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A nuclear pathway for α_1-adrenergic receptor signaling in cardiac cells

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α_1-Adrenergic agonists and antagonists constitute an important class of therapeutic agents commonly used for the treatment of various cardiovascular diseases like hypertension, congestive heart failure and supraventricular tachycardia. At the heart level, activation of α_1-adrenergic receptors is associated with marked morphological and genetic changes. These include enhancement of contractility, myocardial growth (hypertrophy) and release of the heart major secretory product, atrial natriuretic factor (ANF). However, the signal transduction pathways which link extracellular activation of the receptors to cellular and genetic changes are not well understood. Using primary cardiocyte cultures from neonate rat hearts, an α_1-adrenergic regulatory sequence has been identified in the 5′ flanking region of the ANF gene. This sequence, which is necessary and sufficient for transcriptional activation in response to the α_1-specific agonist phenylephrine, interacts with novel zinc-dependent proteins which are induced by α_1-adrenergic stimulation. Consistent with a conserved regulatory mechanism, the α_1 response element is highly conserved between rodent, bovine and human ANF genes, and is also present in the promoter region of other α_1-responsive cardiac genes. The identification of a nuclear pathway for α_1-receptor signaling will be useful for elucidating the intracellular effectors of α_1-adrenergic receptors.

Key words: α_1-adrenergic receptors/ANF gene regulation/cardiac hypertrophy/*cis*-regulatory elements

Introduction

The naturally occurring catecholamines, epinephrine and norepinephrine, exert a major influence on cardiovascular homeostasis including regulation of cardiac function and control of arterial blood pressure. These effects are mediated by distinct subtypes of adrenergic receptors, all of which belong to the superfamily of cell surface G protein-linked receptors (Dohlman *et al.*, 1991). The different types of adrenergic receptors—α_1, α_2 and β—have distinct physiological actions which are mediated by different signal transduction pathways. Cardiac myocytes possess both α_1 and β receptors and several lines of evidence suggest a dual myocardial response to adrenergic stimulation (Simpson, 1985; Morgan and Baker, 1991). Heart rate and contractility are regulated mainly through β-adrenergic receptors which are classically coupled to activation of adenylyl cyclase and increase in cellular cAMP content (Raymond *et al.*, 1990; Dohlman *et al.*, 1991). α_1-Adrenergic stimulation can also enhance myocardial contractility and it is associated with an increase in myocyte protein synthesis and cell growth (Simpson, 1985; Morgan and Baker, 1991). Furthermore, activation of the α_1-adrenergic receptor results in the release of the heart major secretory product, atrial natriuretic factor (ANF), both *in vivo* and in isolated cardiac tissues or cells (Manning *et al.*, 1985; Uehlinger *et al.*, 1986; Schiebinger *et al.*, 1987; Shields and Glembotski, 1989). Activation of the α_1-adrenergic receptor is thought to play a major role in the pathophysiology and clinical manifestations of congestive heart failure (Leier *et al.*, 1990), while chronic stimulation of myocardial α_1-adrenergic receptors leads to cardiac hypertrophy *in vivo* and to cardiomyocyte enlargement in cultures *in vitro* (Simpson, 1985; Morgan and Baker, 1991). In fact, α_1-adrenergic antagonists are widely used therapeutically for the treatment of congestive heart failure and hypertension, while α_1-adrenergic agonists are commonly used drugs for the treatment of hypotension and supraventricular tachycardia (Leier *et al.*, 1990). These profound phenotypic effects on the heart are accompanied by alteration in the expression of several cardiac genes like skeletal actin, β-myosin heavy chain and ANF (Long *et al.*, 1989; Waspe *et al.*, 1990; Knowlton *et al.*, 1991; McBride *et al.*, 1993). However, despite its apparent importance, the signal transduction pathways, including the cytoplasmic and nuclear mechanisms which couple α_1-adrenergic receptors to cardiac growth and genetic switching, remain unknown.

To elucidate the subcellular mechanisms of α_1-adrenergic signaling in the heart, an α_1-adrenergic regulatory sequence has been identified in the 5′ flanking region of the ANF gene. This sequence, which is necessary and sufficient for transcriptional activation in response to the α_1-specific agonist phenylephrine (PE), is not related to any previously identified regulatory elements that mediate genetic responses to activation of intracellular second messengers.

