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Differential Regulation of Phospholipase C-β Isozymes in Cardiomyocyte Hypertrophy

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Cardiac hypertrophy is a major predictor of heart failure and of morbidity and mortality in developed countries. Many hormones and growth factors induce cardiac hypertrophy via activation of members of the phospholipase C (PLC) family. The expression pattern of the PLCβ isozyme subfamily was investigated in neonatal rat cardiomyocytes after stimulation with different hypertrophic stimuli. Under control conditions and after stimulation with norepinephrine, cardiomyocytes expressed similar amounts of PLCβ3 mRNA. In the presence of fetal calf serum (FCS), additional expression of PLCβ1 was induced. Growth hormone (GH) and insulin-like growth factor-I (IGF-I) both induced a substantial increase in PLCβ3 mRNA expression. The response to GH could not be abolished by the IGF-I receptor blocker IGF-I analogue indicating an IGF-I-independent action of GH. The upregulation of PLCβ3 by IGF-I was abolished by preincubation of cardiomyocytes with the IGF-I receptor antagonist IGF-I analogue, the tyrosine kinase inhibitor genistein, the extracellular signal-related kinase (ERK) inhibitor PD 98059, the phosphatidylinositol-3-(PI-3) kinase inhibitor wortmannin and the p70 S6 kinase inhibitor rapamycin. Induction of the immediate early genes c-myc, c-fos, and c-jun by IGF-I was abolished by preincubation with antisense oligos against PLCβ3. It is concluded that the expression of PLCβ isozymes in cardiomyocytes is differentially regulated by different hypertrophic stimuli. The upregulation of PLCβ3 by IGF-I is dependent on the activity of tyrosine kinase, ERK, PI3 kinase, and p70 S6 kinase and PLCβ3 expression seems to be required for the induction of immediate early genes by IGF-I. The involvement of the PLCβ subfamily in signal transduction of receptors other than G-protein-coupled receptors is suggested. © 2000 Academic Press

Abbreviations used: DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; ERK, extracellular signal-related kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GH, growth hormone; IGF-I, insulin-like growth factor I; IRS-1, insulin receptor substrate 1; Jak, Janus kinase; MAP kinase, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PI3-kinase, phosphatidylinositol-3-kinase; PLC, phospholipase C; PtdIns (3,4,5)P_3, Phosphatidylinositol (3,4,5)-trisphosphate; RT-PCR, reverse transcription and polymerase chain reaction; SDS, sodium dodecyl sulfate; Stat, signal transducer and activator of transcription; mTOR, target of rapamycin.

In the mammalian heart, three major receptor families are involved in hypertrophic responses [1, 2]: (i) G protein-coupled receptors such as the α1-adrenoceptor [3], (ii) tyrosine kinase receptors such as the IGF-I receptor [4] and (iii) cytokine receptors such as the GH receptor [5]. The intracellular signal cascades of these receptors include phospholipase C. The PLC enzyme family consists of three families, PLCβ, PLCγ, and PLCδ, each of which comprising several members [6, 7]. The PLCβ family includes four distinct isozymes designated PLCβ1–4 and has been implicated in Gq protein-coupled signaling, the activation of which induces cardiac hypertrophy and failure [7–9]. Is has not yet been investigated which isozymes of the PLCβ family are involved in cardiac hypertrophy. The increase in PLC activity assumed to be important for the hypertrophic response might be due to activation of preexisting enzyme, to increased biosynthesis of the isozyme present or to a change of the isozyme expression pattern. In the present study, expression of PLCβ isozymes in response to norepinephrine, and GH and IGF-I was investigated.

MATERIALS AND METHODS

Materials. IGF-I and IGF-I analogue were purchased from Bachem (Heidelberg, FRG). Genistein and PD 98059 were from Calbiochem (Schwalbach, FRG). Wortmannin and rapamycin were purchased from Sigma (Deisenhofen, FRG). Oligonucleotide primers for PCR and antisense oligonucleotides were synthesized by MWG-Biotech (Ebersberg, FRG). All other materials were obtained from standard vendors or sources previously described [11].

