The present study was designed to determine if \( \alpha \)- and \( \beta \)-adrenergic stimulation of neonatal rat myocardial cells might induce common and/or distinct members of the immediate early gene program and to assess whether their induction might correlate with the differential effects of these adrenergic agonists on cardiac gene expression, sarcomere assembly, and several features of myocardial cell hypertrophy. \( \alpha \)- and \( \beta \)-adrenergic stimulation of neonatal rat myocardial cells both produce an increase in the assembly of an individual contractile protein (myosin light chain-2) into organized sarcomeric units and also rapidly induce mRNAs for the immediate early genes \( c-fos \) and \( c-jun \), thereby suggesting a potential role for these protooncogenes in sarcomerogenesis. \( \alpha \)-Adrenergic stimulation results in the co-induction of mRNAs encoding a zinc finger protein gene (\( Egr-1 \)). However, \( \beta \)-adrenergic stimulation does not produce a significant increase in the levels of \( Egr-1 \) mRNA, providing the first evidence in any cell system that \( c-fos \) and \( Egr-1 \) expression can regulated separately. Studies with norepinephrine in combination with various adrenergic receptor antagonists revealed that the induction of \( Egr-1 \) is primarily a \( \alpha \)-mediated, pertussis toxin-insensitive response. These studies provide the first evidence for the induction of immediate early genes following adrenergic stimulation of myocardial cells and demonstrate that \( \alpha \)- and \( \beta \)-adrenergic stimulation can rapidly activate the expression of common and distinct subsets of these transcriptional regulators. Since \( \alpha \)- and \( \beta \)-adrenergic agonists have differential effects on cardiac gene expression and on the acquisition of several features of myocardial cell hypertrophy, the induction of \( Egr-1 \) provides a potential mechanism for the induction of genes that are exclusively induced during \( \alpha \)-adrenergic-mediated myocardial cell hypertrophy.

\( \alpha \)- and \( \beta \)-Adrenergic Stimulation Induces Distinct Patterns of Immediate Early Gene Expression in Neonatal Rat Myocardial Cells

\( Egr-1 \) induction is primarily an \( \alpha \)-mediated response.

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The abbreviations used are: MLC, myosin light chain; bgh, bovine growth hormone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.
Adrenergic Induction of fos/jun/Egr-1 in Myocyte Hypertrophy

The purpose of this study was to determine if α- and β-adrenergic stimulation of neonatal rat myocardial cells might induce common and/or distinct members of the immediate early gene program and to assess whether their induction might correlate with the differential effects of these adrenergic agonists on cardiac gene expression, sarcomere assembly, and several features of myocardial cell hypertrophy. In neonatal rat myocardial cells, α- and β-adrenergic stimulation both produce an increase in the assembly of an individual contractile protein (myosin light chain-2) into organized sarcomeric units and also rapidly activate the immediate early gene program, which includes the induction of the c-fos and c-jun protooncogenes, thereby suggesting a potential role for these protooncogenes in sarcomerogenesis. Although α-adrenergic stimulation results in the co-induction of a zinc finger protein gene (Egr-1), β-adrenergic stimulation does not produce a significant increase in the levels of Egr-1 mRNA, thereby providing the first evidence in any cell system that c-fos and Egr-1 expression can be coordinately regulated. Studies with norepinephrine in combination with various adrenergic receptor antagonists revealed that the induction of Egr-1 is primarily an α1-mediated, pertussis toxin-insensitive response. These studies provide evidence for the induction of immediate early genes following adrenergic stimulation of myocardial cells and demonstrate that α- and β-adrenergic stimulation lead to the expression of common and distinct subsets of these transcriptional regulators rapidly. Since α- and β-adrenergic agonists have differential effects on cardiac gene expression (6,7,11), the induction of Egr-1 provides a potential mechanism for the induction of genes that are exclusively induced during α-adrenergic-mediated myocardial cell hypertrophy.

