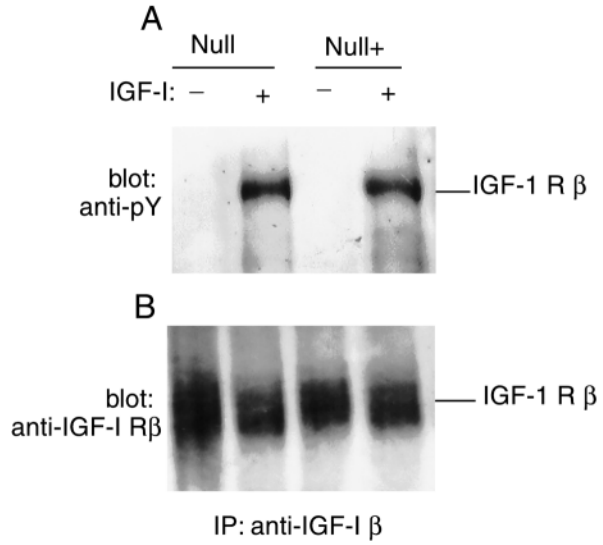

Fig. 3. The level of IGF-I receptor and its autuphosphorylation in Null and Null+ cells. Adherent Null and Null+ cells were incubated at 37°C in medium containing 10% FBS. At a confluence level of 50-70%, the cells were serum-starved by incubating overnight in medium containing 0.5% FBS. After trypsinization the cells were placed in suspension culture by plating on poly-HEMA coated dishes. IGF-I (100 ng/ml) was added as indicated and incubated at 37°C for 15 minutes. The cells were then lysed in TGH buffer, and the cell extracts were subjected to immunoprecipitation. (A) An aliquot (1 mg) of lysate was immunoprecipitated with IGF-I receptor antibody and analyzed by western blotting with anti-phosphotyrosine. (B) The phosphotyrosine blot was stripped and re-probed with IGF-I receptor antibody.

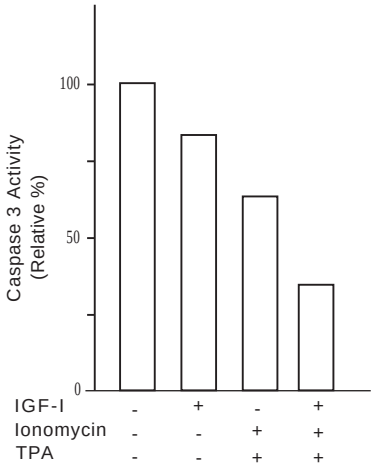

Fig. 4. Capacity of ionomycin and TPA to rescue IGF-I-mediated survival in Null cells. Null cells were placed in suspension culture as described in Fig. 2. 10% FBS, IGF-I (100 ng/ml), or ionomycin (2 μM) and TPA (100 ng/ml) were added as indicated. Caspase 3 activity was measured 20 hours later.

death induced by suspension culture. Also, comparable results in both assays were obtained for TV-I Null and Null+ cells (Fig. 2A,C) and the independently derived TV-II Null and Null+ cells (Fig. 2B,D). These cell lines are also responsive to mitogenic growth factors, such as EGF and PDGF, which may also act as survival factors. When each of these growth factors was tested in the anoikis protocol, they promoted only a low level of survival (approximately 20%) compared with IGF-I.

Since IGF-I-mediated cell survival signaling in suspension culture is impaired in the Null cells, the IGF-I receptor level and IGF-I induced receptor phosphorylation in Null and Null+ cells were compared. The results are depicted in Fig. 3 and demonstrate that Null and Null+ cell lines have comparable levels of IGF-I receptors and that the receptors are autophosphorylated in a ligand-dependent manner in both cell lines.

