Inositol Polyphosphate 1-Phosphatase Is a Novel Antihypertrophic Factor*

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Activation of Gα-coupled α-adrenergic receptors leads to hypertrophic growth of neonatal rat ventricular cardiomyocytes that is associated with increased expression of hypertrophy-related genes, including atrial natriuretic peptide (ANP) and myosin light chain-2 (MLC), as well as increased ribosome synthesis. The role of inositol phosphates in signaling pathways involved in these changes in gene expression was examined by overexpressing inositol phosphate-metabolizing enzymes and determining effects on ANP, MLC, and 4S ribosomal gene expression following co-transfection of appropriate reporter gene constructs. Overexpression of enzymes that metabolize inositol 1,4,5-trisphosphate did not reduce ANP or MLC responses, but overexpression of the enzyme primarily responsible for metabolism of inositol 4,5-bisphosphate (Ins(1,4)P2), inositol polyphosphate 1-phosphatase (INPP), reduced ANP and MLC responses associated with α-adrenergic receptor-mediated hypertrophy. Similarly overexpressed INPP reduced ANP and MLC responses associated with contraction-induced hypertrophy. In addition, overexpression of INPP reduced the increase in ribosomal DNA transcription associated with both hypertrophic models. Hypertrophied cells from both cell models as well as ventricular tissue from mouse hearts hypertrophied by pressure overload in vivo contained heightened levels of Ins(1,4)P2, suggesting reduced INPP activity in three different models of hypertrophy. These studies provide evidence for an involvement of Ins(1,4)P2 in hypertrophic signaling pathways in ventricular myocytes.

Cardiac myocytes respond to stressors or to growth stimuli by increasing cell size in the absence of substantial cell division (1). Such hypertrophic growth in the in vivo situation is initially advantageous in allowing the heart to compensate for such factors as loss of myocytes or increased aortic pressure. However, sustained hypertrophy eventually leads to the development of heart failure, currently a leading cause of death in western societies (2). For this reason, the mechanisms involved in regulating hypertrophic growth are the subject of intense investigation.

Signaling pathways involved in cardiac hypertrophic growth appear to be similar to those that mediate growth and division in other cell types (3). Hypertrophy can be initiated by cytokines, growth factors, or factors that activate G protein-coupled receptors, principally those that activate the Gq class of heterotrimeric G proteins (1). Activation of Gq, either following receptor activation or directly by the use of constitutively active Gqα mutants leads to hypertrophic responses involving mitogen-activated protein kinase pathways as well as monomeric G proteins, although the details of all the intermediates are not fully understood (4). Currently the only well established substrates for activated Gqα are the isofoms of PLC-β (5), thus activation of Gq would be expected to lead to sn-1,2-diacylglycerol generation and protein kinase C (PKC) activation as well as Ins(1,4,5)P3 production and increases in cytosolic Ca2+. While activation of PKC can initiate hypertrophic responses, PKC does not appear to be solely responsible for all aspects of Gq-initiated hypertrophic responses (6, 7), implying a role for the inositol phosphate (InsP) arm of the pathway. Recent studies have identified mechanisms whereby raised cytosolic Ca2+ could lead to hypertrophic responses via the Ca2+-dependent phosphatase calcineurin or possibly via pathways initiated by calmodulin-activated kinase IV (8–10). However, Ins(1,4,5)P3 has little effect on global Ca2+ levels in myocardial preparations, and such effects would need to be considered against the large increases and decreases in cytosolic Ca2+ that occur during each beat in contracting myocytes. There is no evidence that Ins(1,4,5)P3 can cause sustained increases in diastolic Ca2+ levels as would be required for calcineurin or calmodulin-activated kinase mechanisms (11, 12).

We have previously presented evidence that activation of Gq-coupled receptors in cardiomyocytes leads to an InsP response that involves principally the generation of Ins(1,4)P2 from PtdIns(4)P independently of any Ins(1,4,5)P3 production (13, 14). Such a mechanism might serve to reduce the generation of Ins(1,4,5)P3 because that is potentially arrhythmogenic (12, 15–17). However, it is also possible that products of these
Ins(1,4)P$_2$-generating pathways have a functional significance of their own. In the current paper we present evidence that inositol polyphosphate 1-phosphatase (INPP), the enzyme primarily responsible for metabolism of Ins(1,4)P$_2$, reduces hypertrophic responses in neonatal rat cardiomyocytes. This is the first report of a functional importance of Ins(1,4)P$_2$ in cardiac signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Cell Isolation and Culture—**Neonatal cardiomyocytes (NCMs) were isolated from 1–3-day-old rats and maintained in serum-free modified Eagle’s medium supplemented with 10 μg/ml insulin and 10 μg/ml transferrin exactly as described previously (18). Bromodeoxyuridine (BrdUrd, 0.1 μM) was included for the first 3 days.

