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Role of Heterotrimeric G Protein and Calcium in Cardiomyocyte Hypertrophy Induced by IGF-1

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ABSTRACT

In the heart, insulin-like growth factor-1 (IGF-1) is a peptide with pro-hypertrophic and anti-apoptotic actions. The pro-hypertrophic properties of IGF-1 have been attributed to the extracellular regulated kinase (ERK) pathway. Recently, we reported that IGF-1 also increases intracellular Ca^{2+} levels through a pertussis toxin (PTX)-sensitive G protein. Here we investigate whether this Ca^{2+} signal is involved in IGF-1-induced cardiomyocyte hypertrophy. Our results show that the IGF-1-induced increase in Ca^{2+} level is abolished by the IGF-1 receptor tyrosine kinase inhibitor AG538, PTX and the peptide inhibitor of Gβγ signaling, βARKct. Increases in the activities of Ca^{2+}-dependent enzymes calcineurin, calmodulin kinase II (CaMKII), and protein kinase Cα (PKCα) were observed at 5 min after IGF-1 exposure. AG538, PTX, βARKct, and the dominant negative PKCα prevented the IGF-1-dependent phosphorylation of ERK1/2. Participation of calcineurin and CaMKII in ERK phosphorylation was discounted. IGF-1-induced cardiomyocyte hypertrophy, determined by cell size and β-myosin heavy chain (β-MHC), was prevented by AG538, PTX, βARKct, dominant negative PKCα, and the MEK1/2 inhibitor PD98059. Inhibition of calcineurin with CAIN did not abolish IGF-1-induced cardiac hypertrophy. We conclude that IGF-1 induces hypertrophy in cultured cardiomyocytes by activation of the receptor tyrosine kinase activity/βγ-subunits of a PTX-sensitive G protein/Ca^{2+}/PKCα/ERK pathway without the participation of calcineurin. J. Cell. Biochem. 115: 712–720, 2014.

KEY WORDS: INSULIN-LIKE GROWTH FACTOR-1; CARDIAC MYOCYTE; HYPERTROPHY; HETEROTRIMERIC G PROTEIN; CALCIUM; EXTRACELLULAR-SIGNAL-REGULATED KINASE

Cardiac hypertrophy is an adaptive mechanism to enhance cardiac output in response to several cardiovascular disorders, including hypertension, atherosclerosis, myocardial infarction, vascular disease, and contractile abnormalities resulting from sarcomeric protein mutations [Sugden, 2001; Wilkins and Molkentin, 2004]. During the hypertrophic response, cardiomyocytes increase in size without undergoing in cell division, assemble additional sarcomeres, and activate a fetal program of cardiac gene expression [Sugden, 2001; Wilkins and Molkentin, 2004]. Prolonged cardiac hypertrophy is often accompanied by fibrosis and cardiomyocyte dropout, resulting in dilated cardiomyopathy, heart failure, and sudden death due to arrhythmias [Sugden, 2001; Wilkins and Molkentin, 2004].

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Insulin-like growth factor-1 (IGF-1) is a well-known pro-hypertrophic agent [Ebensperger et al., 1998; McMullen and Izumo, 2006; Catalucci et al., 2008; Kim et al., 2008; Munoz et al., 2009]. IGF-1 also has protective and anti-apoptotic properties in different models of myocardial ischemia and infarction [Ito et al., 1993; Morales et al., 2000; Sun et al., 2000; Maldonado et al., 2005; O′sullivan et al., 2011]. We have previously reported that binding of IGF-1 to its receptor (IGF-1R) activates two main signaling pathways in cardiomyocytes, the extracellular regulated kinase (ERK) and the phosphatidylinositol-3 kinase (PI3-K)/Akt [Foncea et al., 1997]. Pro-hypertrophic properties of IGF-1 had been attributed to the MEK–ERK pathway rather than the PI3K/Akt pathway [Lavandero et al., 1998]. Recently, we also described that IGF-1 induced a rapid nuclear Ca^{2+} signal via the pertussis toxin (PTX)-sensitive G protein/phospholipase C (PLC)/inositol-1,4,5-trisphosphate (IP3)/IP3 receptor (IP3R) pathway [Ibarra et al., 2004, 2013].

The capacity of IGF-1 to increase Ca^{2+}i strongly suggests that Ca^{2+} could be a potential second messenger in the IGF-1 signaling network to stimulate cardiac hypertrophy. However, whether activation of the G protein/PLC/IP3/Ca^{2+} pathway plays a critical role in cardiomyocyte hypertrophy induced by IGF-1 remains unresolved. We investigate here whether the G protein-dependent Ca^{2+} pathway participates in the transcriptional and morphological hypertrophic responses triggered by IGF-1 in cultured cardiomyocytes.

MATERIALS AND METHODS

ANIMALS

Rats were bred in the Animal Breeding Facility from the Facultad Ciencias Quimicas y Farmaceuticas, Universidad de Chile (Santiago, Chile). We performed all studies with the approval of the Institutional Bioethics Committee at the School of Chemical & Pharmaceutical Sciences, University of Chile, Santiago, Chile. This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication, 8th edition, 2011).

PRIMARY CULTURES OF CARDIOMYOCYTES

Cardiomyocytes were dissociated from neonatal Sprague–Dawley rat ventricles (Animal Breeding Facility, Universidad de Chile) as described previously [Foncea et al., 1997]. After enzymatic dissociation, cardiomyocytes were plated on gelatin-coated 60 mm tissue-culture dishes at a density of $1–4 \times 10^6$ cells/dish (350 cells/mm^2) for Western blot and at a density of 5×10^5 cells per 60 mm dish for assessment of cell size. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Serum was withdrawn for 24 h before the cells were further treated with IGF-1 (0–100 nM) in serum-free medium (Dulbecco′s modified Eagle′s medium [DMEM] and medium 199 in a 4:1 ratio) at 37°C.