PLC-γ1 function in suspension culture

The data presented in this paper suggest that PLC-γ1 is necessary for IGF-I-mediated cell survival in suspension culture. If this is a critical difference between Null and Null+ cells in these assays, then 12-O-tetradecanoyl phorbol-13-acetate (TPA) and ionomycin, pharmacological agents that replace downstream PLC-γ1 functions, should promote survival of cells that do not contain PLC-γ1. Ionomycin raises intracellular Ca^{2+} levels, whereas TPA constitutively activates protein kinase C. To test this possibility, Null cells were placed in suspension and treated with IGF-I or ionomycin and TPA or IGF-I together with ionomycin and TPA. Subsequently, caspase 3 activity was measured as an index of cell death. The results are shown in Fig. 4. As expected from previous data, the presence of IGF-I did not significantly lower caspase 3 activation when the Null cells were placed in suspension. Treatment with ionomycin and TPA, however, decreased caspase 3 activation by almost 40%, suggesting that pharmacological replacement of PLC-γ1 function does enhance cell survival. To test whether IGF-I signaling might further increase cell survival in the presence of TPA and ionomycin, all three agonists were added to the suspension of Null cells. Under these conditions, caspase 3 activation was reduced by approximately 70%. The results of this experiment are consistent with the conclusion that PLC-γ1 activity contributes to IGF-I-induced cell survival pathways. The increased level of reduction of caspase 3 activity by ionomycin and TPA in the presence of IGF-I indicates that IGF-I contributes signals to cell survival that are not entirely replaced by ionomycin and TPA.

If PLC-γ1 is involved in IGF-I-dependent survival of cells placed in suspension, then it would be expected that IGF-I might induce the tyrosine phosphorylation of PLC-γ1 under these conditions. This has been tested in the experiment shown in Fig. 5 using Null+ that are adherent or placed in suspension. In either situation, the addition of IGF-I did not provoke a detectable level of PLC-γ1 phosphorylation. However, in the presence of the phosphotyrosine phosphatase inhibitor pervanadate and IGF-I the tyrosine phosphorylation of PLC-γ1 was readily detected. Pervanadate by itself did not yield detectable phosphorylation of this protein.

When cells are placed in suspension culture, recent evidence indicates that the cellular content of some signaling proteins, for example, Ras (Gatzka et al., 2000) and insulin receptor substrate-1 (IRS-I) (Leburn et al., 2000) are dramatically decreased within a few hours. Also, it has been reported that the induction of apoptosis by etoposide in Molt-4 cells results in the caspase-dependent cleavage of PLC-γ1 (Bae et al., 2000). In view of these reports we have examined the

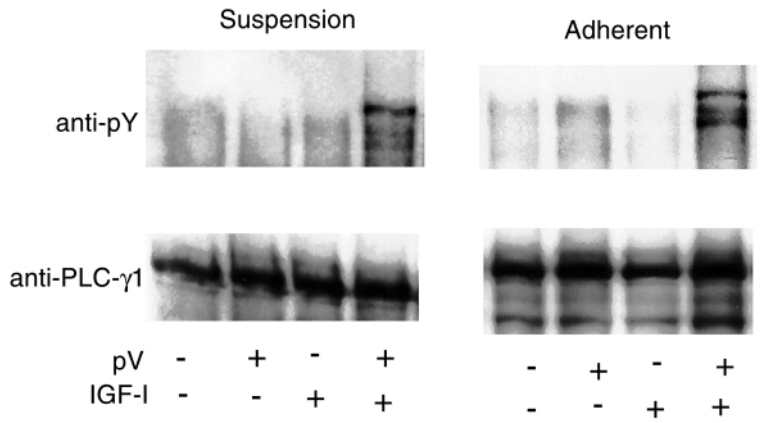

Fig. 5. Detection of IGF-I-dependent tyrosine phosphorylation. Null+ cells were either untreated or treated with 100 μM pervanadate (pV) for 15 minutes at 37°C. Then the cells were incubated with IGF-I (100 ng/ml) at 4°C for 45 minutes in suspension culture or in adherent condition as indicated. The cells were lysed in TGH buffer, and the cell extracts were subjected to immunoprecipitation. (A) An aliquot (1 mg) of lysate was immunoprecipitated with PLC-γ1 antibody and analyzed by western blotting with anti-phosphotyrosine. (B) The phosphotyrosine blot was stripped and re-probed with PLC-γ1 antibody.

metabolic stability of PLC-γ1 in Null+ cells placed in suspension. The results showed that during a 10 hour incubation in suspension culture, in serum-free medium without IGF-I, the cellular level of PLC-γ1 was unaltered (data not shown). Therefore, these results do not suggest that PLC-γ1 is metabolically destabilized by suspension culture.

