The 1,4,5-inositol trisphosphate pathway is a key component in Fas-mediated hypertrophy in neonatal rat ventricular myocytes

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Abstract

Objective: Cardiac hypertrophy is a compensatory response to increased mechanical load. Since Fas receptor activation is an important component in hypertrophy induced by pressure- and volume-overload, deciphering the underlying signaling pathways is of prime importance. Based on our previous work showing that in mice and rats ventricular myocytes the electrophysiological disturbances and diastolic [Ca2+]i-rise caused by 3 h of Fas activation are dependent on the Fas→phospholipase C (PLC)→1,4,5-inositol trisphosphate (1,4,5-IP3)→sarcoplasmic reticulum (SR) [Ca2+]i release pathway, we tested the hypothesis that this pathway is also critical for Fas-mediated hypertrophy.

Methods: The effects of 24 h Fas activation in cultured neonatal rat ventricular myocytes (NRVM) were analyzed by means of RT-PCR, Western blot, immunofluorescence and fura-2 fluorescence.

Results: Fas activation increased nuclei surface area, atrial natriuretic peptide and connexin43 (Cx43) mRNA, the protein levels of total Cx43 and non-phosphorylated Cx43, and sarcomeric actin, all indicating hypertrophy. Concomitantly, Fas activation decreased mRNA of SERCA2a, the ryanodine receptor (RyR) and nuclear IP 3R3. Further, Fas activation caused NFAT nuclear translocation. The hypertrophy was abolished by U73122, xestospongin C (blockers of the 1,4,5-IP3 pathway), genistein and by the PI3K blocker LY294002.

Conclusions: Fas-mediated hypertrophy is dependent on the 1,4,5-IP3 pathway, which is functionally inter-connected to the PI3K/AKT/GSK3β pathway. Both pathways act in concert to cause NFAT nuclear translocation and subsequent hypertrophy.

Keywords: Calcium (cellular); Cell culture; Hypertrophy; Myocytes; Signal transduction

1. Introduction

Recent studies have shown that activation of the cardiac Fas receptor (Fas) causes both apoptotic and non-apoptotic deleterious effects [1–4]. In contrast to many Fas-baring cell lines and tissues, Fas activation in ventricular myocytes does not cause apoptosis, indicating that Fas expression per se does not necessarily commit cells to apoptosis. Specifically, we and others have demonstrated that whereas normoxic myocytes are resistant to apoptosis induced by Fas activation, hypoxia predisposes myocytes to Fas-mediated apoptosis [1,3,4]. In a series of studies we found that in adult murine and neonatal rat ventricular myocytes (NRVM), 3 h of Fas activation caused a wide range of functional disturbances, including action potential changes, early- and delayed after depolarizations, arrhythmias, intracellular Ca2+ ([Ca2+]i) rise, decreased Ito and increased ICa,L [5,6]. Our key finding was that these effects were exclusively dependent on the Fas→phospholipase C (PLC)→1,4,5-IP3→SR [Ca2+]i release pathway and on tyrosine kinases (TK) phosphorylation [5,6]. In brief, we showed that: (1) the above mentioned effects were blocked...
by the PLC inhibitor U73122 and by xestospongin C, a specific blocker of the SR 1,4,5-IP₃-operated Ca²⁺ channels, and were reproduced by intracellular application of 1,4,5-IP₃; (2) Fas activation increased 1,4,5-IP₃ levels.

Recently, Dimmelé’s group has shown that 4 h of Fas activation in NRVM caused hypertrophy, and that lpr mice lacking functional Fas demonstrated rapid-onset of left ventricular dilatation and failure, absence of compensatory hypertrophy and increased mortality [1], indicating that the hypertrophy was Fas-dependent. Because of the key role of hypertrophy in major heart diseases, and based on our previous studies, we tested the hypothesis that the 1,4,5-IP₃ pathway is critical for Fas-mediated hypertrophy. This work shows for the first time the involvement of the ubiquitous 1,4,5-IP₃ pathway in ventricular hypertrophy, suggesting that modulators of this pathway specifically targeted to cardiac muscle may have an important therapeutic value.