RECOMBINANT ADENOVIRUSES

Adenoviral vectors (Ad) were propagated and purified as previously described [Shah et al., 2001]. βARKct adenovirus (AdβARKct), a peptide inhibitor of Gβγ signaling, was a gift from Dr. W.J. Koch, Duke University Medical Center, Durham, NC [Koch et al., 1994]. Adenovirus encoding wild-type protein kinase C α (AdwtPKCα), dominant negative PKCα (AddnPKCα), and calcineurin inhibitor (AdCAIN) were gifts from Dr. J. D. Molkentin, University of Cincinnati, Children′s Hospital Medical Center, Cincinnati, OH. Cardiomyocytes were transduced with adenoviral vectors at a multiplicity of infection (MOI) of 300.

MEASUREMENT OF INTRACELLULAR CALCIUM

Cellular Ca^{2+} images were obtained from cultured cardiomyocytes pre-loaded with fluo3-AM, using an inverted confocal microscope (Carl Zeiss Axiovert 135 M-LSM Microsystems) or a fluorescence microscope (Olympus Diaphot-TMD, Nikon Corporation) equipped with a cooled CCD camera and image acquisition system (Spectra Source MCD 600) as previously described [Ibarra et al., 2004, 2013].

WESTERN BLOT ANALYSIS

Electrophoresis and electrotransferences were performed as described [Ibarra et al., 2004]. Antibodies against total phosphorylated ERK (pERK) and total ERK were used at 1:1,000 dilution (Cell Signaling Technology, Beverly). Anti β-MHC (Novocastra, Newcastle, UK) and β-actin antibodies (Sigma Chemical Co, St. Louis, MO) were used 1:1,000 and 1:5,000 dilution, respectively. Blots were quantified by scanning densitometry.

ENZYMATIC ACTIVITIES

Calcineurin activity was determined using the Calcineurin Assay Kit (EMD Millipore Bioscience Calbiochem). Calmodulin kinase II (CaMKII) and PKCα activity were determined using CaMKII and PKC assay kits (Upstate) according to the manufacturer′s directions.

ASSESSMENT OF CARDIOMYOCYTE AREA

To assess the area, cardiomyocytes were transfected with the β-galactosidase (β-gal)-encoding plasmid pON249 (provided by Dr. K. R. Chien, Department of Medicine, University of California, San Diego, CA) and evaluated as described [Finn et al., 1999].

STATISTICS

Results are expressed as mean ± SEM for the number of independent experiments indicated (n) or as examples of representative experiments performed on at least three separate occasions. Data were analyzed by ANOVA, and comparisons between groups were performed using a protected Tukey′s *t*-test. A value of $P < 0.05$ was set as the limit of statistical significance.

RESULTS

IGF-1 ACTIVATES THE CALCIUM SIGNALING PATHWAY BY A HETEROTRIMERIC G PROTEIN IN CULTURED CARDIOMYOCYTES

We recently showed that IGF-1 triggered a fast increase in intracellular Ca^{2+} in cultured cardiomyocytes [Ibarra et al., 2004, 2013]. Figure 1 shows that this Ca^{2+} increase depends not only on a βγ subunit of a PTX-sensitive G protein but also on IGF-1 receptor intrinsic tyrosine kinase activity. AG538, a potent competitive inhibitor of IGF-1 receptor kinase [Blum et al., 2000], completely abolished the Ca^{2+} increase induced by IGF-1 (Fig. 1A). To assess whether the IGF-1-dependent Ca^{2+} increase activates downstream signaling, we assayed enzymatic activities of calcineurin, CaMKII,