Experimental Procedures

Myocardial Cell Cultures—Cultured neonatal rat myocardial cells were prepared as described previously (30) with minor modifications. Myocytes were dispersed from the ventricles of 1-2-day-old Sprague-Dawley rats by digestion with collagenase II (Worthington) and pancreatic (GIBCO) at 37 °C. Purification of the cell suspensions on buffer (116 mM KCl, 0.8 mM NaCl, 20 mM HEPES, 1 mM NaH2PO4, 5.5 mM glucose, 5.4 mM KCl, 0.85 mM MgSO4, pH 7.35) and centrifugation at 3000 rpm for 30 min in a Beckman tabletop centrifuge resulted in nocytofluorescence with MLC antisera. The myocytes were plated in Dulbecco’s modified Eagle’s medium/medium 199 (GIBCO), supplemented with 5% horse serum, 5% fetal calf serum, and antibiotics (84 µg/ml ampicillin and 3 µg/ml gentamicin). Following incubation in serum for 24 h, the cultures were washed and incubated in Dulbecco’s modified Eagle’s medium/medium 199 with various agents.

Isolation and Hybridization of RNA—Total RNA was isolated from myocardial cells by a guanidine HCl technique (31). Northern blot hybridizations were performed as described previously (32) with minor modifications. Briefly, the RNA was size fractionated on a discontinuous Percoll gradient (1.060/1.086 g/ml) made up in Ads buffer (116 mM NaCl, 20 mM HEPES, 1 mM NaH2PO4, 5.5 mM glucose, 5.4 mM KCl, 0.85 mM MgSO4, pH 7.35) and centrifugation at 3000 rpm for 30 min in a Beckman tabletop centrifuge resulted in myocardial cell cultures with >95% myocytes, as assessed by immunocytofluorescence with MLC antisera. The myocytes were plated in 10-cm culture dishes (Falcon) at a density of 1.6 × 10⁶ cells in 6 ml of 4:1 Dulbecco’s modified Eagle’s medium/medium 199 (GIBCO), supplemented with 5% horse serum, 5% fetal calf serum, and antibiotics (84 µg/ml ampicillin and 3 µg/ml gentamicin). Following incubation in serum for 24 h, the cultures were washed and incubated in Dulbecco’s modified Eagle’s medium/medium 199 with various agents.

Immune-blotting and Immunofluorescence Techniques—To obtain antibodies that would specifically recognize MLC-2 antigenic determinants, we synthesized a TrpE/MLC-2 fusion protein employing a well-characterized TrpE expression vector pATH-3 (35). A full-length rat cardiac MLC-2 cDNA (36) was obtained as an EcoRI fragment and cloned into a TrpE fusion protein expression vector (pATH-3) that maintains an open reading frame between the TrpE and the MLC-2 cDNA, resulting in the construction of the plasmid pTrpE-MLC2 (Fig. 1). The pTrpE-MLC2 plasmid was transformed into Escherichia coli host cells followed by growth in tryptophan-free M9 medium, resulting in the induction of the TrpE gene. Under these conditions, the TrpE product was the predominant cellular protein detected following analysis of bacterial cell extracts by polycrylamide gel electrophoresis and Coomassie staining (Fig. 2). Bacterial cells that were not transformed with the pATH vectors lacked the single predominant TrpE protein band. As can be readily seen, transformation of bacterial host cells with the pATH-3 vector resulted in a TrpE protein band of 37 kDa. Bacteria cells transformed with the pTrpE-MLC2 fusion construct displayed a consequent increase in the size of the predominant band to approximately 54 kDa, the expected size of the TrpE/MLC-2 fusion protein. The band corresponding to the TrpE/MLC-2 fusion protein was excised, homogenized, and immunized into rabbits. The resulting antisera were harvested and subsequently utilized for immunoblotting with extracts derived from purified myocardial cell cultures. As can be seen in Fig. 2, the antibody directed against the TrpE/MLC-2 fusion protein was capable of recognizing MLC-2 (both atrial and ventricular forms) but did not recognize MLC-1. In contrast, antisera directed against purified bovine MLC recognized both MLC-1 and MLC-2 in immunoblotting studies as reported previously (6). Rabbits were subsequently immunized with a TrpE/MLC-2 fusion protein, and the antisera were utilized for immunoblotting, which was performed by a method described previously (6). Briefly, myocardial cells were harvested by scraping into phosphate-buffered saline and collected by centrifugation for 5 min at 1000 × g. The resulting cellular pellet was solubilized with 600 µl of 6 M urea, 10 mM Tris, pH 6.8, and was centrifuged for 30 min at 10,000 rpm. The protein content of the cellular extracts was assayed by the method of Bradford (37), and equal aliquots (4–7 µg) of cellular protein were taken for SDS-polycrylamide gel electrophoresis and immunoblotting as described previously (6). Quantitation was performed by densitometry of samples in comparison with a purified myosin light chain standard, and the results were linear within the protein concentration employed in the assay procedure. The indirect immunofluorescence assays were performed by a minor modification of a procedure described previously (38). Briefly, the myocytic cells were grown on Lab-Tek plastic chamber slides that were precoated with laminin, and the cells were rinsed with buffer A