2. Methods

2.1. Cultures preparation, Fas activation, apoptosis determination and [Ca²⁺]i transients measurement

NRVM cultures were prepared from ventricles of 1–2-day-old Sprague–Dawley rats [4], and experiments performed on cultures 4–6 days after plating. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). To inhibit non-cardiac muscle growth, BrdU was included in the culture medium. Fas was activated by recombinant Fas ligand (rFasL) (10 ng/mL) and an enhancing antibody (1 μg/mL) (Alexis, San Diego, CA, USA). This concentration was chosen based on a study [1] showing that in the concentration range of 1–100 ng/mL, maximal hypertrophy (indicated by [³H] leucine incorporation) was attained at 10 ng/mL. For each protocol apoptosis was measured by the DAPI assay [4], in two wells (500 myocytes measured in each well), prepared from 3 different litters, altogether counting 3000 myocytes. [Ca²⁺]i transients were measured by means of fura-2 fluorescence from cultures maintained at 37 °C and stimulated at 0.5 Hz [4].

2.2. Immunoblotting

NRVM were harvested in lysis buffer, and 40 μg of total protein were separated by 12% SDS-PAGE gels, transferred to nitrocellulose membranes, and immunoblotted [4]. The primary antibodies were anti-Cx43 (Chemicon International, Temecula, CA, USA) and anti-non-phosphorylated (NP) Cx43 antibody (Zymed Laboratories, San Francisco, CA, USA). Immune complexes were detected using the Enhanced Chemiluminescence detection system (Perkin Elmer Life Sciences, Boston, MA, USA) [7].

2.3. Immunofluorescence staining

Actin measurements: NRVM were stained with Alexa Phalloidin (Alexis, Cornerstone Court, San Diego, CA, USA). Actin was quantified by measuring the fluorescence intensity using custom-made “Open View” software, from 12 different areas in each slide, from 4 experiments. Nuclei were visualized by DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). NFAT translocation: NRVM were stained using indirect immunofluorescence staining with polyclonal anti-NFAT (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA), monoclonal anti-α-actinin (Sigma, St. Louis, MO, USA) antibodies, and anti mouse-FITC-C+anti-rabbit rhodamin (Jackson ImmunoResearch Laboratories, Inc., Baltimore, PA, USA). Nuclei were visualized by Topro stain (Jackson ImmunoResearch Laboratories). The slides were visualized using a Provis AX70 fluorescence microscope at ×40 magnification (Olympus, Tokyo, Japan).

Table 1

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<th>Genes</th>
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| ANP   | Up: 5'-ATG GGC TCC TTC TCC ATC ACC-3'  
        Down: 5'-GTA CCG GAA GCT GTT GCA GCC-3'| 94 °C 30 s, 58 °C 30 s, 72 °C 1 min  
40 cycles |
| Cx43  | Up: 5'-AAAGGGCTTAAAGGTCGCGT-3'  
        Down: 5'-GTTCATCAAGGGGAGGCT-3'| 94 °C 30 s, 58 °C 30 s, 72 °C 1 min  
40 cycles |
| RYR2  | Up: 5'-GAATCAGTGATGATTGACGATGG-3'  
        Down: 5'-CTGCTTCCACGTCATCTCCAAGAGC-3'| 94 °C 45 s, 60 °C 1 min, 72 °C 1.5 min  
32 cycles |
| SERCA2a | Up: 5'-ATGGAATCAGCAAGATGACATGGTG-3'  
        Down: 5'-GACACTGACCATCTCATATGGACTAG-3'| 94 °C 45 s, 60 °C 1 min, 72 °C 1.5 min  
32 cycles |
| IP₃R2 | Up: 5'-CTTCTTGGCCTCGGTATT-3'  
        Down: 5'-CCAAGCTCTCCATTTCTCAAG-3'| 94 °C 30 s, 58 °C 30 s, 72 °C 1 min  
35 cycles |
| IP₃R3 | Up: 5'-CTGCGAGAACAGGAGGAAAG-3'  
        Down: 5'-GAACAGCGCGGCAATGGAGAAG-3'| 94 °C 30 s, 58 °C 30 s, 72 °C 1 min  
35 cycles |
| β-actin | Up: 5'-GCCATGTGCTATGCAATCC-3'  
        Down: 5'-GAACCGCTATGCGCGATAG-3'| 94 °C 30 s, 58 °C 30 s, 72 °C 1 min  
28 cycles |
2.4. RT-PCR assay

Total RNA was isolated using EZ-RNA™ kit (Biological Industries, Beit-Haemek, Israel) and Reverse Transcriptase (RT) reaction was conducted [8]. The nucleotides used and the cycling conditions are presented in Table 1. All PCR products were given a final elongation stage at 72 °C for 10 min. Products were resolved on 1.5% agarose gel.