Results

Identification of α_1-adrenergic response element on the ANF promoter

Previous *in vitro* and *in vivo* studies suggested that ANF gene transcription may be responsive to α_1-adrenergic agonists. Therefore, we first tested whether endogenous ANF gene expression and transfected ANF promoter activity were induced by α_1-adrenergic stimulation of cardiomyocyte cultures. Primary cardiocyte cultures were prepared from 1 day-old rats and exposed to the α_1-adrenergic agonist PE for 48 h. Consistent with previous findings (Simpson, 1985), this treatment leads to more synchronous beating and enlargement of myocytes but has no effect on DNA synthesis or myocyte proliferation, thus mimicking phenotypic changes

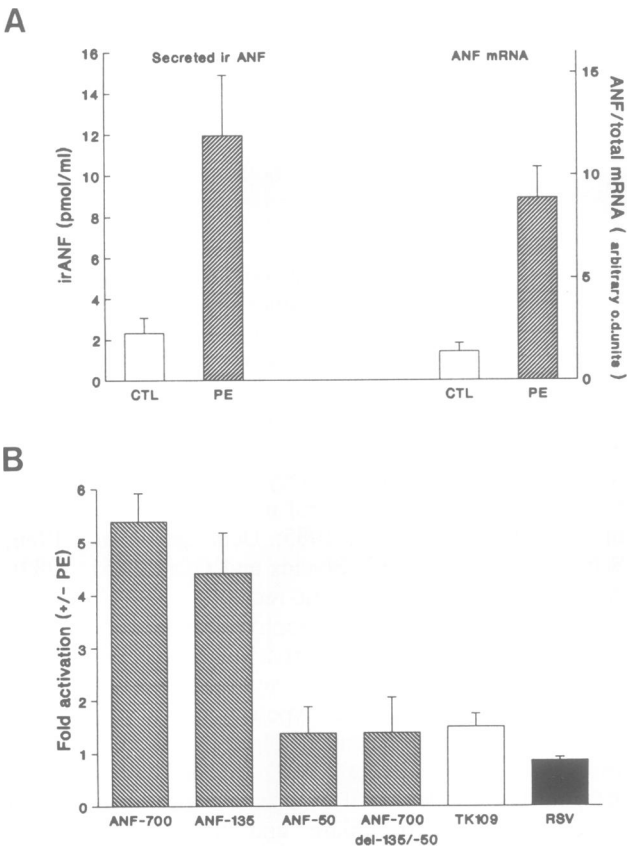


Fig. 1. Modulation of endogenous (**A**) and transfected (**B**) ANF gene expression by phenylephrine (PE). (A) Primary cardiocyte cultures were prepared from ventricles of 1 day-old rats, maintained in serum-free medium and treated with 10^{-4} M PE for 48 h as previously described (McBride *et al.*, 1993). ANF mRNA levels were quantitated using Northern blot hybridization and secreted immunoreactive (ir)ANF was measured in culture media using a specific radioimmunoassay. The results are the mean ± SD of six to eight independent determinations. (**B**) The effect of PE treatment on promoter activity was determined using constructs containing ANF or viral promoters linked to the human growth hormone (hGH) gene and RSV-luciferase was included in all transfections as internal control. Promoter activity was assessed by measuring ir-hGH in the media as previously described (McBride *et al.*, 1993). The results are expressed as the ratio (fold activation) of hGH/luciferase activity in PE-treated versus untreated ventricular myocytes. The results (mean ± SD) are from three to six independent experiments each carried out in duplicate. Similar results were also obtained using ANF promoter constructs linked to the luciferase reporter gene.