Influence of PI-3 kinase and MAP kinase pathways on IGF-I survival signaling

The two major cell signaling pathways utilized by IGF-I in protection from apoptosis are the PI-3 kinase and mitogen-activated protein (MAP) kinase pathways. In view of the potential role of PLC-γ1 signaling in IGF-I-dependent cell survival, the capacity of pharmacologic inhibitors of PI-3 kinase and MAP kinase activation were tested for their influence on IGF-I-mediated survival of Null+ cells in suspension culture. Null+ cells were treated with LY294002, a stable inhibitor of PI-3 kinase, and/or PD98059, an inhibitor of MAP kinase activation, prior to the addition of IGF-I. Caspase 3 activity was then measured 20 hours after plating. The results, presented in Fig. 6, show that in the presence of LY294002 or PD98059, the capacity of IGF-I to prevent caspase 3 activation was reduced by approximately 50%. However, when the two inhibitors were added together, the protective effect of IGF-I on caspase 3 induction was completely prevented. This suggests that the IGF-I-dependent survival of these cells in suspension is also dependent on signaling through PI-3 kinase and MAP kinase.

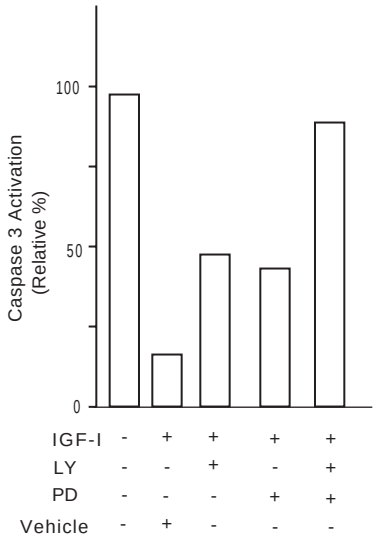

Fig. 6. Pharmacological evaluation of signaling pathways that mediate the IGF-I-dependent cell survival of Null+ cells. Null+ cells were placed in suspension and IGF-I (100 ng/ml), LY294002 (50 μM), or PD 98509 (50 μM) were added as indicated. The cells were then incubated at 37°C for 20 hours, lysed, and caspase 3 activity present in the lysates was measured.

Discussion

The major conclusion from these studies is that PLC-γ1 is necessary for IGF-I to exert a survival influence on fibroblasts placed in suspension. This implies that at least under these conditions, which in the absence of IGF-I result in a form of apoptosis termed anoikis, that PLC-γ1 has an anti-apoptotic or survival function. Using adherent cells, it has been reported that *Plcg*1-null cells are more sensitive to cell death than wild-type cells following the application of oxidative stress (Wang et al., 2001) or heat stress (Bai et al., 2002). One report has presented data indicating that overexpression of PLC-γ1 in PC-12 cells inhibits ultraviolet-irradiation-induced apoptosis, which is also suggestive of an anti-apoptotic function for this protein (Lee et al., 1999). The same group, however, observed that in NIH 3T3 cells overexpression of PLC-β, but not PLC-γ1, suppressed apoptosis induced by oxidative stress (Lee et al., 2000). In B cells genetically deficient in PLC-γ2, surface immunoglobulin M-induced apoptosis is abrogated (Takata et al., 1995), but radiation-induced apoptosis is not affected (Uckun et al., 1996). Therefore, the participation of PLC-γ isoforms in apoptotic or anti-apoptotic signaling may be dependent on cell type and/or the nature of the stimulus.

The major signaling pathways activated by ligand occupation of the IGF-I receptor are the Ras/MAP kinase pathway and the PI-3 kinase-dependent activation of Akt, and both of these pathways are reported to be necessary for the anti-apoptotic function of IGF-I (Kulik et al., 1997; Kulik et al., 1998; Parrizas et al., 1997; Peruzzi et al., 1999). This is consistent with our data showing that inhibitors of these pathways prevent IGF-I from protecting Null+ cells from cell death in suspension culture. Recently, a third IGF-I signaling pathway that participates in cell survival has been identified in

32 D cells (Peruzzi et al., 1999). This pathway depends on raf activation and translocation to mitochondria. All three of these pathways are known to promote the phosphorylation of BAD and thereby, at least in part, prevent apoptosis.