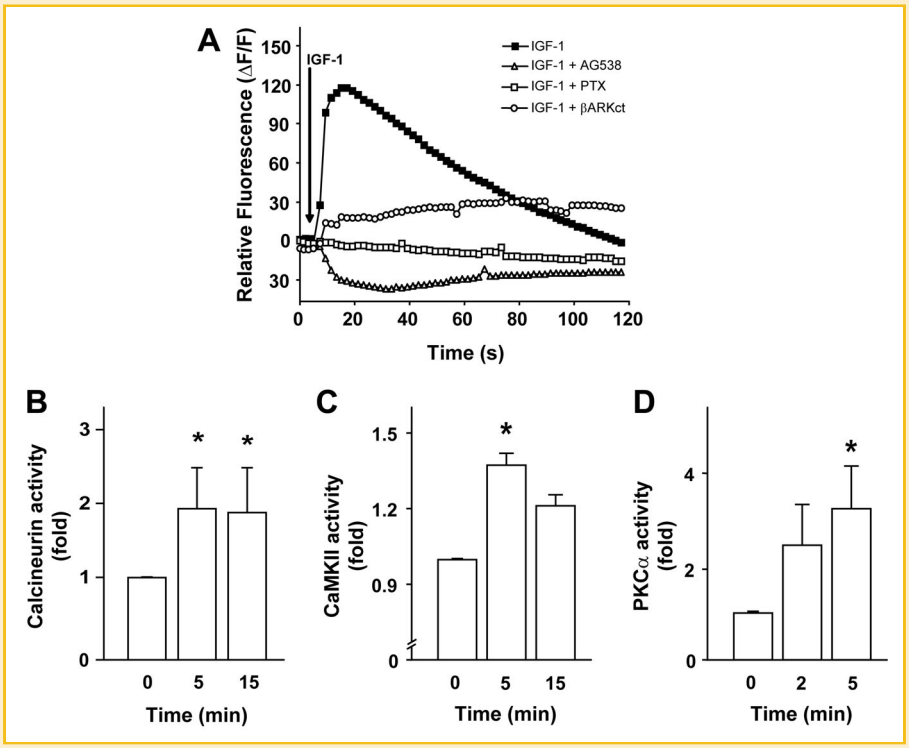

Fig. 1. IGF-1 activates Ca^{2+} signaling in cultured cardiomyocytes. A: Cells, maintained in Ca^{2+}-free resting media, were pre-treated with AG538 (50 μM, 24 h), pertussis toxin (PTX, 1 μg/ml, 90 min) or transduced with adenovirus encoding βARKct (MOI 300, 24 h). Then cardiomyocytes were pre-loaded with fluo3-AM and stimulated with IGF-1 (1 nM). Using a fluorescence microscope equipped with a CDD camera, serial Ca^{2+} images of fluo3 fluorescence in a single cardiomyocyte were recorded, and relative total fluorescence [ratio of fluorescence difference, stimulated–baseline (Fi–Fo), to baseline value (Fo)] as a function of time of each image were calculated. Cardiomyocytes were stimulated with IGF-1 (10 nM) and total protein extracts were obtained at indicated times. Enzymatic activities of (B) calcineurin, (C) calmodulin kinase II (CaMKII), and (D) PKCα were determined as described in the Materials and Methods section. Values are presents as mean ± SEM of three to five independent experiments. $^*P < 0.05$ versus time 0 min.

and PKCα. IGF-1 induced a 1.9 ± 0.5-fold increase in calcineurin activity after 5 min incubation (Fig. 1B). At the same time, a 1.36 ± 0.05-fold activation of CaMKII was observed (Fig. 1C). At 15 min, calcineurin activity was still activated (Fig. 1B,C). IGF-1 also triggered a 3.2 ± 0.9-fold increase in PKCα at 5 min (Fig. 1D). We have previously described that IGF-1 also activates PKC activity at 15 min [Foncea et al., 1995]. These results showed that IGF-1 induces an intracellular Ca^{2+} increase requiring both the IGF-1 receptor intrinsic tyrosine kinase and Gβγ-subunits of a PTX-sensitive heterotrimeric G protein. Moreover, the IGF-1-dependent Ca^{2+} increase activates downstream signaling including calcineurin, CaMKII, and PKCα.

IGF-1 ACTIVATES ERK THROUGH BETA/GAMMA-SUBUNITS OF A HETEROTRIMERIC G PROTEIN

IGF-1 receptor tyrosine kinase activity is required for both ERK and Akt activation because AG538 blocked ERK1/2 and Akt phosphorylation induced by IGF-1 (Fig. 2A,B). However, PTX as well as βARKct blocked ERK1/2 but not Akt phosphorylation induced by IGF-1 (Fig. 2C–F). These results suggest that IGF-1 receptor tyrosine kinase activity is required for both ERK1/2 and Akt phosphorylation, while βγ-subunits of a PTX-sensitive G protein are involved in ERK1/2 but not Akt activation.

IGF-1-INDUCED ERK PHOSPHORYLATION DEPENDS ON PKCA

Because a PTX-sensitive G protein blockade suppressed both the Ca^{2+} increase and ERK1/2 phosphorylation, we assessed whether ERK activation requires IGF-1-induced Ca^{2+} signaling. Calcineurin and CaMKII inhibition with cyclosporin A (CsA) and KN62, respectively, did not abrogate IGF-1-dependent ERK1/2 phosphorylation (Fig. 3A). However, overexpression of wtPKCα increased basal levels of ERK1/2 phosphorylation, and dnPKCα suppressed IGF-1-dependent ERK1/2 activation (Fig. 3B). Taken together, these data indicate that ERK phosphorylation induced by IGF-1 requires PKCα activity, while CaMKII and calcineurin are not involved.

IGF-1 INDUCED HYPERTROPHY REQUIRES THE G PROTEIN/CALCIUM/PKCA/ERK PATHWAY

β-Myosin heavy chain (β-MHC) protein levels and cardiomyocyte size were evaluated to assess transcriptional and morphological hypertrophic responses induced by IGF-1, respectively. Treatment of cultured cardiomyocytes with IGF-1 increased β-MHC by 105 ± 10% and cell area by 120 ± 8% (Fig. 4). Inhibition of IGF-1 receptor intrinsic tyrosine kinase activity with AG538 blocked the IGF-1-dependent increase in both β-MHC and cell size (Fig. 4A). Pre-treatment with PTX or βARKct overexpression also completely abolished the increase in β-MHC and cell size induced by IGF-1