**Expression Constructs and Cloning of Human INPP—**INPP was cloned from a human cDNA library (Stratagene) by PCR using primers corresponding to positions 1–22 and 1177–2000, sense and antisense, respectively, of the published human INPP cDNA sequence (19). The 5’-primer contained an additional 24 nucleotides coding for the FLAG epitope (Sigma) inserted in-frame between the start ATG and the second codon. The PCR product was ligated into the pcR3.1 mammalian expression vector (Invitrogen) resulting in the construct pcR3.1-FLAG-INPP that drives expression of FLAG-tagged INPP (sense) under control of the cytomegalovirus promoter. Plasmids containing full-length antisense inserts were also selected. Orientation of the INPP insert was determined by sequencing and endonuclease mapping.

The atrial natriuretic peptide (ANP)-luciferase plasmid (pANP–638S/LS5), the myosin light chain-2 (MLC)-luciferase plasmid (pMLC–250LJ35), the AP-1-luciferase plasmid (TRE2PRL–36), the CMV-β-galactosidase, and the reporter construct for ribosomal gene transcription, sPMSECAT, have been described previously (20, 21). The pcR3.1 vector containing the chloramphenicol acetyltransferase (CAT) gene or the β-galactosidase gene was used as control in experiments involving INPP. The expression plasmid encoding the type 1 Ins(1,4,5)P$_3$, 5-phosphatase (22) was obtained from Prof. Christina Mitchell (Monash University). The plasmid encoding the A isoform of Ins(1,4,5)P$_3$ 3-kinase was obtained from Dr. Christophe Ermens (Free University of Brussels, Belgium). Plasmids encoding constitutively active JNK-1 and c-Jun were obtained from Dr. N. Dhanasekaran (Temple University School of Medicine, Philadelphia, PA), and the plasmid expressing the AT-1$_r$ receptor was supplied by Dr. Walter Thomas (Baker Institute).

**SDS-PAGE and Western Blotting—**Samples containing 30 μg of protein were separated electrophoretically on 10% SDS-PAGE gels under reducing conditions and were subsequently transferred to nitrocellulose membranes. Membranes were incubated with monoclonal anti-FLAG antibody (M2, Sigma) at a dilution of 1 in 2000 or polyclonal anti-rabbit IgG (Bio-Rad) antibodies (Bio-Rad). Detection was achieved using the enhanced chemiluminescence method (Amersham Biosciences) according to the manufacturer’s instructions. The molecular sizes of the immunodecorated proteins were verified by comparison to the migration of prestained protein markers (Bio-Rad) electrophoresed in parallel.

**Antibody Preparation—**The entire coding region of the INPP cDNA was amplified using primers that introduced a His 6 leader after the ATG and then cloned into the inducible bacterial expression vector pET17b (Novagen). Positive clones were verified by sequencing. Recombinant protein was induced with isopropyl-1-thio-β-d-galactopyranoside and purified using Ni$^{2+}$ affinity resin as recommended by the supplier (Qiagen). The purified protein was used to generate antisera in rabbits, and the sera were purified as described elsewhere (23).

**Verification of INPP Expression and Activity—**Expression of recombinant INPP was confirmed by Western analysis and enzyme assays on transfected cells. CHO cells transfected with pcR3.1-FLAG-INPP (LipojectAMINE, Invitrogen) expressed a protein of molecular mass 47 kDa recognized by anti-FLAG antibody that was not observed in cells transfected with pcR3.1-CAT, a construct that expresses the non-mammalian protein chloramphenicol acetyltransferase (Fig. 1A). The identity of the immunoreactive protein as INPP was further verified by blotting with affinity purified anti-INPP antibody that also identified an immunoreactive protein of a size consistent with the molecular mass predicted from the amino acid sequence of INPP. The expression of this protein was increased by transfection with pcR3.1-FLAG-INPP. Transfection of CHO cells with pcR3.1-FLAG-INPP also increased INPP activity, which was measured as previously described (24) (Fig. 1A). To ensure that overexpression of INPP perturbed InsP metabolism, we transfected HEK-293 cells with Rc-CMV-AT$_1$, to overexpress AT$_1$ receptors as well as with pcR3.1-FLAG-INPP or vector control. Cells were subsequently labeled with 3H[inositol (20 μCi/ml) for 24 h and then stimulated with 20 μM with angiotensin II (1 μM) in the presence of LiCl (10 mM). [3H]InsPs were extracted and quantified using anion exchange HPLC as described below. Overexpression of INPP caused a selective decrease in Ins(1,4)P$_2$ content. Thus, transfection with pcR3.1-FLAG-INPP causes increased expression of active INPP that leads to reduced Ins(1,4)P$_2$ content.