![Fig. 1](https://academic.oup.com/cardiovascres/article-abstract/68/1/75/288328)

Fig. 1. Fas activation in NRVM causes hypertrophy but not apoptosis. (A) NRVM were treated with rFasL or with 50 μM H₂O₂, and apoptosis assessed by the DAPI assay. (B) Fas activation for 24 h increased actin immunofluorescence. Next to the graph, corresponding actin immunofluorescence pictures are depicted. Actin fluorescence is expressed in arbitrary units (n=4). (C) Time dependent changes in ANP mRNA. The upper panel depicts representative gels from control, 3, 6 and 24 h of Fas activation, and from a culture treated for 24 h with endothelin-1 (50 nM) (n=6). (D) Time dependent changes in Cx43 mRNA. The upper panel depicts representative gels from control, 3, 6 and 24 h of Fas activation (n=6). Each value was divided by its corresponding β-actin value. Values were normalized to control cultures, which were set as 1.0. (E) Effect of Fas activation on nuclear area. Altogether, 60 nuclei were measured from two different litters for each group. *P<0.05.
2.5. Nuclear measurements

For nuclear staining, slides were stained with hematoxylin and eosin. Morphometric determination of myocytes nuclear dimensions was performed using image analysis software (analySIS docu®3.0, Soft Imaging System, Munster, Germany). The system consists of an Olympus CH-40 bright field microscope (×100 objective) fitted with a CCD camera. The peripheries of selected nuclei were traced with the mouse cursor and the values (in μm²) were determined. Special care was taken to ensure that the areas selected for analysis on the slides were comparable between the treated and control samples. Altogether, 60 nuclei were measured from two different litters for each group.

2.6. Chemicals

Unless otherwise indicated, chemicals were purchased from Sigma (Sigma Chemical Co, St. Louis, MO, USA).

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Fig. 2. The PLC blocker U73122 and genistein block the increase in actin immunofluorescence and Cx43 protein expression caused by 24 h Fas activation. U73122 (2 μM) or genistein (50 μM) were added to the culture 30 min before, and throughout exposure to rFasL. (A) U73122 blocks the increase in actin immunofluorescence caused by Fas activation. Above the graph, corresponding actin immunofluorescence pictures are depicted (n = 4). (B) Representative Western blots of total Cx43, NP-Cx43 and β-actin under different experimental conditions. The increase in total and NP-Cx43 protein expression induced by Fas activation was blocked by U73122 or genistein. U — U73122; Gen — Genistein. (C) and (D): Summary of the effects of U73122 and genistein on Fas-mediated increase in total and in NP-Cx43, respectively (n = 4). The equivalency of loading was verified with an antibody against β-actin. Each value was divided by its corresponding β-actin value. Values were normalized to control cultures, which were set as 1.0. *P<0.05.
2.7. Statistical analysis

Results were expressed as mean ± SEM. Means of two populations were compared using Student’s t-test for unpaired observations. A value of \( P < 0.05 \) was considered significantly different.

3. Results

3.1. Fas-induced hypertrophy

Firstly, we confirmed that Fas activation in NRVM does not cause apoptosis, although the apoptotic machinery is responsive, as evidenced by the ability of \( \text{H}_2\text{O}_2 \) to cause apoptosis (Fig. 1A). Next, we characterized the time course (3, 6 and 24 h) of the hypertrophy induced by Fas activation. As shown in Fig. 1C, rFasL increased ANP mRNA in a time-dependent fashion, to the extent comparable to that caused by endothelin-1 (ET-1). Cx43 mRNA levels (known to be affected by hypertrophic stimuli [9–11]) were also increased by rFasL in a time-dependent fashion, reaching a 2-fold change at 24 h (Fig. 1D). Since both ANP and Cx43 mRNA levels peaked at 24 h, subsequent experiments were performed at this time point. In agreement with the Cx43 mRNA data (Fig. 1D), total and non-phosphorylated (NP) Cx43 protein expression were increased at 24 h Fas activation (Fig. 2B–D). Two additional markers of hypertrophy, nuclear area [12, 13] and sarcomeric actin were also augmented by Fas activation (Fig. 1B and E). It is noteworthy that due to the pronounced overlap between myocytes in culture, accurate measurement of cell area in confluent NRVM cultures is practically impossible.