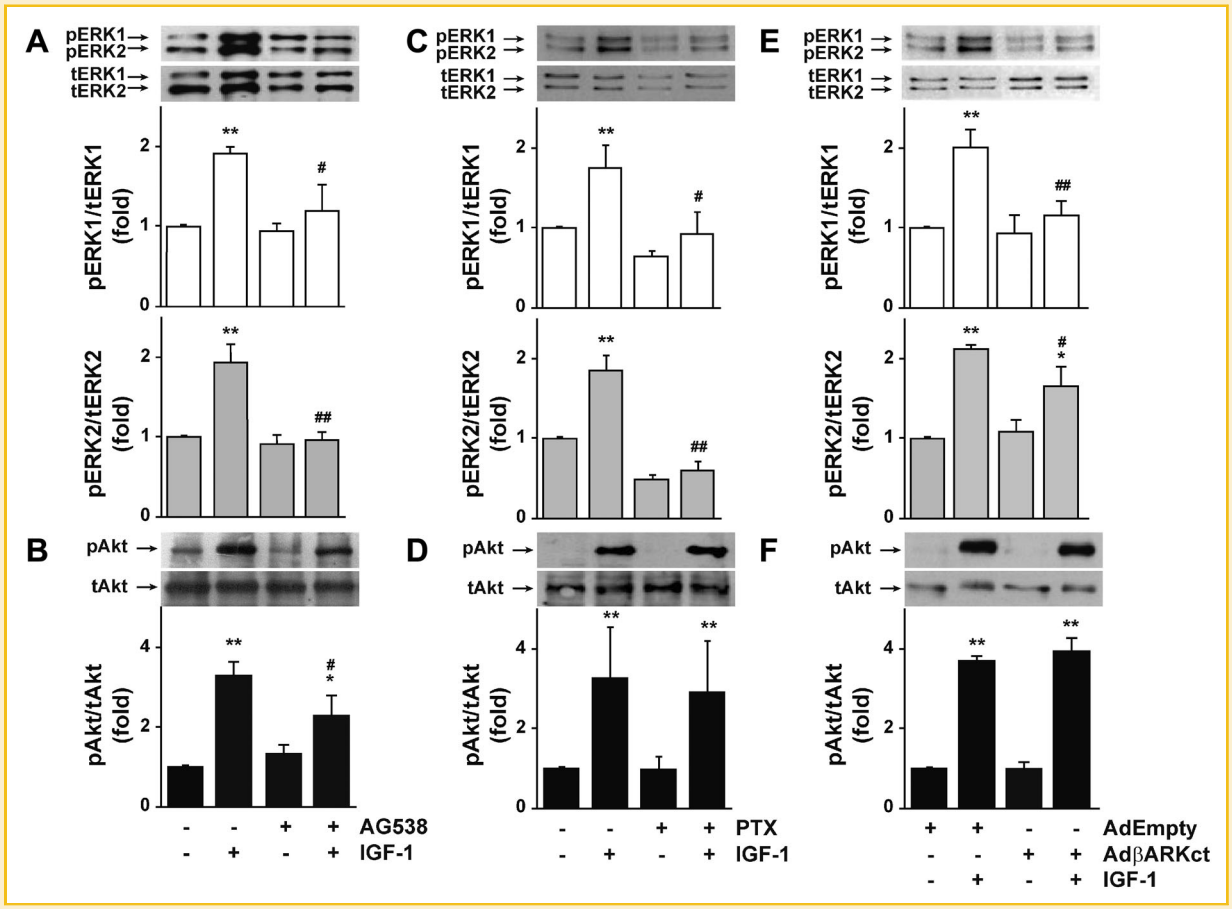

Fig. 2. IGF-1-dependent ERK1/2 phosphorylation requires receptor tyrosine kinase and βγ-subunits of a PTX-sensitive G protein. Cultured cardiomyocytes, maintained in Ca^{2+}-free resting media, were pre-incubated with AG538 (50 μM) for 24 h, PTX (1 μg/ml) for 90 min or transduced with an empty adenovirus (MOI 300) or adenovirus encoding βARKct (MOI 300) for 24 h, and then stimulated with IGF-1 (1 nM) for 5 min. A, C, E: Phosphorylated to total ERK1 and ERK2 ratio (pERK/ERK). B, D, F: Phosphorylated to total Akt ratio (pAkt/Akt) were quantified from Western blots of total protein extracts as described in the Materials and Methods section. Values are presented as mean ± SEM (n = 3 independent experiments), $^{**}P < 0.01$ and $^{*}P < 0.05$ versus control, $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ versus IGF-1.

(Fig. 4B,C). Cardiomyocyte transduction with wtPKCα basally increased β-MHC protein levels and cell size at a magnitude to similar that obtained with IGF-1 treatment (Fig. 4D). Moreover, overexpression of dnPKCα completely suppressed the cardiomyocyte hypertrophy stimulated by IGF-1. Inhibition of calcineurin with CAIN did not abolish IGF-1-dependent increases in β-MHC protein level and cell size (Fig. 4E). These data suggest that IGF-1-dependent cardiomyocyte hypertrophy requires the participation of the IGF-1 receptor intrinsic tyrosine kinase, βγ-subunits of a PTX-sensitive heterotrimeric G protein, PKCα and ERK. Although IGF-1 activates calcineurin, this phosphatase is not involved in IGF-1-dependent cardiomyocyte hypertrophy.

DISCUSSION

Our data showed that IGF-1 stimulates cardiomyocyte hypertrophy mediated by βγ-subunits of a PTX-sensitive G protein coupled to the IGF-1R, increasing intracellular Ca^{2+}, followed by PKCα activation, which is involved in the phosphorylation of ERK1/2 (Fig. 5).

IGF-1R was first reported to be coupled to heterotrimeric G proteins by Nishimoto et al. [1987; Patel, 2004]. The activation of the IGF-1R associated heterotrimeric G protein, specifically Gαi, and the release of βγ-subunits, have been linked to ERK1/2 phosphorylation [Luttrell et al., 1995; Dalle et al., 2001]. IGF-1-induced ERK1/2 activation in rat1 fibroblasts was inhibited by either PTX or overexpression of the βγ scavenger βARKct, suggesting that the release of Gβγ-subunits, rather than the Gα subunit, is responsible for IGF-1-dependent ERK activation [Luttrell et al., 1995]. Similar results have been obtained using human intestinal smooth muscle cells [Kuemmerle and Murthy, 2001], 3T3-L1 mouse pre-adipose cells [Dalle et al., 2001], and rat cerebellar granule neurons [Hallak et al., 2000]. Here we described that ERK1/2 activation by IGF-1 in cardiomyocytes, but not Akt activation, also depends on βγ-subunits of a PTX-sensitive heterotrimeric G protein.