**Hypertrophic Models—**For phenylephrine (PE)-induced hypertrophy, NCMs were plated at 4000 cells/cm$^2$ on 35-mm wells with a 10 μM mixture of ketamine (8 mg/100 g), xylazine (2 mg/100 g), atropine (0.6 mg/100 g), and carprofen (0.5 mg/100 g). The chest was opened along the midline of the upper sternum. The segment of aortic arch between the right innominate and the left main carotid arteries was dissected, and the aortic diameter was narrowed by 70% (28). Sham-operated animals underwent a similar operation except that the aortic arch was not constricted. All experiments were carried out 6 weeks after surgery when hypertrophy was well established (28) (Table I).

**Transient Transfection and Reporter Gene Activity—**Transfection experiments were performed in triplicate using cardiomyocytes on 35-mm wells 1 day after isolation. Transient transfection using a total of 4.8 μg of DNA/well was performed by the calcium phosphate method. As described elsewhere, this results in transfection efficiency between 1 and 2% (29). Cells were harvested into Lysis Buffer containing 0.1 M K-HPO$_4$, 1% Triton X-100, 1 mM dithiothreitol, pH 7.9 (luciferase assays) or 0.25 mM Tris-Cl, pH 8.0 (CAT assays). Luciferase activity was measured in 100 μl Tricine, 10 mM MgSO$_4$, 2 mM EDTA, 2 mM ATP, 75 μM luciferin, pH 7.8 for 2 min in a Lumat LB9507 Lumimeter. CAT activity was assayed by the method of Fujisawa (30) (14C)chloramphenicol, analyzed by TLC as described previously (30), and quantified using a Fujix BAS 1000 phosphorimaging system. β-Galactosidase activity was measured using o-nitrophenyl-β-d-galactoside (0.25 mM) as substrate and determining absorbance at 410 nm. Protein concentration was assayed using the Bradford method. To control for any differences in transfection efficiency, data were expressed as luciferase activity relative to the activity of β-galactosidase co-expressed under a CMV promoter.

**[3H]InsPs Responses in NCMs and in Mouse Left Ventricle—**NCMs on 35-mm wells were labeled with [3H]inositol (15 μCi/ml) in isositol-free Dulbecco’s modified Eagle’s medium supplemented with insulin, transferrin, and BrdUrd for 48 h. Cells were then washed with non-radioactive medium and incubated in Dulbecco’s modified Eagle’s medium containing 1 μM propranolol and 10 mM LiCl for 10 min prior to addition of 100 μM norepinephrine (NE) for 20 min. [3H]InsPs were extracted using 5% trichloracetic acid, 5 mM phytic acid, 2.5 mM EDTA and centrifuged, and the supernatant was extracted with 0.75 volumes of trichloroethane:triethylamine:hexane (1:1:1, v/v) as described previously (31). Aqueous phases were treated with proteinase K (2.5 μg/ml), passed through Dowex-50 columns, and lyophilized. [3H]InsPs were separated using anion exchange HPLC and quantified using an on-line β-counter as described previously (32).

**Mouse left ventricle strips—**Mouse left ventricle strips (15–20 mg of tissue) were mounted in 3-ml organ baths in Heps-buffered Krebs’ medium and labeled with [3H]inositol (20 μCi/ml) as described previously (28). Labeled strips were stimulated with 100 μM NE in the presence of propranolol and LiCl for 20 min. Cells were collected by rapid perfusion with liquid N$_2$. [3H]InsPs were extracted and quantified as described above.

**Treatment of Data—**Differences between treatment groups were as assessed by one-way analysis of variance with Tukey’s test for multiple comparisons and accepted as statistically significant at a family error rate of p < 0.05 (individual pairwise comparisons were significant at p < 0.05). Unless otherwise noted, results shown are from representa...
Materials—Fetal calf serum specially selected for low endotoxin was obtained from the Commonwealth Serum Laboratories, Parkville, Australia. Dulbecco’s modified Eagle’s medium, Hepes, and other materials for the preparation of cell culture solutions and media were cell culture grade, obtained from Sigma, and dissolved in milliQ H2O. Norepinephrine bitartrate and phenylephrine were purchased from Sigma. [3H]Inositol (18.00 Ci/mmol) was obtained from Amersham Biosciences. Other reagents were obtained from Sigma or BDH/AnalaR and were of analytical reagent grade.