Neonatal rat cardiomyocytes. Cardiomyocytes from neonatal Sprague–Dawley rats were isolated and cultured as described elsewhere [10, 12]. Plating density was about 3×10^5 cells/cm^2, cardiomyocytes were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) fetal calf serum (FCS) at 37°C at 5% (v/v) CO_2. The cardiomyocytes showed spontaneous contraction. Medium was changed after 24, 48, and 72 h. 96 h after isolation,

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complete medium was replaced by serum-free medium and cultivation was continued for 24 h before application of IGF-I. In case of incubation for more than 24 h in the presence of IGF-I, medium was changed after 24 h.

RT-PCR. Total RNA from rat left ventricular myocardial tissue and from neonatal rat cardiomyocytes was prepared according to the modified method of Chomczynski and Sacchi [13] using the RNA Clean Kit from AGS (Heidelberg, FRG). Aliquots of 1.25–3 μg of total RNA were subjected to Moloney Mouse Leukemia Virus Reverse Transcriptase (Gibco-BRL, Bethesda, MD; 200 U) for 10 min at 23°C, 45 min at 42°C and 5 min at 95°C as described [11] in the presence of 100 pmol random hexamer (Boehringer, Mannheim, FRG) and 20 U RNase inhibitor (Promega, Heidelberg, FRG). Subsequent PCR amplifications of the first strand cDNA was performed in a 100 μl reaction using *Thermus aquaticus* DNA polymerase (2.5 U; Boehringer, Mannheim, FRG). The reaction mixture was made up of the same components previously described. For the amplification of the 1002 bp PLCβ3 fragment the primers 5′-cagcaggcc-aagatggcagagtactgc-3′ and 5′-accaaacatgtccacctcgac-3′ were used. The 450 bp GAPDH fragment was amplified using the primers 5′-accacagtccatgccatcac-3′ (upstream) and 5′-tccaccaccctgttgctgta-3′ (downstream). The 548 bp c-myc fragment was amplified using the primers 5′-agtgcattgatccctcagtggtctttcccta-3′ (upstream) and 5′-cagctcgttcctcctctgacgttccaagacgtt-3′ (downstream). The 548 bp c-fos fragment was amplified using the primers 5′-gagctgacaga-tacactccaagcg-3′ (upstream) and 5′-cagtctgctgcatagaaggaaccg-3′ (downstream). Each reaction contained a negative control without cDNA template to exclude contamination of the reaction with DNA. For semi-quantification, PCR conditions were chosen so that the reaction was within the exponential range with respect to the amount of cDNA template and number of cycles performed. The same cDNA samples were subjected to PCR with primers complementary to GAPDH and the signals were equal in the samples on the same gel. All PCR products were subcloned into pBluescript and subjected to DNA sequencing. No differences between the amplified and the known sequences occurred. The experiments shown were representative of at least three experiments.

Antisense oligonucleotides. Antisense oligonucleotides containing a phosphothioate modification were synthesized by MWG-Biotech, Ebersberg, FRG. The sequences used were 5′-cagggtctccaccacggc-3′ (PLCβ3 oligo 1) and 5′-cgcccgccatggccaagc-3′ (PLCβ3 oligo 2) as well as the respective sense oligos. Oligo 1 was located in the amino terminal region of the molecule, oligo 2 spanned the start codon. Antisense oligos against c-myc were 5′-aacgttgaggggcat-3′ according to [14] and the respective sense oligo. Cells were preincubated with 5 μM or 15 μM oligonucleotide for 1 h before application of IGF-I.

RESULTS AND DISCUSSION

PLC isozymes have been implicated in the hypertrophic response of the heart to a variety of stimuli including growth factors, hormones and mechanical stretch [1, 2, 15]. In neonatal rat cardiomyocytes, norepinephrine induces a hypertrophic response via α1-adrenoceptors, Gq proteins and PLCβ isozymes [10, 16]. The pathophysiological importance of this pathway is underlined by the findings that cardiac-specific transgenic overexpression of Gqα results in cardiomyocyte hypertrophy, increased apoptotic cell death and dilated cardiomyopathy [8, 9]. In addition, the expression of Gqα, PLCβ1 and PLCβ3 is increased in viable and scar tissue after myocardial infarction and a role for this signaling pathway in cardiac remodeling has been suggested [17]. In order to investigate whether