3.2. The effect of Fas activation on \([\text{Ca}^{2+}]_i\) handling proteins

Following a previous study showing that hypertrophy caused by aortic stenosis [14] is associated with alterations in key components of the intracellular \( \text{Ca}^{2+} \) handling...
machinery, we tested whether similar changes occur in Fas-mediated hypertrophy. Firstly, we tested the mRNA levels of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), responsible for pumping Ca\(^{2+}\) back into the SR. Of the three cardiac SERCA isoforms, SERCA2a, SERCA2b, and SERCA3, the first is the major isoform in cardiomyocytes. As depicted in Fig. 3A, Fas activation decreased SERCA2a mRNA level by 24%. Next, we tested the effect of Fas activation on the cardiac ryanodine receptor (RyR) SR isoform-RyR2, which is central to the Ca\(^{2+}\)-induced release of Ca\(^{2+}\) (CIRC) machinery. As shown in Fig. 3B, RyR2 was decreased by Fas activation to a similar extent as SERCA2a (~20%). Finally, we determined the effect of Fas activation on the intracellular IP\(_3\) receptors (IP\(_3\)R). Of the three known isoforms, we focused on the two that exist in neonatal and adult rat ventricular myocytes [15]. Whereas mRNA level of the type 2 cytosolic-localized IP\(_3\)R [15] was unchanged by Fas activation (Fig. 3C), that of the nuclear IP\(_3\)R type 3 was decreased by 25% (Fig. 3D).

### 3.3. The involvement of the 1,4,5-IP\(_3\) pathway in Fas-mediated hypertrophy

The first step in testing the 1,4,5-IP\(_3\) hypothesis was to determine whether the specific PLC blocker U73122 [4] interferes with Fas-mediated hypertrophy. In support of the hypothesis, U73122 blocked rFasL-induced increase in actin fluorescence (Fig. 2A), as well as total and NP Cx43 protein (Fig. 2B–D), and ANP and Cx43 mRNA (Fig. 4A–C). Furthermore, blocking the SR 1,4,5-IP\(_3\)-operated Ca\(^{2+}\) release channel with xestospongin C [4] also prevented rFasL-mediated increase in ANP and Cx43 mRNA levels (Fig. 4A–C). In support of our notion that 1,4,5-IP\(_3\)-mediated Ca\(^{2+}\) release is the main source triggering the hypertrophic program, we found that the RyR blocker ryanodine (10\(^{-5}\) mmol/l) did not prevent rFasL-induced increase in ANP mRNA level (Fig. 4D).

Since the only known function of the 1,4,5-IP\(_3\)-operated SR channel is to release Ca\(^{2+}\), it is conceivable that this increase in [Ca\(^{2+}\)], occurring at a certain point in time during Fas activation is instrumental to the hypertrophic response. To decipher this key issue we measured [Ca\(^{2+}\)] transients, and determined diastolic [Ca\(^{2+}\)], at 0, 3 and 24 h of Fas activation (Fig. 5). The effects of Fas activation were arrhythmias (as we previously reported [4]) occurring throughout the test period, and time-dependent changes in diastolic [Ca\(^{2+}\)] firstly increasing and then decreasing towards control levels (Fig. 5). In agreement with our previous report (focusing on 3 h of Fas activation) [4], the arrhythmias occurring at 24 h of Fas activation were also

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Fig. 4. Inhibitors of the 1,4,5-IP\(_3\) pathway and genistein block the increase in ANP and Cx43 mRNA caused by 24 h rFasL-induced. U73122 (2 μM), xestospongin C (10 μM) or genistein (50 μM) were added to the culture medium 30 min before, and throughout exposure to rFasL. (A) Representative gels of ANP and Cx43 mRNA under the different experimental conditions. A summary of the effect of U73122, xestospongin C and genistein on Fas-mediated increase in ANP mRNA (B, \(n=7\) experiments) and Cx43 mRNA (C, \(n=4\) experiments). (D) The increase in ANP mRNA induced by Fas activation was not blocked by ryanodine (10 μM). \(n=4\) experiments. Each value was divided by its corresponding β-actin value. Values were normalized to control cultures, which were set as 1.0. *\(P<0.05\).
blocked by U73122 and genistein (Fig. 5D and E). The persistence of the arrhythmias and the increased ANP and Cx43 mRNA levels (Fig. 5F) in the presence of a transient increase in diastolic $[\text{Ca}^{2+}]_i$ will be addressed in the Discussion.