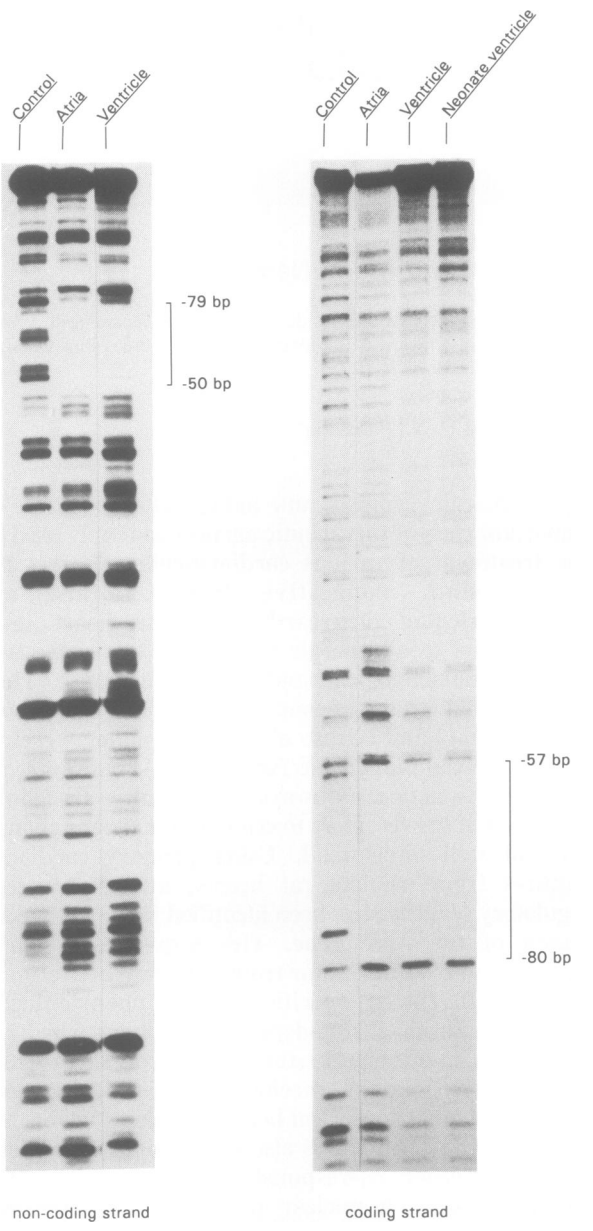


Fig. 2. DNase I footprinting of the proximal rat ANF promoter. A 5′ end-labeled *Hin*dIII–*Sau*3A fragment spanning nucleotides −135 to +63 bp was used. Nuclear extracts were prepared as detailed in Materials and methods. The results shown are from one representative experiment. Identical results were obtained using three different preparations of nuclear extracts. No other footprint was detected between −135 and −50 bp, even when footprinting reactions were carried out using a longer (−300 to +64 bp) fragment to allow detection of any protein–DNA interaction overlapping, or close to the −135 bp *Hin*dIII site.

associated with *in vivo* cardiac hypertrophy. ANF mRNA and secreted immunoreactive (ir) ANF levels were increased 6-fold in PE-treated myocytes (Figure 1A). The increase in endogenous ANF gene expression was also observed at the level of transfected ANF promoter activity which increased 5-fold following PE stimulation, while activity of the Rous sarcoma virus (RSV) and herpes simplex thymidine kinase (TK) promoters was not affected by PE treatment (Figure 1B). These results suggest that adrenergic stimulation modulates ANF transcription, and thus the ANF promoter may be used to define an α_1-adrenergic response element. Mapping of this element was carried out using nested 5′ deletion mutants (Argentin *et al.*, 1991; McBride *et al.*, 1993) which revealed that 135 bp of upstream ANF gene sequences were sufficient for PE responsiveness; however, an ANF promoter containing sequences up to −50 bp was no longer induced by PE suggesting that a PE response element (PERE) is present between −50 and −135 bp. Consistent with this, internal deletion of sequences between −50 and −135 bp markedly reduced PE responsiveness (Figure 1B).

To define further the PE regulatory element(s), DNase I footprinting experiments were performed using cardiac nuclear extracts. As shown in Figure 2, a footprint was observed between −80 and −50 bp. Further characterization of this putative PERE was carried out using electrophoretic mobility shift assays (EMSA). Binding of cardiocyte extracts to a 36 bp double-stranded oligonucleotide corresponding to the footprint produced several specific complexes