RESULTS

PE-stimulated ANP Responses Require Gq and PLC—NCMs were transfected with expression plasmids encoding Gq or Gq mutants together with the reporter gene ANP-luciferase as a measure of hypertrophic signaling and CMV-β-galactosidase to control for transfection efficiency. 20 h after transfection, PE (50 μM) was added for a further 40 h. Cells were harvested, and luciferase and β-galactosidase activities were measured. PE caused a substantial increase in ANP expression as indicated by luciferase activity in transfected cells (Fig. 2). As reported previously, the PE response was increased by overexpression of Gq-WT, while basal activity was not altered (29). Expression of the constitutively active mutant Gq(Q209L) increased ANP expression, and there was no further increase with added PE. In marked contrast, Gq(Q209L) with alanine substitutions D243A, N244E, and E245A (QL,DNE) rendering it unable to stimulate PLC (33) had no detectable effect on ANP responses in the presence or absence of PE. Thus both Gq and PLC appear to be involved in mediating ANP responses to PE in cardiomyocytes.

PE-stimulated ANP and MLC Responses Do Not Require PKC Activation—PLC activation generates two partially independent signaling pathways, one initiated by sn-1,2-diacylglycerol generation and activation of various isoforms of PKC and
the other initiated by Ins(1,4,5)P_3 and inositol phosphate metabolites. To examine the contribution of PKCs to PE-induced hypertrophic signaling, cells were treated with the selective PKC inhibitor bisindolylmaleimide (1 μM). Isolated NCMs were transfected with ANP- or MLC-luciferase constructs and subsequently treated with PE (50 μM) with or without bisindolylmaleimide (1 μM). As shown in Fig. 3, inhibition of PKCs with bisindolylmaleimide did not significantly reduce signaling from PE to either ANP or MLC reporter genes. However, expression of both ANP- and MLC-luciferase was increased by direct activation of PKCs by phorbol 12-myristate 13-acetate (PMA, 10 μM), although the stimulation was less than with PE, and bisindolylmaleimide (1 μM) was inhibitory. The inactive isomer of bisindolylmaleimide, bisindolylmaleimide 5, was ineffective in reducing the PMA response, attesting to the specificity of the inhibition (Fig. 3).

Another series of experiments was performed using a reporter construct encoding AP-1 response elements (2XTRE-luciferase) (34). Both PE (50 μM) and PMA (10 μM) increased AP-1 activity as indicated by increased luciferase activity, although in contrast to the ANP and MLC responses, PE was relatively weak compared with PMA. Responses to both effectors were inhibited by bisindolylmaleimide (1 μM) (Fig. 3).

**Fig. 2. Stimulation of ANP promoter activity by PE involves G_q and PLC activation.** NCMs were transfected with G_q-WT, constitutively active G_q(Q209L), constitutively active G_q that is unable to activate PLC (G_q(QL,DNE)), or control vector and subsequently stimulated with 50 μM PE. Luciferase and β-galactosidase activities were measured after 40 h. Values shown are luminescence readings per unit of β-galactosidase activity expressed as absorbance at 410 nm (mean ± S.E.) of triplicate estimations. The experiment was performed three times with similar results. Open bars, no additions; black bars, PE. *, p < 0.01 relative to no additions. †, p < 0.01 relative to PE stimulation in cells transfected with control vector. RLU, relative luminescence units.

**Fig. 3. Inhibition of PKC reduces AP-1 responses, but not the ANP and MLC responses, to PE.** NCMs were transfected with ANP-, MLC-, or 2XTRE (AP-1)-luciferase constructs and subsequently stimulated with PE (50 μM) or PMA (1 μM) together with bisindolylmaleimide 1 (active) or bisindolylmaleimide 5 (inactive). Luciferase activity was assayed 40 h later. Values shown are luminescence units per unit of β-galactosidase, mean ± S.E. of triplicate estimations. Black bars, no inhibitor; gray bars, bisindolylmaleimide 1; open bars, bisindolylmaleimide 5. *, p < 0.01 relative to no additions. †, p < 0.01 relative to PMA control. The experiment was performed three times. RLU, relative luminescence units; NA, no additions.