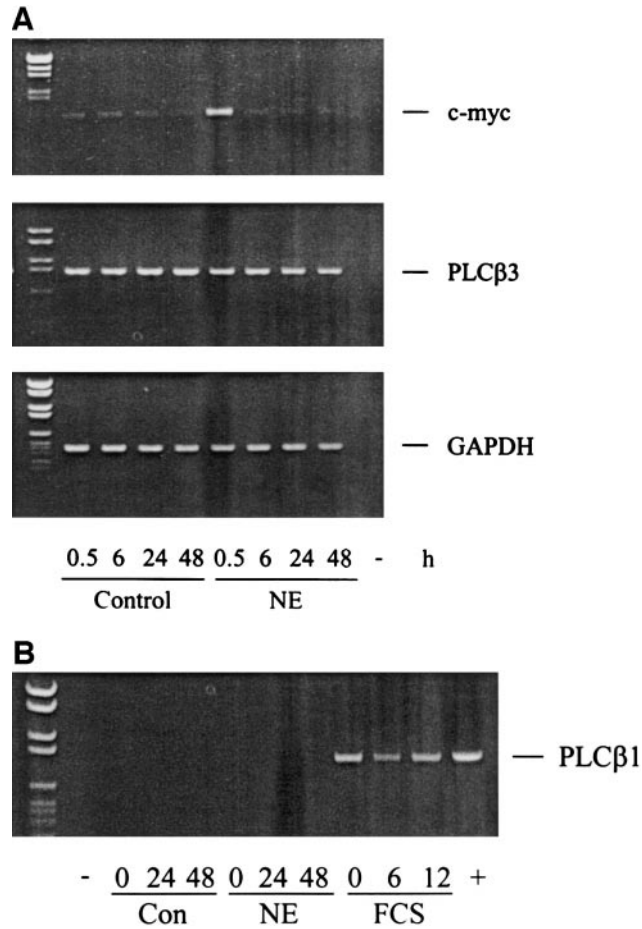

FIG. 1. (A–B) Effect of norepinephrine (NE) and fetal calf serum (FCS) on PLCβ expression. Neonatal rat cardiomyocytes were incubated in the absence (Control) or presence of norepinephrine (NE; 2 μM) for the time indicated (A). RT-PCR of PLCβ3 was performed as described under Materials and Methods. (B) Effect of norepinephrine and fetal calf serum on expression of PLCβ1. Neonatal rat cardiomyocytes were cultured in serum-free medium for 48 h before addition of norepinephrine (NE; 2 μM) or further incubation under the same conditions (Con) for the time indicated. Alternatively, cells were cultured in the presence of fetal calf serum (FCS; 10% (v/v)) for 48 h followed by further incubation with FCS for the time indicated. RT-PCR was performed using gene-specific primers for PLCβ1 as described under Materials and Methods. PLCβ1 cDNA was amplified as a positive control (+).

the hypertrophic response to norepinephrine was accompanied by a change in PLCβ isozyme expression pattern, we performed RT-PCR with mRNA from norepinephrine-treated neonatal rat cardiomyocytes (Fig. 1). Under control conditions, cardiomyocytes expressed PLCβ3, but not PLCβ1 mRNA. This expression pattern was not affected by treatment of the cells with norepinephrine. The amount of PLCβ3 mRNA was similar in cardiomyocytes treated with norepinephrine and control incubations over a time period of 48 h (Fig. 1A). In the experiment shown in Fig. 1B using gene-specific primers for PLCβ1, cardiomyocytes were cultured for 48 h either in serum-free medium

(Con; NE) or in medium containing fetal calf serum (FCS; 10%) before addition of the stimuli. Incubation in the presence of FCS lead to expression of PLCβ1 mRNA (Fig. 1B), while incubation with norepinephrine (NE; 2 μM) did not. These findings indicate that the hypertrophic response of cardiomyocytes to norepinephrine is not associated with a PLCβ isozyme shift or a marked increase in PLCβ3 mRNA expression, but rather with an activation of preexisting PLCβ3 enzyme. Fetal calf serum induces a growth response in many cell types and it is tempting to speculate that expression PLCβ1 might contribute to this response.