We have previously shown that IGF-1 induced a transient intracellular Ca^{2+} increase in cardiomyocytes through βγ-subunits of a PTX-sensitive G protein, followed by PI3K and PLC activation, IP_3 generation, and IP_3 receptor activation (Fig. 5) [Ibarra et al., 2004]. Recent findings from our group have also shown that the activation of intracellular Ca^{2+} release by IGF-1 in cardiomyocytes is a compartmentalized and complex process that relies on the strategic localization of the IGF-1R to perinuclear sarcolemmal signaling

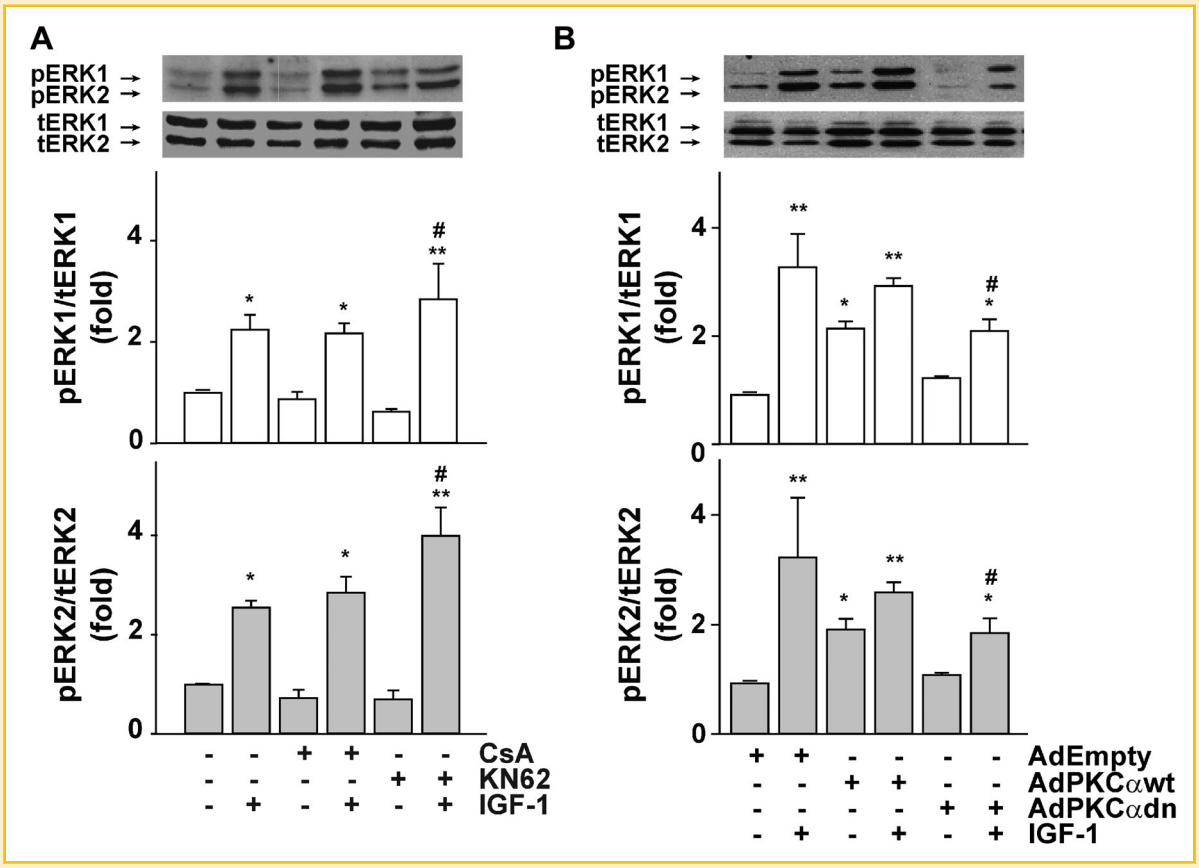

Fig. 3. IGF-1-dependent ERK1/2 phosphorylation is mediated by PKCα without the participation of calcineurin and CaMKII. A: Cultured cardiomyocytes were pre-incubated with cyclosporin A (CsA, 250 nM) or KN62 (1 μM) for 30 min and then stimulated with IGF-1 (10 nM) for 5 min. B: Cultured cardiomyocytes were transduced with empty adenovirus (AdEmpty), adenovirus encoding wild-type PKCα (AdwtPKCα) or dominant negative PKCα (AddnPKCα) using MOI 300 for 24 h and then stimulated with IGF-1 (10 nM) for 5 min. Phosphorylated to total ERK1 and ERK2 ratio (pERK/ERK) was quantified from Western blots of total protein extracts as described in the Material and Methods section. Values are mean ± SEM (n = 3). $^{**}P < 0.01$ and $^{*}P < 0.05$ versus control or AdEmpty, $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ versus IGF-1.