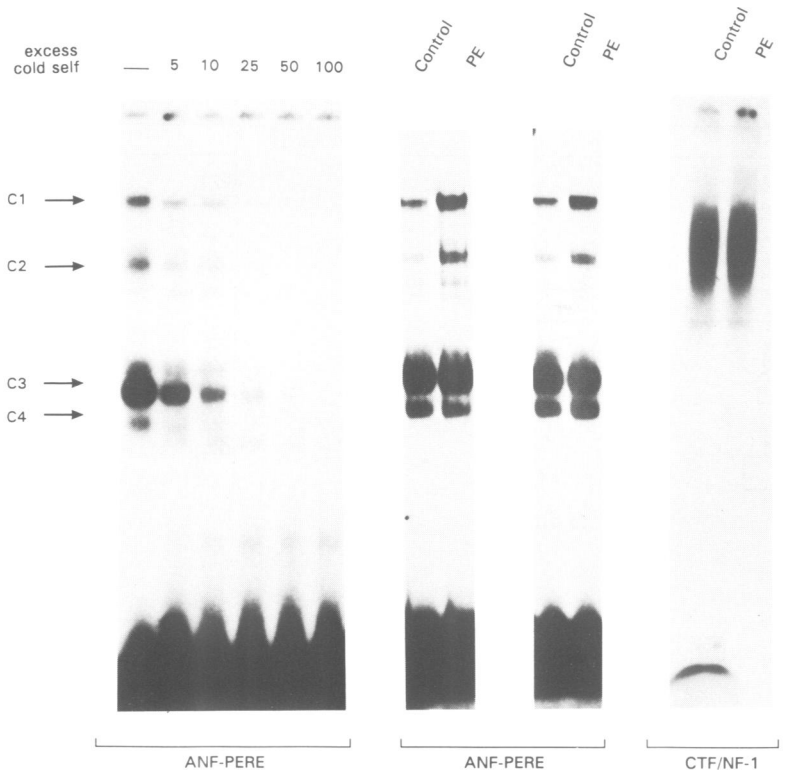
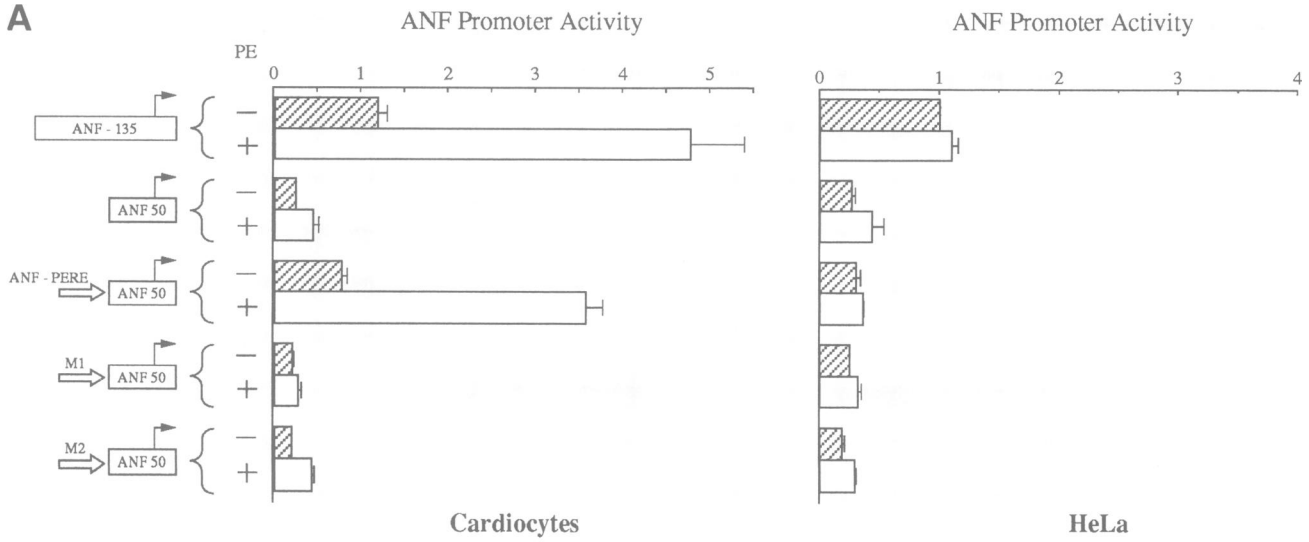


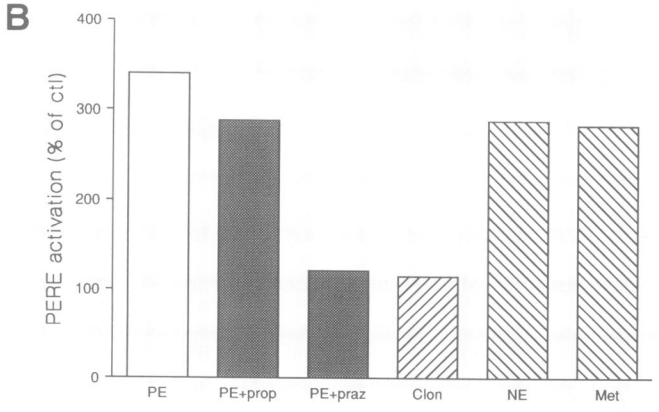
Fig. 3. Electrophoretic mobility shift assay (EMSA) using a 36 bp double-stranded oligonucleotide corresponding to the footprint and representing the putative PE response element (PERE). Cell extracts were prepared from primary ventricular myocyte cultures treated (PE) or not (control) with 10^{-4} M PE for 48 h according to Schöler *et al.* (1989). Binding reactions were carried out at room temperature for 30 min using 2 μg of extract in 60 mM KCl, 10 mM Tris−HCl pH 7.9, 5 mM $MgCl_2$, 1 mM EDTA pH 8.0, 1 mM DTT and 4% Ficoll in a final volume of 20 μl. In at least six different cultures, PE treatment produced a 3- to 5-fold increase in C1 and C2 binding but did not affect the specific C3 and C4 complexes. The results shown are from two different experiments. Binding over the PERE was already increased following 12 h exposure to PE (the earliest time point examined). An oligonucleotide corresponding to a CTF/NF-1 site (Chodosh *et al.*, 1988) showed no change in cardiac nuclear protein binding following PE treatment. Binding over either a tissue-specific ANF element or a putative ANF serum response element was also unaffected by PE treatment as already shown (McBride *et al.*, 1993).