**Fig. 4. Inositol phosphate metabolism.** Substrates and products of enzymes used in the current study are shown. 1, Ins(1,4,5)P_3 3-kinase; 2, Ins(1,4,5)P_3 5-phosphatase; 3, INPP.

**Fig. 5. ANP and MLC responses to PE are not reduced by increased metabolism of Ins(1,4,5)P_3.** NCMs were transfected with ANP- (upper panels) or MLC-luciferase (lower panels) together with plasmids encoding Ins(1,4,5)P_3 3-kinase (IP_3kinase) or Ins(1,4,5)P_3 5-phosphatase (IP_3Pase) as indicated. PE (50 μM) was added, and luciferase activity was measured after 40 h. Values shown are luminescence units per unit of β-galactosidase activity, mean ± S.E. of triplicate estimations. The experiment was performed three times with similar results. Open bars, no additions; black bars, PE. *, p < 0.01 relative to no additions. †, p < 0.01 relative to control vector. RLU, relative luminescence units.
Thus, PE-stimulated activation of AP-1 response elements requires PKC activity, whereas transcriptional activation of ANP and MLC by PE is PKC-independent in this model (35).

Metabolism of Ins(1,4,5)P₃ Does Not Reduce ANP or MLC Responses to PE—The finding of a requirement for PLC activity without PKC involvement suggests a role for the other arm of the PLC response, the inositol phosphates. To investigate the possible role of Ins(1,4,5)P₃ in the signaling pathways that link β₂-adrenergic receptor activation to increased expression of ANP and MLC, Ins(1,4,5)P₃-metabolizing enzymes were overexpressed, and responses to PE were evaluated. Pathways involved in the metabolism of Ins(1,4,5)P₃ are shown in Fig. 4. Overexpression of the enzyme that phosphorylates Ins(1,4,5)P₃ to Ins(1,3,4,5)P₄, Ins(1,4,5)P₃ 3-kinase (Fig. 4, enzyme 1), did not cause any change in ANP or MLC expression in the presence or absence of PE (Fig. 5, left panels). This argues against a major role for Ins(1,4,5)P₃ or InsPs derived from Ins(1,3,4,5)P₄, including Ins(1,3,4)P₃ and its metabolites. Overexpression of an enzyme that dephosphorylates Ins(1,4,5)P₃ to Ins(1,4)P₂, the 43-kDa type 1 Ins(1,4,5)P₃ 5-phosphatase (Fig. 4, enzyme 2), on the other hand, increased PE-stimulated responses (Fig. 5, right panels). The finding that increased dephosphorylation of Ins(1,4,5)P₃ actually stimulated ANP and MLC responses could be interpreted as either Ins(1,4,5)P₃ or the alternative substrate Ins(1,3,4,5)P₄ being inhibitory. However, the lack of effect of overexpression of the Ins(1,4,5)P₃ 3-kinase, which reduces Ins(1,4,5)P₃ while increasing Ins(1,3,4,5)P₄, argues against both possibilities.

Metabolism of Ins(1,4)P₂ Reduces ANP and MLC Responses to PE—Another possible explanation for the stimulatory effect of overexpression of an enzyme that dephosphorylates Ins(1,4)P₂ to Ins(4)P₁ is that Ins(1,4)P₂ itself enhances hypertrophic signaling. To test this possibility, cardiomyocytes were transfected with the enzyme that dephosphorylates Ins(1,4)P₂ to Ins(4)P₁, INPP (Fig. 4, enzyme 3), together with ANP-luciferase or MLC-luciferase constructs. Overexpression of INPP inhibited both ANP and MLC responses to PE in a dose-dependent manner as shown in Fig. 6A. To examine the specificity of the observed inhibitory effect of INPP, transcription from ANP and MLC promoters was stimulated by overexpressing constitutively active JNK-1 together with c-Jun. Increased transcription was not inhibited by INPP. This shows
that the inhibitory action of INPP targets upstream signaling pathways involved in the hypertrophic response and that INPP overexpression is not generally toxic to the cells. To further establish the specificity of the effect of INPP on hypertrophic signaling, we transfected NCMs with full-length antisense INPP and examined effects on ANP responses to 50 μM PE. Antisense INPP reduced INPP content in CHO cells and increased ANP transcription in the presence and absence of PE, providing further evidence that activity of this enzyme can regulate signaling pathways culminating in ANP transcription (Fig. 6B).