![Fig. 1. Construction of the TrpE/MLC-2 expression vector.](image)
and antisera. Host E. coli bacterial cells were transformed with the pATH-3 and pTrpE-MLC2 expression vectors and subsequently were placed under conditions to induce the TrpE gene. Briefly, 5 ml of complete M9 medium supplemented with L-tryptophan to a final concentration of 20 µg/ml was inoculated with a bacterial colony containing the pTrpE-MLC2 plasmid and incubated for 20-24 h at 37 °C with shaking. The 5 ml of preculture was inoculated into 500 ml of complete M9 medium lacking tryptophan and incubated for 14 h at 37 °C with shaking. The bacterial culture was centrifuged at 5000 X g for 30 min, and the bacterial pellet was suspended in 15 ml of SDS-polyacrylamide gel electrophoresis sample buffer. Nucleic acids were sheared by several passages through a 20-gauge needle, and the bacterial extracts were analyzed by SDS polyacrylamide gel electrophoresis followed by Coomassie staining (left panel). The TrpE/MLC2 fusion protein was purified by preparative gel electrophoresis, and the TrpE/MLC2 band was excised, homogenized, and injected into rabbits with serial immunizations. The antisera were subsequently utilized in immunoblotting experiments with total myocardial cell extracts. In lane 1, the positions of MLC-1 and MLC-2 are indicated, in lane 2, the top band is atrial MLC-2, and the lower band is ventricular MLC-2.

and then fixed for 15 min at room temperature in 2 ml of 3% paraformaldehyde in buffer A consisting of 10 mM NaPO₄, 150 mM NaCl, 1 mM MgCl₂, pH 7.4. The slides were incubated in 2 ml of 50 mM NH₄Cl for 10 min, washed twice in buffer A, and permeabilized with 2 ml of 0.2% Triton X-100 in buffer A for 15 min at room temperature followed by additional washing three times with buffer A. The coverslips were blocked with 1% bovine serum albumin for 10 min, incubated with aliquots of appropriately diluted TrpE/MLC2 antisera or bovine growth hormone (bgh)/Egr-1 antisera (raised against a bgh/Egr-1 fusion protein) for 60 min at 37 °C, rinsed, and washed four times with buffer A. Subsequently, the chamber slides were incubated for 10 min in a 1:20 dilution of normal goat serum and then incubated for 60 min at 37 °C with goat anti-rabbit IgG conjugated to fluorescein. After rinsing once and washing four times with buffer A, the slides were mounted on glass coverslips with 90% glycerol in 0.1 M Tris (pH 9.4) and viewed by fluorescence microscopy.