Growth hormone (GH) is a hypertrophic factor which exerts its intracellular effects independently from heterotrimeric G proteins [5]. The non-receptor tyrosine kinase Jak and subsequent activation of Stat are involved in GH receptor-coupled signal transduction. However, most of the biological effects of GH are mediated by secretion of IGF-I in the liver and other tissues such as the heart [18], which also contains IGF-I and GH receptors [19, 20]. PLCγ1 has been implicated in the growth response to GH [5] as well as IGF-I [20]. Surprisingly, in our experiments the mRNA of PLCβ3, an isozyme thus far implicated in G protein-coupled signal transduction, was upregulated after 30 min in response to GH and IGF-I (Fig. 2A). The early growth response gene c-myc was also induced by GH and IGF-I, while the amount of GAPDH mRNA was similar in all samples (Fig. 2A). PLCβ1 expression was not induced by GH or IGF-I. In order to investigate whether the upregulation of PLCβ3 expression was due to GH receptor signaling or autocrine/paracrine activation of the IGF-I signaling cascade, cardiomyocytes were preincubated with the peptide IGF-I receptor antagonist IGF-I analogue [21] before application of GH. Figure 2B illustrates that the upregulation of c-myc and PLCβ3 by GH was not abolished by preincubation with IGF-I analogue. As a positive control for the effect of IGF-I analogue, cardiomyocytes were incubated with the blocker before application of IGF-I. As expected, the IGF-I-induced upregulation of c-myc and PLCβ3 expression was abolished by IGF-I analogue (Fig. 2B). The results suggest that both GH and IGF-I lead to an early upregulation of PLCβ3 by two distinct mechanisms.

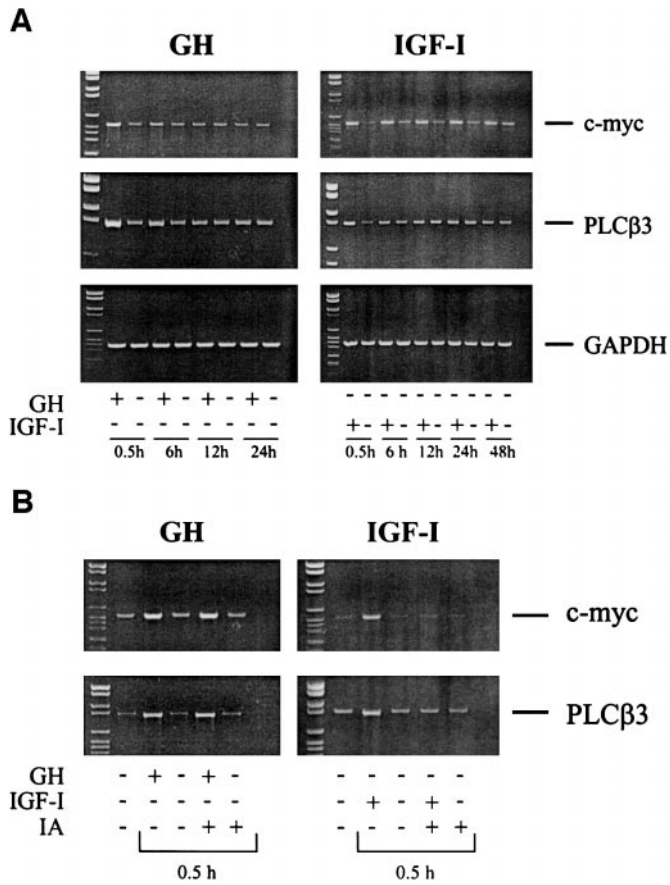

FIG. 2. (A) Effect of growth hormone (GH) and insulin-like growth factor-I (IGF-I) on PLCβ3 expression. Neonatal rat cardiomyocytes were incubated in the absence or presence of GH (100 nM; left panel) or IGF-I (100 nM; right panel) for the time indicated. RT-PCR of PLCβ3 as performed as described under Materials and Methods. c-myc and GAPDH mRNAs were amplified for comparison. (B) Effect of IGF-I analogue on GH- and IGF-I-stimulated c-myc and PLCβ3 expression. Neonatal rat cardiomyocytes were incubated in the absence or presence of GH (100 nM; left panel) or IGF-I (100 nM; right panel) for 30 min after preincubation in the absence or presence of IGF-I analogue (IA; 1 μg/ml; 1 h). RT-PCR was performed as described under Materials and Methods.