To further examine possible cytotoxic effects of INPP overexpression, we transfected NCMs with pCR3.1-FLAG-INPP together with pEGFP-C1 so that transfected cells could be identified by EGFP fluorescence. Fluorescent cells were viable, and there was no indication of cell rounding or cytotoxicity (Fig. 6C).

**Metabolism of Ins(1,4)P2 Reduces rDNA Transcription—**Increases in ribosomal gene transcription and ribosome biogenesis are a prerequisite for PE-mediated cardiac hypertrophy (26, 27). In experiments similar to those described above, the effect of overexpression of INPP on rDNA transcription was assessed using pSMECAT, an accurate reporter for rDNA (26). NCMs were transfected with pCR3.1-FLAG-INPP together with pSMECAT and subsequently stimulated with PE for 40 h. Overexpression of INPP reduced pSMECAT activity in response to PE, as shown in Fig. 7, suggesting a role for INPP in signaling pathways directly related to cell growth.

**Hypertrophied Myocytes Contain Heightened Ins(1,4)-P2 Levels—**The inhibitory activity of INPP suggested that its substrate, Ins(1,4)P2, was in some way involved in the hypertrophic response. To provide further evidence for this, we looked for evidence of perturbed INPP activity in hypertrophied myocytes by measuring the levels of the substrate of INPP, Ins(1,4)P2, relative to the product, Ins(4)P1. NCMs were treated with PE to induce hypertrophy in medium containing [3H]inositol (15 μCi/ml) to label the inositol phospholipids. [3H]Inositol-labeled cells, control and hypertrophied, were subsequently stimulated for 20 min with 100 μM NE in the presence of 10 mM LiCl (to inhibit breakdown of the isomers of InsP1) as described under “Experimental Procedures.” [3H]InsPs were extracted, and the different isomers were separated and quantified. As shown in Fig. 8, hypertrophied myocytes contained heightened levels of [3H]Ins(1,4)P2 and showed increased ratios of Ins(1,4)P2 to Ins(4)P1 suggesting reduced INPP activity in this hypertrophic model.

**INPP Activity Influences Contraction-induced Hypertrophy in NCMs—**As PE itself is a direct activator of InsP generation, chronic treatment may have influenced InsP metabolism. For
this reason, we sought to repeat the above studies using the contraction-induced hypertrophic model of neonatal cardiomyocyte hypertrophy as described under “Experimental Procedures.” [3H]Inositol-labeled contracting (hypertrophied) and contraction-arrested (non-hypertrophied) cells were stimulated with 100 μM NE for 20 min, and [3H]InsPs were extracted, separated, and quantified. [3H]Ins(1,4)P2 content was substantially higher in the contracting, hypertrophied myocytes (Fig. 9A), and the ratio of Ins(1,4)P2 to Ins(4)P1 was heightened (Fig. 9B).

As described previously, contracting NCMs showed heightened expression of ANP and increased rDNA transcription (26, 27). In experiments similar to those described above, INPP was overexpressed in arrested or contracting NCMs, and markers of ANP expression (ANP-luciferase) or rDNA transcription (pSMECAT) were measured. As shown in Fig. 9, C and D, overexpression of INPP reduced both the ANP and rDNA responses induced by spontaneous contraction. Thus, the inhibitory activity of INPP is not restricted to PE-induced hypertrophy.

**FIG. 9.** Ins(1,4)P2 levels are increased in NCMs hypertrophied by spontaneous contraction, and overexpression of INPP reduces hypertrophic responses in this model. A and B, [3H]inositol-labeled hypertrophied (Contracting) and control (Arrested) cells were stimulated with 100 μM NE for 20 min in the presence of propranolol and LiCl. A, [3H]InsPs were extracted and quantified by HPLC. B, content of Ins(1,4)P2 in control and hypertrophied NCMs expressed as the ratio of Ins(1,4)P2 to Ins(4)P1 in percent. Values shown are mean ± S.E., n = 3. *, p < 0.01 relative to non-hypertrophied cells. C and D, NCMs were plated at high density and allowed to contract spontaneously (hypertrophied cells) or arrested with 50 mM KCl (non-hypertrophied cells) and were transfected with ANP-luciferase or pSMECAT as described under “Experimental Procedures.” After 40 h, luciferase and CAT activities were measured. Gray bars, arrested NCMs; black bars, contracting NCMs. All values are mean ± S.E. of triplicate estimation and all experiments were performed three times. *, p < 0.01 relative to arrested cells. †, p < 0.01 relative to control vector. RLU, relative luminometer units.