RESULTS

α- and β-adrenergic Effects on MLC-2 Content and Assembly into Sarcomeric Units by Indirect Immunofluorescence with TrpE/MLC2 Antiserum—Previous studies have documented a quantitative increase in MLC-2 content (2-3-fold) of neonatal rat myocardial cells during α-adrenergic stimulation (6) whereas β-adrenergic stimulation has a minimal effect on the accumulation of MLC-2 (6). To study further the effects of adrenergic stimuli on the assembly of MLC-2 into organized contractile units, we generated MLC-2-specific antisera by first synthesizing a bacterial TrpE/MLC-2 fusion protein (Figs. 1 and 2). Utilizing the TrpE/MLC2 antisera for indirect immunofluorescence, the assembly of MLC-2 into organized sarcomeres was examined in cells following stimulation of α- or β-adrenergic receptor-dependent pathways with phenylephrine or combined isoproterenol and cholera toxin treatment, respectively. As shown in Fig. 3, stimulation of either the α- or β-adrenergic pathway resulted in an increase in the organization of MLC-2 into organized contractile units, as evidenced by the presence of organized, parallel arrays of striations on indirect immunofluorescence. Since both α- and β-adrenergic stimuli can influence the assembly of MLC-2 in organized contractile units in these neonatal rat myocardial cells, we sought to determine if α- and β-adrenergic pathways might activate common immediate early genes that are associated with this response.

Induction of Immediate Early Genes following α- and β-Adrenergic Stimulation of Myocardial Cells—To establish the appropriate hybridization conditions of rat mRNAs with the heterologous probes derived from conserved regions of the v-fos, human c-jun, and mouse Egr-1 genes, we first examined the kinetics of induction of c-fos, c-jun, and Egr-1 mRNAs in cultured rat nonmyocardial cells that consist largely of fibroblasts (Fig. 4). Consistent with previous results obtained in many cell lines, serum stimulation of rat nonmyocardial cells resulted in the rapid induction of c-fos, c-jun, and Egr-1 mRNAs, with peak levels at 30 min of stimulation, followed by a return toward baseline levels after 2 h. These results validate the hybridization conditions utilized in Northern blotting analyses with the heterologous probes.

Following treatment with the α-adrenergic agonist phenylephrine (0.1 mM), neonatal rat myocardial cells displayed a rapid increase in the levels of c-fos (15-fold), c-jun (3-fold), and Egr-1 (15-fold) mRNAs (Fig. 5). Time course studies revealed a maximal induction at 30 min of continuous agonist stimulation (data not shown). Consistent with previous reports that document the co-regulation of Egr-1 with the c-fos gene during mitogen stimulation of many cell types (14, 16, 20, 39), the induction and decay of the c-fos, c-jun, and Egr-1 mRNAs have returned toward baseline levels (Fig. 5).

Stimulation of the β-adrenergic pathway with combined isoproterenol and cholera toxin treatment also induced a severalfold increase in the levels of c-fos and c-jun mRNAs. However, there was no statistically significant increase (p > 0.05) in the level of Egr-1 mRNA (Figs. 5 and 6). Since previous reports have found Egr-1 to be co-regulated with c-fos in multiple cell types and stimuli (16, 27), β-adrenergic stimulation of neonatal rat myocardial cells provides the first evidence that Egr-1 and c-fos can be coordinately regulated. Furthermore, it is apparent that α- and β-adrenergic stimuli must generate signals that not only activate the expression of similar sets of transcriptional factors, such as c-fos and c-jun, but also must produce divergent signals that result in the differential induction of individual members of the immediate early gene program, such as Egr-1.