3.4. Fas-mediated hypertrophy is blocked by tyrosine kinases inhibition

Because of the clinical significance of Fas-mediated hypertrophy, identifying drug-responsive elements in this pathway which can serve as potential targets for attenuating Fas-based myocardial dysfunction is of prime importance. Based on our recent study showing that the omnipotent tyrosine kinases (TK) inhibitor genistein prevented Fas-mediated acute effects [6], we tested whether inhibiting TK will also attenuate Fas-mediated hypertrophy. Indeed, genistein blocked the hypertrophy, as evidenced by lack of change in ANP and Cx43 mRNA (Fig. 4A–C), and total Cx43 protein (Fig. 2B–C). Concomitantly, genistein increased the NP-Cx43 level, which is probably due to its inhibitory effect of TK phosphorylation site(s) located on the Cx43 molecule [16].

3.5. The effect of Fas activation on the nuclear factor of activated T cell (NFAT)

The NFAT family of transcription factors is dephosphorylated upon activation of the $\text{Ca}^{2+}$/CaM-dependent phosphatase calcineurin, resulting in unmasking of their nuclear localization signals thereby permitting nuclear import [17]. The requirement for NFAT translocation in calcineurin-mediated hypertrophy was confirmed [18], as well as the fact that cardiac hypertrophy is suppressed by
activated glycogen synthase-3β (GSK3β) which is upstream to NFAT and causes its nuclear export [19]. Based on previous studies showing that hypertrophic signals such as endothelin-1 [18] cause NFAT nuclear translocation and hypertrophy, we tested whether NFAT is involved in Fas-mediated hypertrophy. As seen in Fig. 6 (panel B, left side), while in non-stimulated myocytes, NFATc1 (an isoform translocated by endothelin-1) displayed a predominant cytosolic localization (the stained nuclei are shown in the lower panel), Fas activation for 3 h caused intense NFATc1 nuclear translocation (panel B, right side), as evidenced by the Topro-stained nuclei (panel C, right side).

3.6. The interaction between the 1,4,5-IP3 and PI3K/AKT pathways

Earlier studies have shown that activation of the hypertrophic program by Fas activation, and β-adrenergic and Gq-coupled receptors leads to GSK3β inactivation, predominantly through the PI3K/Akt pathway [1,19–22]. To test whether the 1,4,5-IP3 and the PI3K/Akt pathways operate independently or in concert to cause Fas-mediated hypertrophy, we determined whether Fas activation can induce hypertrophy in the presence of LY294002, a PI3K/Akt inhibitor. In agreement with Schlüter et al. [23], LY294002 blocked Fas-mediated hypertrophy, indicated by ANP mRNA level (Fig. 7A). Importantly, LY294002 also prevented the development of Fas-mediated arrhythmias (Fig. 7B), and the diastolic [Ca2+]i rise commonly seen at 3 h Fas activation (4 different experiments, total of 20 myocytes measured). As will be discussed below, these findings suggest that the 1,4,5-IP3 and the PI3K/Akt pathways are functionally inter-connected.

4. Discussion

In support of our working hypothesis that the Fas → PLC → 1,4,5-IP3 → SR [Ca2+]i release pathway is responsible for Fas-mediated hypertrophy, we found that the hypertrophy was blocked by U73122 — a PLC inhibitor, and xestospongin C — a specific inhibitor of the 1,4,5-IP3-operated SR Ca2+ release channels. Importantly, we found that the 1,4,5-IP3 and the PI3K pathways are inter-connected, causing NFAT nuclear translocation.