**DISCUSSION**

Ventricular myocytes increase in size under pathological conditions in vivo and in vitro. Such hypertrophic growth is associated with increased expression of a number of genes, including ANP and MLC, and these gene are often used as “markers” for pathophysiological hypertrophic responses. The importance of pathways downstream of Gq in initiating hypertrophic growth in cardiomyocytes is well established in in vivo studies (38) as well as in experiments using rat neonatal cardiomyocyte models (4). Currently the only well established targets for activated Gq are the β-isomers of PtdIns-specific PLC (5). As PLC activation generates both inositol phosphates...
and sn-1,2-diacylglycerol, either PKC family members, inositol phosphates, or both might be involved in activating downstream hypertrophic signaling molecules. There is currently substantial evidence for PKC involvement in aspects of the hypertrophic response (3), but the possible involvement of the inositol phosphate arm of the pathway has been less thoroughly investigated.

In the current experiments, we found that the PKC inhibitor bisindolylmaleimide inhibited ANP and MLC responses to PMA but was unable to prevent responses to /H9251-adrenergic receptor stimulation, suggesting a lack of requirement for PKC in this particular aspect of hypertrophy. This is consistent with findings in a recent study where modifying the activity of PKC while altering some aspects of cardiac growth responses to Gq overexpression in vivo did not perturb the increased expression of either ANP or MLC (7). Bisindolylmaleimide was able to inhibit PE-induced increases in transcription from an AP-1 reporter construct and also inhibited ANP and MLC responses to PMA. This shows that bisindolylmaleimide is able to access PKC isoforms effectively in NCMs under our experimental conditions. The PMA data also show that activation of at least one of the isoforms of PKC can increase ANP and MLC transcriptions. However, it also implies that this isoform either is not activated by PE or that PE stimulation of this isoform is exactly balanced by activation of an isoform that inhibits the response. The apparent lack of necessity for PKC in PE-dependent activation of ANP and MLC gene expression does not preclude a role for PKC in other aspects of the hypertrophic response to PE, including increases in cell size.

The lack of involvement of PKC in signaling pathways linking PE to ANP and MLC promoters pointed to a role for the inositol phosphate arm of the pathway. Given its well established association with Ca2+/Ins(1,4,5)P3 was the most likely contender (12, 39). Increased cytosolic Ca2+ could contribute to hypertrophic signaling by activating conventional PKC isoforms and by stimulating Ca2+-activated phosphatases such as calcineurin (40) or calmodulin-activated kinase IV (10). However, overexpression of Ins(1,4,5)P3-metabolizing enzymes did not reduce PE-stimulated increases in ANP or MLC transcription. Overexpression of Ins(1,4,5)P3 5-phosphatase actually increased transcription of both reporter genes, which might suggest an inhibitory role for one of its two substrates, Ins(1,4,5)P3 or Ins(1,3,4,5)P4. However, overexpression of Ins(1,4,5)P3 3-kinase, which metabolizes Ins(1,4,5)P3 to Ins(1,3,4,5)P4 and would thus be expected to decrease Ins(1,4,5)P3 and increase Ins(1,3,4,5)P4, had no effect on signaling, arguing that neither of these InsPs is important in signaling pathways culminating in ANP or MLC transcription.

The other possible explanation for the enhanced ANP and MLC responses in cells overexpressing Ins(1,4,5)P3 5-phosphatase is that the product of the Ins(1,4,5)P3 5-phosphatase, Ins(1,4)P2, is stimulatory. In agreement with this, overexpression of the enzyme primarily responsible for metabolizing Ins(1,4)P2, INPP, inhibited the responses (Figs. 6, 7, and 9). In addition to Ins(1,4)P2, INPP removes the 1-phosphate from Ins(1,3,4,5)P4 (19), but our data suggest that increased Ins(1,3,4,5)P4 is unlikely to be responsible for the observed inhibitory action of INPP as it is generated by pathways initiated by Ins(1,4,5)P3 3-kinase, and overexpressing this enzyme had no effect on ANP and MLC responses. However, it remains

![Diagram](http://www.jbc.org/)

**FIG. 10.** Hypertrophied mouse hearts contain reduced Ins(1,4)P2. Mouse hearts were hypertrophied by thoracic aortic constriction and harvested 6 weeks after surgery. Left ventricle strips were labeled with [3H]inositol and subsequently stimulated with 100 μM NE for 20 min. A, [3H]InsPs were extracted and quantified by HPLC. B, content of Ins(1,4)P2 in control and hypertrophied ventricles expressed as the ratio of Ins(1,4)P2 to Ins(4)P1. Gray bars, sham; black bars, TAC. Values shown are mean ± S.E., n = 6. *, p < 0.01 relative to sham-operated animals.
possible that the hydrolysis product of INPP, Ins(4)P, is inhibitory to hypertrophic signaling rather than the substrate, Ins(1,4)P, being stimulatory.