microdomains and on the downstream activation of a perinuclear signalosome located in direct apposition to the juxtanuclear receptor [Ibarra et al., 2013]. In this context, PTX-sensitive heterotrimeric G protein was demonstrated to form part of this mechanism. Both Gαi and Gβ subunits co-immunoprecipitate with the IGF-1Rβ subunit in unstimulated neonatal rat ventricular cardiomyocytes, suggesting there is an association between the proteins at rest. Moreover, stimulation with IGF-1 rapidly (60 s) increased the amount of Gαi and Gβ subunits recovered from IGF-1Rβ immunoprecipitates, indicating that activation of IGF-1R (i.e., intrinsic tyrosine kinase activity) further promotes an association between the receptor and heterotrimeric G protein [Ibarra et al., 2013]. In our view, recruitment of heterotrimeric G protein to the activated IGF-1R is an obligatory step for subsequent activation of the non-canonical PLC–IP3–IP3R–Ca^{2+}–ERK pathway [Ibarra et al., 2004]. The observation that a Gβγ dimmer blocker completely abrogated the downstream activation of phospholipase C activity argues in favor of a mandatory role for Gβγ in the activation of the non-canonical IGF-1R pathway; however, it remains unresolved whether this relationship relies on a direct protein–protein interaction or on an indirect interaction through scaffold proteins.

Here we showed that both the increase in Ca^{2+} and the ERK1/2 phosphorylation induced by IGF-1 also depended on IGF-1R tyrosine kinase activity, because both events were blocked by AG538 [Blum et al., 2000], a specific inhibitor of IGF-1R kinase (Fig. 5). However, a contradictory result was described by Perrault et al. [2011]; in smooth muscle cells, IGF-1-stimulated ERK1/2 phosphorylation through Gβγ-subunit signaling but without the participation of IGF-1R tyrosine kinase activity.

The IGF-1-dependent Ca^{2+} increase activates downstream signaling pathways in cardiomyocytes, particularly calcineurin, CaMKII, and PKCα (Fig. 5). Activation of calcineurin, CaMKII, and PKCα by IGF-1 have been described in stem cells, astrocytes, neurons, skeletal muscle, renal cells, and cardiomyocytes [Gooch et al., 2001; Miyashita et al., 2001; Zheng et al., 2004; Maniar et al., 2005; Gao et al., 2006; Fernandez et al., 2012; Lu et al., 2012]. Activation of ERK by PKCα has been well described in MAPK activation by G-coupled protein receptor [Sugden, 2001; Rozengurt, 2007]. Moreover, ERK activation by IGF-1 involves PKCα both in adult and neonatal cardiomyocytes [Pecherskaya and Solem, 2000; Vijayan et al., 2004]. IGF1-induced activation of ERK1/2 was blocked by pre-incubation with the PKC inhibitors bisindolylmaleimide and Gö6976