Assessment of Receptor Specificity of Egr-1 Induction by Indirect Immunofluorescence with Egr-1 Antisera—To evalu-
ate further the adrenergic receptor specificity for the induction of the Egr-1 gene, we assessed the expression of Egr-1 protein by indirect immunofluorescence with antisera directed against a bgh/Egr-1 fusion protein (38). Since Egr-1 contains a zinc finger domain and is a putative transcriptional factor, the expression of Egr-1 should be detectable by positive nuclear staining. As depicted in Fig. 7A, the nuclei of control myocardial cells were visualized as negatively staining bodies, with a faint, nonspecific background staining in the cytoplasm. As a positive control, the myocardial cells were exposed to the tumor-promoting phorbol ester (phorbol 12-dimyrystate 13-acetate), a strong stimulus for the induction of Egr-1 in multiple cell types (16, 39), which resulted in positive nuclear staining within 1 h of stimulation (Fig. 7B). Following treatment with the combined α- and β-adrenergic agonist norepinephrine, the cultured myocardial cells also displayed positive nuclear staining, indicating the expression of the Egr-1 protein in greater than 90% of the cells within a random field (Fig. 7D). Preincubation of the cells with the α₁-adrenergic antagonist prazosin inhibited Egr-1 expression within myocardial cell nuclei (Fig. 7F) whereas pretreatment with the β-adrenergic antagonist propranolol was ineffective (Fig. 7E). Treatment with the α-adrenergic agonist phenylephrine resulted in the detectable expression of Egr-1 by nuclear immunofluorescence (Fig. 7C), which was also inhibited by prazosin and unaffected by pretreatment with propranolol (data not shown). Combined treatment with isoproterenol and cholera toxin also resulted in no evidence of Egr-1 expression as assessed by immunocytofluorescence (data not shown), providing further evidence that β-adrenergic stimulation alone is not sufficient to induce the expression of Egr-1 in neonatal rat myocardial cells. Taken together, these results clearly demonstrate that the induction of Egr-1 expression in neonatal rat myocardial cells is primarily an α₁-adrenergic recep-
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FIG. 5. Induction of c-fos, c-jun, and Egr-1 mRNAs following α- and β-adrenergic stimulation of neonatal rat myocardial cells. Neonatal rat myocardial cells were obtained as described under "Experimental Procedures" and plated for 24 h in medium containing 15% serum. The cells were rinsed and then plated in defined medium for various time periods and subsequently treated with either phenylephrine (PE, 0.1 mM) or isoproterenol (ISO, 10 μM) and cholera toxin (CTX, 1 μg/ml). After various time periods, the cells were harvested for Northern blot analyses with specific cDNA probes, encoding the c-fos, c-jun, Egr-1 and 28 S ribosomal RNA genes as described in Fig. 4. Results are representative of four additional experiments, which yielded similar results.

FIG. 6. Increase in c-fos, c-jun, and Egr-1 mRNAs following 30 min of α- and β-adrenergic stimulation of cultured myocardial cells. Experimental details are similar to those in Fig. 5. Results represent the fold increase versus control in the c-fos, c-jun, and Egr-1 mRNAs as assessed by Northern blotting with specific probes. For each analysis, the hybridization signal was normalized to the signal obtained with a 28 S ribosomal RNA probe to correct for loading and transfer effects. Results represent the mean ± standard error of the mean for five separate experiments. PE, phenylephrine; CTX, cholera toxin.