4.1. The hypertrophy markers

Fas-mediated hypertrophy was associated with increased ANP mRNA, sarcomeric actin and nuclear area. At 24 h of Fas activation the first two markers increased by ~2–2.5-fold, resembling the effects of the hypertrophy inducers norepinephrine, and the renin-angiotensin system activation [24,25]. Nuclear area was increased by 50%, similar to the effect seen in cardiac hypertrophy caused in rats by aortocaval fistula or pulmonary artery constriction [12,13]. A novel effect of Fas activation is a marked increase in Cx43, the main gap junctional protein in ventricular myocytes [26]. At 24 h of Fas activation, Cx43 mRNA, total Cx43 and NP Cx43 increased by 80% (Fig. 1D), 70% (Fig. 2C) and 130% (Fig. 2D), respectively. These results resemble those reported by Saffitz’s group who showed that in NRVM, angiotensin II and the membrane-permeant cAMP analogue db-cAMP (hypertrophy inducers) caused a 2–4 fold increase in Cx43 protein expression, which resulted from increased synthesis [26]. Since in our study both Cx43 mRNA and protein were augmented, it is likely that Fas activation also increased Cx43 synthesis. These changes in Cx43 concomitant with the hypertrophy can
contribute under pathophysiological conditions to development of anatomical and functional substrates of arrhythmias, frequently seen in the hypertrophic myocardium [28]. In addition to the changes in the hypertrophy markers, Fas activation decreased the expression of three key proteins associated with [Ca²⁺]i handling: SERCA (24%, Fig. 3A), RyR2 (Fig. 3B, 18%) and IP₃R type 3 (Fig. 3D, 25%). In agreement with our findings, in cultures treated for 24 h with angiotensin II, SERCA and RyR were decreased by 50% and 45% respectively [29]. Similarly, in a volume-overload model in rats, the decrease in SERCA mRNA was associated with a decrease in RyR mRNA and with a progressive left ventricular dysfunction [30]. Our IP₃R findings differ from those of Go et al. who showed that in failing human hearts RyR mRNA decreased whereas IP₃R mRNA increased [31]. While we did not determine how Fas activation affected RyR, SERCA and the IP₃R, we propose that the combined decrease in these proteins may contribute to Fas-mediated cardiac dysfunction.

4.2. The involvement of the 1,4,5-IP₃ pathway in Fas-induced hypertrophy

Our working hypothesis that 1,4,5-IP₃ is a key participant in Fas-mediated hypertrophy is supported by the findings that both U73122 and xestospongin C prevented the prominent arrhythmias and the diastolic [Ca²⁺]i rise in NRVM cultures treated for 3 h with rFasL. Thus, increased 1,4,5-IP₃ and the consequent SR Ca²⁺ release are responsible for both the acute and chronic effects of Fas activation. To further elucidate the signaling events (downstream of the 1,4,5-IP₃-mediated SR Ca²⁺ release) leading to hypertrophy, we determined whether the well established calcineurin-mediated, NFAT nuclear translocation is involved in Fas-mediated hypertrophy. Indeed, as shown in Fig. 6, Fas activation caused nuclear translocation of NFATc1. The NFAT proteins contain an N-terminal regulatory domain that controls their nuclear import in response to calcineurin activation [32]. While in non-stimulated cells this regulatory domain is phosphorylated and masks the nuclear localization sequence, causing NFAT proteins to be sequestered in the cytoplasm, calcineurin activation causes NFAT nuclear localization which is sufficient to induce cardiac hypertrophy [33]. Our findings are in close agreement with previous reports, such as that by Van Rooij et al., showing that endothelin-1 caused NFATc1 nuclear translocation and hypertrophy [18].

In the present work we identified two serially-connected signaling elements involved in Fas-mediated hypertrophy: the 1,4,5-IP₃ and the NFAT; the issue which should be addressed is the link between the two pathways. According to our proposed model (Fig. 8), the link is 1,4,5-IP₃-mediated Ca²⁺ release, which activates calcineurin, subsequently triggering NFAT nuclear translocation and the consequent hypertrophy [32]. In this regard, it is noteworthy that diastolic [Ca²⁺]i peaked at 3 h of Fas activation, and then returned back to control levels at 24 h. At the same time, the extent of hypertrophy steadily increased during the 24 h period (Fig. 5). This lack of complete association between
Despite the decline of diastolic $[Ca^{2+}]_i$ to control levels.

Later on, diastolic $[Ca^{2+}]_i$ declined towards control levels, the calcineurin (which triggered hypertrophy), and, although diastolic $[Ca^{2+}]_i$ changes and the increase in the hypertrophic response persisted and even gradually increased (compared to 3 h). This concept is supported by the occurrence of arrhythmias (which were blocked by U73122 and genistein, Fig. 5) at 24 h of Fas activation despite the decline of diastolic $[Ca^{2+}]_i$ to control levels. Therefore, we propose that 1,4,5-IP$_3$-mediated $Ca^{2+}$ release is the trigger that initiates the hypertrophic program.