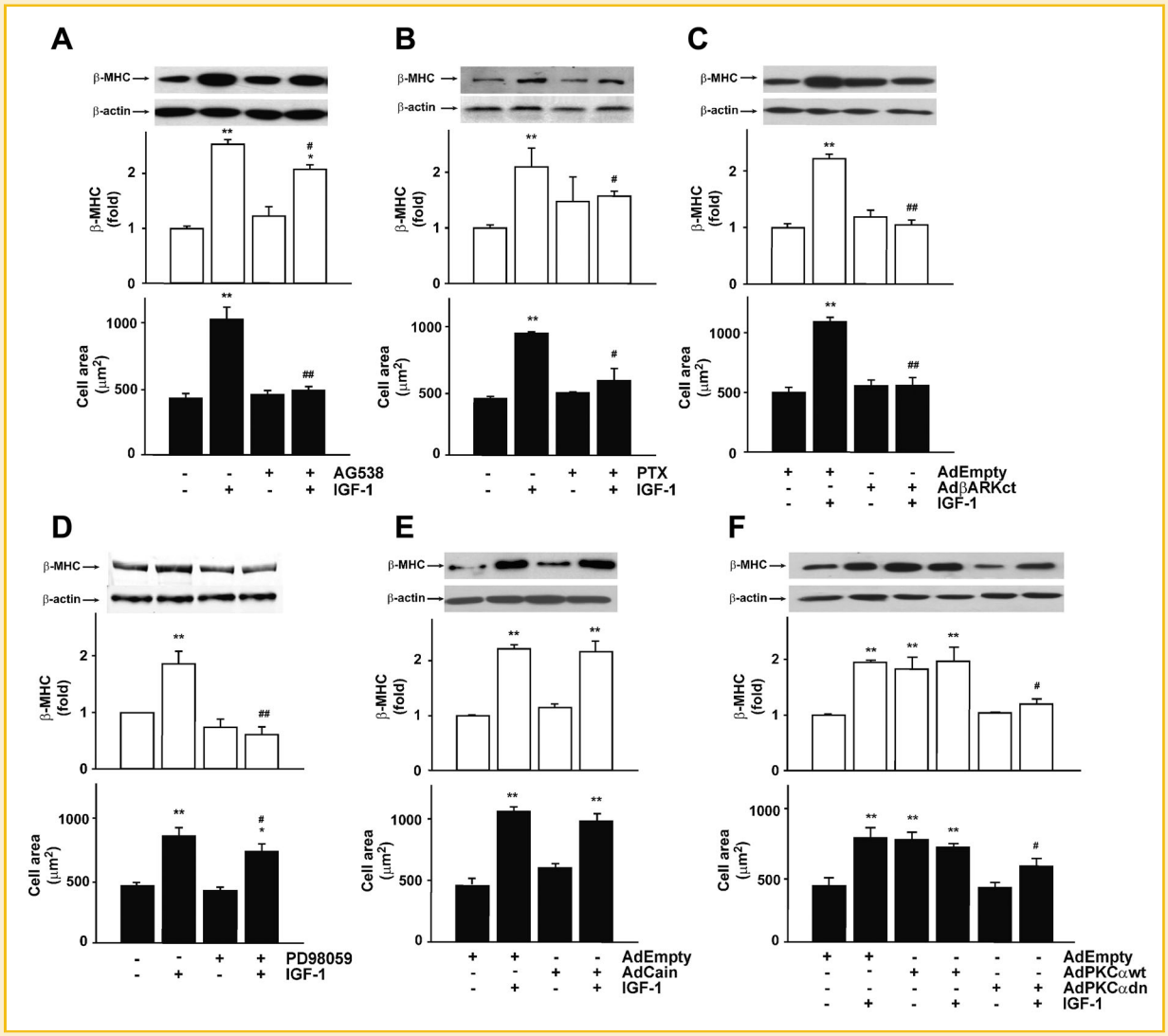

Fig. 4. IGF-1 stimulates cardiomyocyte hypertrophy by a mechanism involving receptor tyrosine kinase, βγ-subunits of a PTX-sensitive G protein, PKCα and ERK. Cultured cardiomyocytes were pre-incubated with (A) AG538 (50 μM, 24 h), (B) PTX (1 μg/ml, 90 min), (D) PD98059 (100 μM, 30 min) or transduced with an empty adenovirus (AdEmpty) or adenovirus encoding (C) βARKct (AdβARKct), (E) CAIN (AdCAIN), (F) wild-type PKCα (AdwtPKCα) or dominant negative PKCα (AddnPKCα) (MOI 300, 24 h), and then stimulated with IGF-1 (10 nM) for 48 h. β-Myosin heavy chain (β-MHC) and β-actin were quantified from Western blots of total protein extracts as described in the Materials and Methods section. Values are presented as mean ± SEM of at least three different experiments $^{**}P < 0.01$ and $^{*}P < 0.05$ versus control or AdEmpty, $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ versus IGF-1.

[Pecherskaya and Solem, 2000], as well as the adenoviral over-expression of dnPKCα [Vijayan et al., 2004]. Our results agree with these previous findings. Our data showed that the PKCα-dependence of ERK activation in cardiomyocytes was also supported by the fact that expression of wtPKCα basally increased ERK1/2 phosphorylation.

PI3K and its downstream target Akt have been described as critical components of heart size regulation [Crackower et al., 2002]. Transgenic mice expressing a constitutively active PI3K-α construct have increased heart size, due to an increase in myocyte size. However, in those mice, little change was observed in β-MHC levels [Crackower et al., 2002]. These results indicate that cell size and β-MHC expression are regulated by different pathways. Our results showed that AG538 does not effectively diminish the IGF-1-dependent increase in β-MHC levels, yet it completely blocks cell size enlargement. These results suggest that IGF-1R tyrosine kinase mainly regulates cell size, while the $G\beta\gamma$–Ca^{2+}–PKCα–ERK1/2 pathway is strongly involved in the regulation of β-MHC expression.

In cardiomyocytes, there is abundant evidence that ERK1/2 and PKCα are important in mediating the transcriptional and morphological responses to hypertrophic agonists, including IGF-1 [Sarbassov et al., 1997; Pecherskaya and Solem, 2000; Haddad and Adams, 2004; Molkentin, 2004; Vijayan et al., 2004; Maniar et al., 2005]. Both signaling pathways can act independently to signal hypertrophy in cardiomyocytes [Sugden, 2001]. Here we showed that both pathways are linked, as PKCα was necessary for