The central role of Ins(1,4,5)P in regulating intracellular Ca responses in non-excitable cells is well established (41), although its functional importance in excitable tissues such as heart is less clear (12). Functional roles have also been ascribed to a number of other InsPs. Ins(3,4,5,6)P can regulate Cl channels in some cell types (42), and a role in transcriptional regulation has also been suggested (43). Ins(3)P has been assigned roles in nuclear export of mRNA (44) and in repair of DNA double strand breaks (45). In the case of Ins(1,4)P, a role in cellular growth responses was reported in studies where exogenously expressed INPP localized to the nucleus and reduced DNA synthesis (46). Furthermore, Ins(1,4,5)P itself has been shown to activate DNA polymerase- (47). However, given that postnatal cardiomyocytes are terminally differentiated and do not undergo substantial cell division, such effects on DNA synthesis are unlikely to be involved in the antihypertrophic action of INPP demonstrated in these experiments. In addition to reducing transcription from ANP and MLC promoters, overexpression of INPP was found to inhibit RNA transcription. As increased ribosome synthesis has previously been shown to be a prerequisite for both PE- and contraction-mediated hypertrophic growth of cardiomyocytes (27, 48), this suggests that Ins(1,4)P may be involved in pathways regulating cellular growth in addition to its effect on ANP and MLC transcription.

The inhibitory effect of overexpressing INPP on transcription of genes associated with hypertrophic growth pointed to a role for Ins(1,4)P in hypertrophic signaling pathways. In keeping with this, heightened levels of Ins(1,4)P were observed not only in PE-induced hypertrophy but also in hypertrophy associated with contraction and more importantly in hypertrophied left ventricle in vivo. The finding of increased Ins(1,4)P relative to Ins(4)P in three different hypertrophic models implies that reduced activity of INPP is a common feature of hypertrophy. While such changes might be secondary to the hypertrophy, the finding that increased INPP expression can reduce at least some components of the hypertrophic response suggests that Ins(1,4)P has a functional role. In contrast to these models of hypertrophy where Ins(1,4,5)P levels are increased, we have previously reported reduced levels of Ins(1,4)P in heart tissue from rats chronically fed diets enriched in n-3 fatty acids (49). We have also observed an acute decrease in Ins(1,4)P in cardiomyocytes, in isolated atria, and in perfused rat hearts under conditions of ischemia or hypoxia (36, 50). Lowered Ins(1,4)P implies increased activity or expression of INPP as opposed to the situation in hypertrophy where activity appears to be decreased.

Previous studies from our laboratory have shown that PLC activation via α1-adrenergic receptors in neonatal rat cardiomyocytes generates both Ins(1,4,5)P from PtdIns(4,5)P2 and Ins(1,4)P from PtdIns(4)P (14). Given that Ins(1,4,5)P is potentially arrhythmogenic in heart, the generation of Ins(1,4,5)P might serve primarily to minimize changes in Ins(1,4,5)P while maintaining the ability to produce diacylglycerol and thus to regulate PKC. The current experiments demonstrate that Ins(1,4)P has a function of its own in signaling pathways in cardiomyocytes, and thus direct generation of Ins(1,4)P may be an important aspect of cardiac growth responses.

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REFERENCES

1. Homey, C. J. (1998) Circulation 97, 1890–1892
2. Hofmann, R. (1997) Circulation 95, 3297–3327
3. Bogoyevitch, M., and Sugden, P. (1996) Int. J. Biochem. Cell Biol. 28, 1–12
4. Dorn, G. W., and Brown, J. H. (1999) Trends Cardiovasc. Med. 9, 26–34
5. Rebecchi, M. J., and Pentyala, S. N. (2000) Physiol. Rev. 80, 1293–1335
6. Passier, R., Zeng, H., Frey, N., Naya, F. J., Nicol, R. L., McKinsey, T. A., Robbins, J., Grant, S. R., and Olson, E. N. (1998) Cell 93, 215–228
7. Hanakahi, L. A., Bartlet-Jones, M., Chappell, C., Pappin, D., and West, S. C. (1999) Circulation 93, 167–178
8. Wu, G. Y., Toyokawa, T., Hahn, H., and Dorn, G. W. (2000) J. Biol. Chem. 275, 29927–29930
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