Effects of Pertussis Toxin on the Accumulation of MLC-2 and the Induction of c-fos and Egr-1 mRNAs—In fibroblasts, the induction of the c-myc protooncogene and the consequent proliferative response following mitogen stimulation can be blunted by pertussis toxin pretreatment (40, 41). In addition, previous studies have suggested that α-adrenergic stimulation of phosphatidylinositol hydrolysis in neonatal rat myocardial cells can be partially inhibited by pretreatment with pertussis toxin (42). Accordingly, it became of interest to examine directly the possibility of a pertussis toxin-sensitive pathway for the induction of the immediate early gene program and the accumulation of MLC-2 during α-adrenergic stimulation of neonatal rat myocardial cells. First, a series of studies examined the concentration of pertussis toxin which was required to achieve maximal ADP-ribosylation of the available pertussis toxin substrates within the myocardial cells. As displayed in Fig. 8, in vitro incubation of neonatal rat myocardial cell membranes with pertussis toxin resulted in the ADP-ribosylation of 39-41-kDa substrates, similar to the pertussis toxin substrates found in other cell types described in previous studies (43). Pretreatment of the myocardial cells with 0.5 ng/ml pertussis toxin for 6 h blocked subsequent in vitro ADP-ribosylation following in vitro incubation of membranes with pertussis toxin (Fig. 8), thereby indicating maximal ADP-ribosylation of the available myocardial cell toxin substrates during the prelabeling period. Following pretreatment with various doses of pertussis toxin, it became evident that the adrenergic induction of c-fos and Egr-1 mRNAs as well as the induction of MLC-2 were not sensitive to pertussis toxin (Fig. 9). Thus, it is likely that a pertussis toxin-insensitive G protein substrate links the α1-adrenergic receptor with the induction of c-fos, c-jun, and Egr-1 genes and the subsequent accumulation of MLC-2 in neonatal rat myocardial cells.
Fig. 7. Adrenergic receptor specificity for Egr-1 expression as assessed by immunofluorescence analysis with bgb/Egr-1 antisera in cultured myocardial cells. Cultured neonatal rat myocardial cells were treated with various combinations of agonists for 1 h and subsequently processed for immunofluorescent analyses with antibodies directed against a bgb/Egr-1 fusion protein as described under “Experimental Procedures.” In certain instances, the myocardial cells were pretreated with adrenergic receptor antagonists to define the receptor specificity of the induction of the Egr-1 protein. Results are representative of three independent experiments. Panel A, control myocardial cells; panel B, phorbol ester (100 nM); panel C, phenylephrine (0.1 mM); panel D, norepinephrine (10 μM); panel E, norepinephrine (10 μM) plus propranolol (10 μM); panel F, norepinephrine (10 μM) plus prazosin (10 μM).

Discussion

Adrenergic Induction of the Immediate Early Gene Program in Cardiac Cells—The transient induction of a set of growth-related genes, termed early response or immediate-early genes, is one of the earliest detectable effects of growth factors in diverse cell types (12–26). The immediate-early genes are characterized by their rapid and transient induction following mitogen stimulation, which does not ordinarily require the synthesis of new or additional proteins. Utilizing these criteria, the protooncogenes c-fos and c-jun are central members of the immediate early gene program. Recent studies have implicated a role for the c-fos and c-jun protooncogenes in the transcriptional activation of phorbol-inducible genes by the formation of fos/jun heterodimers, which activate transcription following binding to a consensus AP-1 site in the promoter region of the phorbol-inducible genes (19, 24, 44–49). Utilizing antisense constructs (50, 51) or the microinjection of neutralizing fos antisera into quiescent cells (52), direct evidence has been obtained that c-fos induction is required for the activation of cell proliferation and the onset of DNA synthesis following growth factor stimulation. In addition, utilizing a combination of experimental approaches including differential screening and subtraction cloning of cDNA libraries derived from quiescent and mitogen-stimulated cells, several new members of the fos and jun gene family have been identified (24, 25, 53, 54) as well as a series of related zinc finger-encoding genes (the Egr family, namely Egr-1, also known as zif/268, Krox-24, nerve growth factor IA, and Tis 8 (15, 16, 20, 23); Egr-2 or Krox-20 (18, 22); and Egr-3 and others, which have structural features of the steroid receptor super family (Nur77/nerve growth factor IB; 21, 26). Thus, based upon structural and functional criteria, the immediate-early genes have been shown to encode known or putative transcriptional factors and have been proposed to orchestrate different programs of gene expression in various differentiated cell types (55).