The signal transduction of IGF-I in cardiomyocytes was further investigated by specific inhibitors (Figs. 3 and 4). Preincubation of the cells with the tyrosine kinase inhibitor genistein also blocked the IGF-I-mediated upregulation of immediate early genes and PLCβ3 (Fig. 3A), while the compound alone had no effect. These findings indicate that IGF-I requires tyrosine phosphorylating activity to exert its effects. Tyrosine kinase activity is required for autophosphorylation of the IGF-I receptor, but also for phosphorylation of downstream signal transduction molecules such as IRS-1. In many cell types, IGF-I leads to activation of the ERK isoforms of MAP kinases [2]. In order to test whether ERKs are also required for the early growth response of cardiomyocytes to IGF-I, cells were preincubated with the specific ERK inhibitor PD 98059 (Fig. 3B) before application of IGF-I. PD 98059 abolished the IGF-I-induced upregulation of c-myc (upper panel) and PLCβ3 (middle panel) mRNA, respectively, while expression of GAPDH was constant under all conditions (lower panel). These results indicate that the upregulation of immediate early genes and PLCβ3 by IGF-I require ERK activity. Phosphatidylinositol-3-kinase is also implicated in IGF-I receptor-coupled signal transduction. Figure 3C illustrates the effect of preincubation with the PI3-kinase inhibitor wortmannin on IGF-I-mediated upregulation of immediate early gene and PLCβ3 mRNA. The IGF-I-induced upregulation of