The means by which PLC-γ1 may participate in anti-apoptotic signaling is unclear. Ca^{2+} can induce or prevent apoptosis depending on the cell line and the nature of the calcium signal (Berridge et al., 1998). IGF-I has been shown to promote Ca^{2+} entry into the cells, inositol 1,4,5-trisphosphate formation, the production of 1,2-diacylglycerol and activation of protein kinase C (Kojima et al., 1988; Kojima et al., 1990; Kojima et al., 1993; Takasu et al., 1989; Munaron and Pla, 2000). These intracellular signaling events are consistent with the activation of a PLC isoenzyme. The tyrosine phosphorylation of PLC-γ1 induced by IGF-I, which is constitutively activate IGF-I receptors, or insulin has been reported for adherent cells (Jiang et al., 1996; Foncea et al., 1997; Hong et al., 2001; Eichhorn et al., 2001), and our data extends this to IGF-I-treated cells in suspension.

In our study the addition of pervanadate was necessary to detect PLC-γ1 tyrosine phosphorylation. This seemingly low level of phosphorylation may be influenced by two factors. First, in all cell systems the agonist-dependent tyrosine phosphorylation of PLC-γ1 is transient, and the detected level of tyrosine phosphorylation therefore depends on the level of synchrony in the cell population response to the agonist. Second, others have shown that phosphotyrosine phosphatase activity is increased when cells are placed in suspension (Sabe et al., 1997). Under these conditions the addition of pervanadate is effective in revealing the presence of tyrosine phosphorylated proteins. However, in our experiment pervanadate was also required to detect PLC-γ1 tyrosine phosphorylation in adherent cells treated with IGF-I. This does suggest that IGF-I is not a particularly strong stimulator of PLC-γ1 tyrosine phosphorylation. However, it is possible that the observed level of tyrosine phosphorylation is sufficient to transiently activate PLC-γ1 or that pervanadate only partially overcomes phosphatase activity and in situ PLC-γ1 is actually phosphorylated to a higher extent. Also, several mechanisms have been proposed to enhance the activation of PLC-γ1 in concert with tyrosine phosphorylation or in its absence (Rhee, 2001). These include potential modulatory molecules such as phosphatidic acid, phosphatidylinositol 3,4,5-trisphosphate, arachidonic acid and the 680k Da protein AHNAK. Whether any of these are relevant to the IGF-dependent control of PLC-γ1 activity during anoikis is not known.

A mechanism for IGF-I-induced Ca^{2+} influx in cells is reported to involve the IGF-I-dependent translocation of a calcium-permeable channel to the plasma membrane through a PI-3-kinase-dependent signal (Kanzaki et al., 1999). The means by which the opening of this channel is regulated at the cell surface is not known. However, this channel is structurally related to the transient-receptor-potential (TRP) channel family, and these channels are thought to be activated by the depletion of intracellular stores of calcium by an unclear mechanism (Putney, 1999). Hence, PLC-γ1 activation by IGF-I could provide a means to empty intracellular calcium stores and thereby provoke influx of extracellular calcium through this TRP channel.

Interestingly, cell adhesion is known to regulate the plasma membrane level of PI 4,5-P_2. When cells are placed in suspension culture, cellular PI 4,5-P_2 levels decline significantly and are restored quickly when cells are allowed to adhere to a substratum (McNamee et al., 1993; Oude Weernink et al., 2000). This change is thought to reflect changes in the activity of PI4P-5 kinase. However, suspension also increases the cellular level of inositol phosphates provoked by agonists, such as PDGF, that act on PLC-γ1. Hence, the reductions in PI 4,5-P_2 levels provoked by suspension conditions do not seem to sufficiently abrogate PLC-γ1 activation through the receptor tyrosine kinases.

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