The present study documents the activation of the immediate-early gene program following α- and β-adrenergic stimulation of neonatal rat myocardial cells. Although many of the effects of adrenergic agonists on cardiac structure, function, and metabolism had previously been thought to be mediated solely through the rapid phosphorylation of existing cardiac proteins, the early induction of a program of immediate-early gene expression suggests that the regulation of the transcription of individual cardiac genes might play an unexpectedly important role in the adaptive responses of the myocardium during chronic catecholamine stimulation. This notion is consistent with the enlarging role of autonomic receptors in the control of cellular proliferation, as evidenced by the recent finding that the stimulation of a serotonin or muscarinic receptor can result in the activation of a mitogenic response in several cell types (56, 57). In a similar manner, the induction of the immediate-early gene program of myocardial cells by adrenergic stimuli is consistent with the role of autonomic receptors in the control of growth and differentiation. Interestingly, the α-adrenergic induction of the immediate-early gene program within 30 min precedes the transcriptional activation of cardiac genes which is not detectable until approximately 3 h (6). Accordingly, it is of interest to determine if the induction of the immediate-early gene program is required for the subsequent activation of cardiac gene transcription during α-adrenergic stimulation.
Expression of c-fos/c-jun during α- and β-Adrenergic Stimulation Is Associated with an Increase in the Assembly of an Individual Contractile Protein (MLC-2) into Organized Sarcomeres—In neonatal rat myocardial cells, α- and β-adrenergic pathways induce the expression of the c-fos and c-jun protooncogenes and result in an increase in sarcomerogenesis, as assessed by the assembly of an individual contractile protein (MLC-2), into organized sarcomeric units. Although a cause-effect relationship between these events cannot be presumed, the association of fos/jun expression with sarcomere assembly in cardiac muscle cells stands in contrast to previous results in skeletal muscle cells. In primary skeletal muscle cells, agonists that activate the expression of c-fos, such as tumor-promoting phorbol esters, lead to the selective disassembly of the myofibrillar apparatus and inhibition of the expression of myofibrillar genes (58–60). Constitutive expression of c-fos in skeletal muscle cells leads to a decrease in the expression of endogenous muscle-specific marker genes and a decrease in reporter genes under the control of muscle-specific promoters (28). This effect may be mediated by the downregulation of myoD (28), resulting in the consequent decrease in the expression of muscle-specific genes that are directly or indirectly controlled by this master muscle regulatory gene (61). Expression of myoD in these cells, which are constitutively expressing c-fos, results in the reactivation of exogenous and endogenous muscle-specific marker genes, effectively releasing the inhibition on muscle gene expression. Since the induction of c-fos in cardiac cells by adrenergic agonists is associated with the assembly of MLC-2 into organized contractile units, one might surmise that the cardiac muscle-specific regulatory factor(s) that activate MLC-2 expression and/or sarcomerogenesis in cardiac muscle might be positively regulated by c-fos. Direct proof of this notion will require the identification of the transcriptional factors that activate MLC-2 gene expression. Recent studies have demonstrated that a 250-base pair fragment of the MLC-2 5'-flanking region is sufficient to confer cardiac-specific (62) and adrenergic-inducible expression in cardiac cells, which should allow the future evaluation of the role of the immediate early gene program in the induction of this contractile protein gene during α-adrenergic-induced myocardial cell hypertrophy.