### 4.3. The interaction between the 1,4,5-IP$_3$ and the PI3K/AKT pathway

An important finding was that both the 1,4,5-IP$_3$ pathway (U73122 and xestospongin C) and the PI3K/Akt pathway (LY294002) inhibitors completely blocked Fas-mediated hypertrophy, indicating that these pathways share a common link (Fig. 8). These findings are in accord with a report showing that LY294002 blocked the increase in diastolic $[Ca^{2+}]_i$ and the accompanying arrhythmias in neonatal cardiac myocytes as well as $[Ca^{2+}]_i$ rise in human hepatoma-derived cell line HepG2 [34,35]. According to our model (Fig. 8), the first step in Fas-based hypertrophy is Fas engagement, which via TK phosphorylation leads to PI3K activation. This in turn results in PLC activation by PI2 — a PI3K product, which anchors PLC to the cell membrane [34], leading to increased 1,4,5-IP$_3$ production. Binding of 1,4,5-IP$_3$ to its SR receptors causes $Ca^{2+}$ release, a phenomenon demonstrated by us [4] and others [15,36].

Subsequently, the $[Ca^{2+}]_i$ rise activates calcineurin which causes translocation to the nucleus of NFAT by its dephosphorylation, which is an essential step in the hypertrophic response [37,38]. Concurrently, Fas-mediated PI3K activation leads to inactivation of GSK3β (by its phosphorylation) [1], which is an established target of the PI3K/Akt signaling pathway [20]. Thus, inhibiting GSK3β — a negative regulator of cardiac hypertrophy, prevents the nuclear export of NFAT [19,37], which promotes hypertrophy. This scheme is consistent with a report by Antos et al. who showed that localization of NFATc1 to the nucleus in response to activated calcineurin was diminished in the presence of a GSK3β inhibitor [39]. Further, it was recently shown that an increase in PI3K activity can induce cardiac hypertrophy, whereas transgenic overexpression of a dominant-negative PI3K decreases cardiac mass [40]. Thus, by so doing, Fas-activated PI3K signaling can potentiate the $Ca^{2+}$-activated calcineurin hypertrophic pathway by removing the inhibitory influence of GSK3β.

### 4.4. The involvement of TK phosphorylation in Fas-induced hypertrophy

In agreement with our previous work showing that TK inhibitors block the acute effects of Fas activation, genistein also prevented Fas-mediated hypertrophy. Since a discussion of the potential sites possibly affected by genistein has been presented recently [6], this issue is addressed here only in brief. In principle, genistein can affect the hypertrophic pathway at 3 different sites: (1) The Fas receptor. Although the TK associated with Fas activation have not been fully characterized, they can be inhibited by genistein [41,42] (2) PLC. A number of studies proposed a possible interaction between the TK Fyn and Lck, and PLC. Some studies claim to have identified specific phosphorylation sites on PLCγ [43], while others suggest that PLC activation is related to TK phosphorylation, by forming a stable complex between these two [44,45]. (3) The 1,4,5-IP$_3$ receptor and the PI3K pathway. Gulbins et al. reported that PI3K, shown to be stimulated by Fas activation, [1] is dependent upon TK phosphorylation [46]. Furthermore, it was recently reported that PI3K might act as a regulator of $[Ca^{2+}]_i$ in cardiac cells [34]. PI3 (a PI3K product synthesized from PI2) anchors Tec — a tyrosine kinase and PLCγ to the membrane, thus enabling 1,4,5-IP$_3$ production from PI2 [34]. Accordingly, Jayaraman et al. showed that: (1) during T lymphocyte stimulation the IP$_3$R is physically associated with Fyn, and (2) in Fyn knockout mice, IP$_3$R phosphorylation was reduced [47]. Collectively, these and other studies support the notion that TK phosphorylation participates, probably at multiple sites, in the Fas signaling pathway leading to hypertrophy.
In summary, we have identified the PLC→1,4,5 IP3→SR [Ca2+] release pathway as a novel signaling cascade underlying Fas-induced hypertrophy in ventricular myocytes. Based on these findings, we suggest that modulators of the 1,4,5-IP3 pathway specifically targeted to cardiac muscle may have an important therapeutic value.

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