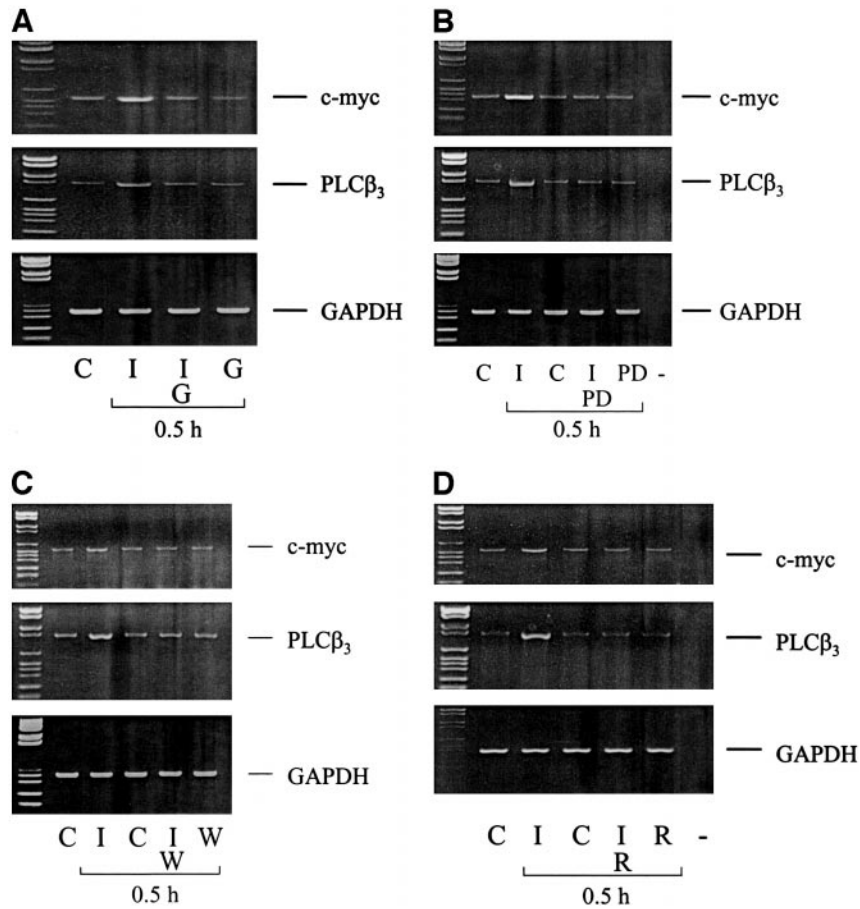

FIG. 3. (A–D) Effect of genistein (10 μM, A), PD 98059 (20 μM; B), wortmannin (25 nM; C), and rapamycin (500 pg/ml; D) on expression of c-myc, PLCβ3 and GAPDH in neonatal rat cardiomyocytes. Neonatal rat cardiomyocytes were incubated in the absence or presence of IGF-I (I; 100 nM; 30 min) after preincubation in the absence or presence of the respective inhibitor for 1 h. RNA was prepared and RT-PCR was performed as described under Materials and Methods. The experiment shown is representative of at least 3 experiments performed.

these mRNAs was abolished by wortmannin. The same holds true for preincubation with rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), a protein which itself activates p70 S6 kinase (Fig. 3D). P70 S6 kinase is a downstream effector of PI3-kinase and phosphorylates the ribosomal S6 protein. It is believed to facilitate translation by this mechanism. However, the prevention of the upregulation of the transcription factors c-myc, c-fos and c-jun by rapamycin in the present study suggests that p70 S6 kinase might also be involved in the regulation of transcriptional activity, maybe by phosphorylation of proteins other than S6 protein.

Many receptor tyrosine kinases activate the PLCγ subfamily of phospholipases C, while the PLCβ subfamily has been implicated in G protein-mediated signaling [6, 7]. In rat cardiomyocytes, Foncea *et al.* [20] demonstrated the activation of PLCγ1 by IGF-I. The present paper provides evidence that PLCβ3 might also be involved in IGF-I receptor signaling. The rapid and substantial upregulation of PLCβ3 mRNA exhibited the same requirements as discussed above for immediate early genes. These findings indicate that PLCβ3 might be a regulator downstream of ERKs, PI3 kinase and p70 S6 kinase. PLCβ3 activity cannot be inhibited pharmacologically in an isozyme-specific manner. Therefore, the inhibition of PLCβ3 expression by specific antisense oligonucleotides was chosen to investigate whether PLCβ3 was required for the induction of immediate early genes by IGF-I. Cardiomyocytes were preincubated with antisense oligonucleotides against two different stretches of sequence of PLCβ3 before addition of IGF-I. Figure 4A shows a representative experiment with PLCβ3 antisense oligo 1. Preincubation with oligo 1 abolished IGF-I-induced upregulation of immediate early genes (upper panel) suggesting that the expression of PLCβ3 mRNA is required for this response. The IGF-I-induced increase of PLCβ3 mRNA was also abolished by the antisense oligo (middle panel). At the highest concentration of oligo 1 (15 μM), PLCβ3 was not detectable anymore suggesting degradation of the oligonucleotide/mRNA hybrids by RNase H [22]. As a control, GAPDH mRNA was amplified (lower panel) and shown to be unaltered