Induction of Egr-1 Is Primarily an α-Mediated Response

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**Fig. 8.** Effect of pertussis toxin pretreatment on \textit{in vitro} and \textit{in vivo} ADP-ribosylation of G protein substrates in cultured myocyte membranes. Cultured myocardial cells were obtained as described under “Experimental Procedures,” and S49 lymphoma cells were the generous gift of Dr. Alan Maisel and Paul Insel (67). A membrane fraction was isolated, and \textit{in vitro} ADP-ribosylation with pertussis toxin (4.4 μg/ml) was performed as described previously in (68). In certain cases, the myocardial cells were pretreated for 6 h with various concentrations of pertussis toxin prior to the isolation of the membrane fraction. Lane 1, S49 lymphoma cell membranes incubated \textit{in vitro} without pertussis toxin; lane 2, S49 lymphoma membrane incubated \textit{in vitro} with pertussis toxin; lane 3, control myocyte membranes incubated \textit{in vitro} without pertussis toxin; lane 4, control myocyte membranes incubated \textit{in vitro} with pertussis toxin; lanes 5–7, membranes derived from myocytes pretreated \textit{in vivo} with various concentrations of pertussis toxin (lane 5, 0.1 ng/ml; lane 6, 0.25 ng/ml; lane 7, 0.5 ng/ml) followed by \textit{in vitro} treatment with pertussis toxin. Results are representative of three independent experiments, which yielded similar results.

**Fig. 9.** Effect of pertussis toxin pretreatment on the induction of c-fos and Egr-1 mRNAs, and the accumulation of MLC-2 protein. Cultured myocardial cells were pretreated with various concentrations of pertussis toxin for 6 h and subsequently incubated with the α-adrenergic agonist phenylephrine (0.1 mM). In certain cultures, the cell were harvested after 30 min of phenylephrine treatment to assess the induction of Egr-1 and c-fos mRNAs by Northern blotting as described under “Experimental Procedures.” Results are expressed as the percent of maximal induction in control versus phenylephrine-treated cells. To assess the effect of pertussis toxin on the accumulation of MLC-2, the cells were treated for 48 h with phenylephrine (0.1 mM) and harvested for the assessment of MLC-2 content by quantitative immunoblotting, as described previously (6). Left panel, MLC-2; middle panel, c-fos; right panel, Egr-1. Results are expressed as the percent maximal induction (phenylephrine versus control) are representative of five separate experiments, and are expressed as the mean ± standard error. There was no statistically significant effect of pertussis toxin on the induction of the c-fos or Egr-1 genes or on MLC-2 accumulation.
and Is Associated with Acquisition of Several Features of Myocardial Cell Hypertrophy—Previous studies in multiple cell types have demonstrated that the immediate-early gene c-fos and Egr-1 are co-regulated following exposure to diverse hormonal and electrical stimuli (14, 16, 20, 27, 40, 55, 63). At the present time the target genes that are activated by Egr-1 expression are unknown although the Egr-1 protein appears to autoregulate its own expression. In the present study we provide evidence that α- and β-adrenergic stimulation can not only induce common sets of immediate-early genes (c-fos/c-jun) but can also differentially regulate the zinc finger immediate-early gene, Egr-1. Since in multiple cell types and diverse stimuli the expression of Egr-1 is co-regulated with c-fos, the demonstration that β-adrenergic stimulation results in the induction of c-fos without an accompanying increase in Egr-1 is of particular interest. Since α- and β-adrenergic stimuli have differential effects on the expression of cardiac genes such as the MLC-2 gene (6), cardiac and skeletal actin genes (7, 9), and the atrial natriuretic factor gene (29), the role of each protooncogene as well as an increase in the assembly of an individual contractile protein (MLC-2) into organized sarcomeric units. The induction of the immediate-early gene Egr-1 appears to be primarily an α-adrenergic response and thereby is correlated with the activation of α-mediated myocardial cell hypertrophy. Although previous studies have demonstrated the induction of the c-fos and c-myc protooncogenes during in vivo hypertrophy (8, 64–66), their role in the acquisition of specific structural and genetic features of the hypertrophic response is largely unknown. In this regard, the neonatal rat myocardial cell system may prove to be a valuable model to study the role of individual protooncogenes and other members of the immediate early gene program in sarcomerogenesis and the expression of cardiac specific genes during adrenergic-induced myocardial cell hypertrophy.

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REFERENCES
5. Brown, J. H., and Jones, L. G. (1986) Phosphoinositides and
6. D. Gius and V. P. Sukhatme, unpublished observations.

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