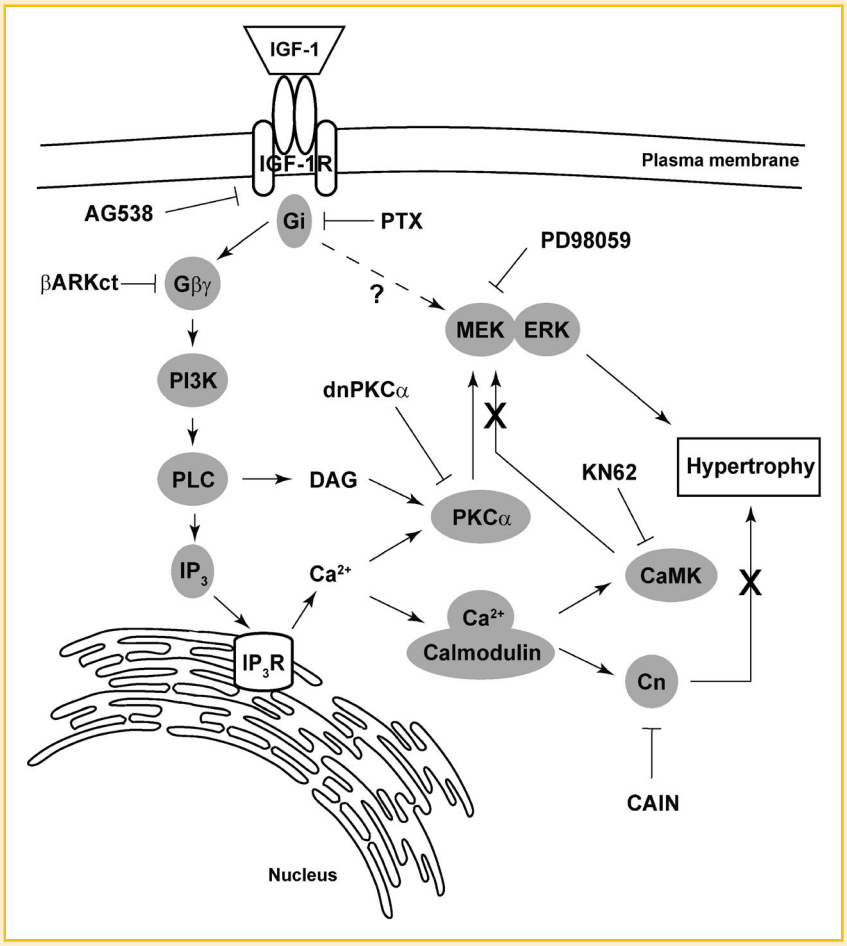

Fig. 5. Model of the molecular mechanism of cardiomyocyte hypertrophy triggered by IGF-1. IGF-1 receptor tyrosine kinase and βγ-subunits of a pertussis toxin (PTX)-sensitive heterotrimeric G protein are required to induce intracellular Ca^{2+} increase through a phosphatidylinositol 3-kinase (PI3K)/phospholipase C (PLC)/inositol-1,4,5-phosphate (IP_3)/IP_3 receptor (IP_3R) pathway. Ca^{2+}/diacylglycerol (DAG) activates PKCα, while Ca^{2+}/calmodulin activates calcineurin (Cn) and calmodulin kinase (CaMK). PKCα phosphorylates ERK, which is responsible for IGF-1-induced cardiomyocyte hypertrophy. CaMK is not involved in ERK activation, and Cn does not participate in IGF-1-dependent hypertrophy.

ERK activation after IGF-1 treatment. However, our results do not discard the possibility that ERK1/2 can be activated by IGF-1R via a PKCα-independent mechanism (Fig. 5).

In reactive hypertrophy, calcineurin has been involved in ERK regulation [Molkentin, 2004]. Calcineurin and ERK signaling pathways have shown to be co-dependent such that activation of calcineurin in cardiomyocytes leads to up-regulation of ERK signaling [Molkentin, 2004]. In our system, although IGF-1 also activated calcineurin and CaMKII, neither enzyme was involved in ERK1/2 activation, because inhibition with CsA and KN62, respectively, failed to block the ERK1/2 phosphorylation triggered by IGF1. We have previously described that IGF-1-dependent induction of calcineurin is involved in CREB activation and mediates anti-apoptotic effects of IGF-1 in cardiomyocytes [Maldonado et al., 2005].

Cardiac hypertrophy can be physiological or pathological [Bernardo et al., 2010]. Physiological hypertrophy occurs during the postnatal period, pregnancy, and exercise; is associated with normal cardiac structure, normal or improved cardiac function; and is reversible [Fagard, 1997; Ellison et al., 2012]. In contrast, pathological growth occurs in response to chronic pressure or volume overload, myocardial infarction, or ischemia associated with coronary artery disease [Weber and Brilla, 1993]. Substantial experimental and clinical evidence suggests that IGF-1 participates in the initiation and development of physiological cardiac hypertrophy [Ebensperger et al., 1998; McMullen and Izumo, 2006; Catalucci et al., 2008; Kim et al., 2008; Munoz et al., 2009]. Although we observed that the Ca^{2+} increase induced by IGF-1 activates calcineurin, inhibition of this phosphatase with CAIN did not suppress IGF-1-induced cardiomyocyte hypertrophy (Fig. 5). Calcineurin activation has been associated with the induction of pathological cardiac hypertrophy [Molkentin, 2004; Wilkins and Molkentin, 2004]. Therefore, the fact that PKCα/ERK but not the calcineurin pathway participates in IGF-1-induced cardiomyocyte hypertrophy suggests that it could be responsible for the physiological hypertrophy induced by IGF-1.

A limitation of our study involved the use of a rather high concentration of AG538 (50 μM). However, a similar concentration was used by Blum et al. [2000] to inhibit IGF-1-dependent ERK and

Akt activations. At that concentration, AG538 can also affect insulin receptor tyrosine kinase activity [Hirano et al., 2007]. Nevertheless, in our experimental setting (i.e., stimulation with 1–10 nM IGF-1), no activation of the insulin receptor was expected [Blakesley et al., 1996]. However, we cannot rule out that other kinases could be also affected. Another limitation was associated with the use of neonatal cardiomyocytes as a model to perform our studies. Chronic heart failure or cardiac hypertrophy is a disease that most often develops in late adulthood. Because there are several differences between neonatal and adult rat cardiomyocytes [Louch et al., 2011], ideally, cardiomyocytes from adult animals should be used to study the mechanism of cardiomyocyte hypertrophy. However, it is difficult to isolate a large quantity of cardiomyocytes from adult animals and to maintain their viability ex vivo over a long time course to perform hypertrophy studies. In contrast, isolation of neonatal rat cardiomyocytes is relatively easy, and the cells can be maintained in culture for a long time. Therefore, because of the difference between neonatal cardiomyocytes and adult cardiomyocytes, caution should be exercised when extrapolating the results from neonatal cardiomyocytes to cases of adult cardiomyocyte hypertrophy.

In summary, taken together these results suggest that activation of IGF-1R triggers an intracellular Ca^{2+} increase requiring both receptor tyrosine kinase and βγ-subunits of a PTX-sensitive heterotrimeric G protein. Ca^{2+} increase activates PKCα which in turn phosphorylates ERK1/2. Therefore, IGF-1-dependent stimulation of cardiomyocyte hypertrophy is mediated by Gβγ-subunits/Ca^{2+}/PKCα/ERK pathway without the participation of calcineurin.

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