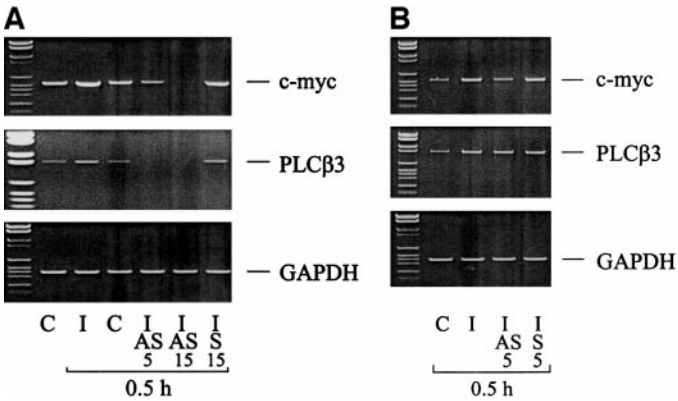

FIG. 4. (A) Effect of PLCβ3 antisense oligonucleotides on expression of c-myc, PLCβ3, and GAPDH in neonatal rat cardiomyocytes. Neonatal rat cardiomyocytes were incubated in the absence (C) or presence of IGF-I (I; 100 nM; 30 min) after preincubation in the absence or presence of antisense oligonucleotides (AS; 5 μM and 15 μM; 1 h) complementary to a stretch of sequence corresponding to the amino terminus of PLCβ3 (oligo 1) or the respective sense oligo (S; 15 μM; 1 h). RNA was prepared and RT-PCR was performed as described under Materials and Methods. The experiment shown is representative of at least 3 experiments performed. (B) Effect of c-myc antisense oligonucleotides on expression of c-myc, PLCβ3 and GAPDH in neonatal rat cardiomyocytes. Neonatal rat cardiomyocytes were incubated in the absence (C) or presence of IGF-I (I; 100 nM; 30 min) after preincubation in the absence or presence antisense oligonucleotides (AS; 5 μM; 1 h) complementary to a stretch of sequence of the c-myc mRNA or the respective sense oligo (S; 5 μM; 1 h). RNA was prepared and RT-PCR was performed as described under Materials and Methods. The experiment shown is representative of at least 3 experiments performed.

by IGF-I treatment and preincubation with antisense oligonucleotides. The same effect was observed with a second PLCβ3 antisense oligonucleotide (oligo 2), while the respective sense oligonucleotides had no effect on immediate early gene and PLCβ3 expression. When GAPDH and c-myc or GAPDH and PLCβ3 were amplified in the same tube, the results were the same as shown in Fig. 4A indicating that treatment with antisense oligonucleotides had no effect on reverse transcription (not shown). As an additional control for possible unspecific effects of antisense DNA/mRNA hybrids cells were incubated with antisense oligos against c-myc (Fig. 4B). Antisense oligos against c-myc lead to a degradation of c-myc mRNA, but had no effect on mRNA levels of GAPDH and PLCβ3. The above results indicate that PLCβ3 is situated downstream of ERK, PI3 kinase and p70 S6 kinase and upstream of immediate early genes in the signaling cascade of IGF-I in cardiomyocytes. This is somewhat surprising as PLCβ isozymes have been shown to be activated by G protein α- and $\beta\gamma$-subunits in several tissues [23, 24] including the heart [25] and would be expected further upstream in the signaling cascade. This observation might be explained by the PLCβ pool being upregulated by IGF-I being located in the nucleus of cardiomyocytes. Different isozymes have been shown to be present and enzymatically active in the nuclei of different cell types [26]. Nuclear PLCβ promotes growth and inhibits differentiation of an erythroleukemia cell line [27, 28]. In Swiss 3T3 cells, nuclear PLCβ1 has been implicated in the growth response to IGF-I [27]. Thus it is tempting to speculate that in cardiomyocytes nuclear PLCβ3 might be important for the hypertrophic response to IGF-I.

The observation that GH and IGF-I induced an upregulation of PLCβ3 while norepinephrine did not might have clinical implications. Cardiac hypertrophy in general is associated with an increased risk of heart failure and an impaired prognosis [29]. The different signal transduction mechanisms and cellular responses to different hypertrophic stimuli such as norepinephrine and IGF-I raise the question whether there are beneficial forms of cardiac hypertrophy. This hypothesis is supported by several animal studies. When large myocardial infarctions were induced in rats, application of growth hormone (GH) and/or its physiological effector IGF-I resulted in an improvement of left ventricular performance [30–33]. This hemodynamic improvement was associated with a hypertrophic response in non-infarcted myocardium. In human healthy volunteers, beneficial hemodynamic effects have been described after acute application of IGF-I [34]. In a number of small studies, patients with heart failure were treated with GH, leading to increased serum and tissue IGF-I levels. While some authors reported improvements in cardiac function [35, 36], others [37, 38] observed a hypertrophic response without hemodynamic improvement. Although large survival studies with GH/IGF-I are not yet available, the above mentioned studies raise the possibility that under special circumstances cardiac hypertrophy might be beneficial. The clinical and molecular biological discrimination of different forms of myocardial hypertrophy might open novel therapeutic opportunities is the treatment of cardiac hypertrophy and failure.

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