(Figure 3). To test whether PE results in qualitative or quantitative changes in binding, EMSA were performed using nuclear extracts prepared from untreated or PE-treated cardiocytes. As seen in Figure 3, PE treatment produced a marked increase of some (C1 and C2), but not all, complexes; binding over a CTF/NF-1 site remained unchanged (Figure 3), as well as binding over a tissue-specific ANF element (McBride *et al.*, 1993 and data not shown), suggesting that the increase in DNA binding to the putative PERE was specific. When this 36 bp element was cloned upstream of the minimal ANF promoter, PE induction was restored to the same level observed with the ANF −135 fragment (Figure 4A). The α_1-adrenergic specificity of this transcriptional response was further confirmed using various adrenergic agonists and antagonists. In addition to PE, the PERE was stimulated by chronic treatment of myocytes with the naturally occurring agonist norepinephrine and with another α_1-adrenergic receptor agonist, methoxamine, but not by the α_2-receptor agonist clonidine; transcriptional activation by PE was not affected by the β-receptor antagonist propranolol but was completely abolished by the α_1-receptor antagonist prazosin (Figure 4B). Interestingly, the presence of the PERE had no effect on basal or PE-stimulated transcriptional activity in HeLa cells (Figure 4A), perhaps reflecting the lack of α_1 receptors in these cells (Schwinn *et al.*, 1991).

The α_1-adrenergic response element interacts with novel zinc-dependent nuclear proteins

The identity of the proteins that interact with the PERE was investigated using methylation interference and competition experiments with double-stranded oligonucleotides corresponding to previously identified transcription factor binding sites. Methylation interference studies revealed that methylation of the G residues in the GGGGAGGG (GAG) motif interfered with formation of the PE-sensitive C1 and C2 complexes (Table I and Figure 4C). The importance of this motif for the PE response was tested by site-directed mutagenesis. Mutations in the first (M1) or second (M2) GGG triplet abolished PE responsiveness (Figure 4A) and eliminated binding of the PE-induced complexes (PEX) (Figure 4D and E). Moreover, an oligonucleotide corresponding to the homologous region of the human ANF promoter (Nemer *et al.*, 1984), which differs by only three nucleotides outside the GAG motif, and a region (G3, Table I) from the human cardiac actin promoter, which contains the GAG motif (Gustafson and Kedes, 1989) but has little homology to the ANF sequence in the flanking nucleotides, were effective competitors of PEX (Figure 4E and data not shown). Thus, the GAG motif appears to be essential for PEX binding and PE response. In competition experiments, PEX were unaffected by the addition of a 50- or 100-fold molar excess of oligonucleotides corresponding


A
ANF Promoter Activity
PE
0 1 2 3 4 5
ANF - 135
ANF 50
ANF - PERE
ANF 50
M1
ANF 50
M2
ANF 50
Cardiocytes
ANF Promoter Activity
0 1 2 3 4
HeLa


B
PERE activation (% of ctl)
400
300
200
100
0
PE
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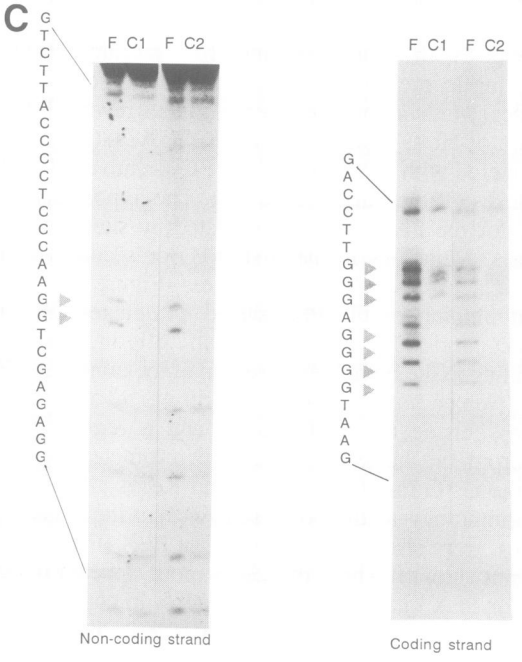
NE
Met


C
G T C T T A C C C C T C C C A A G G T C G A G A G G
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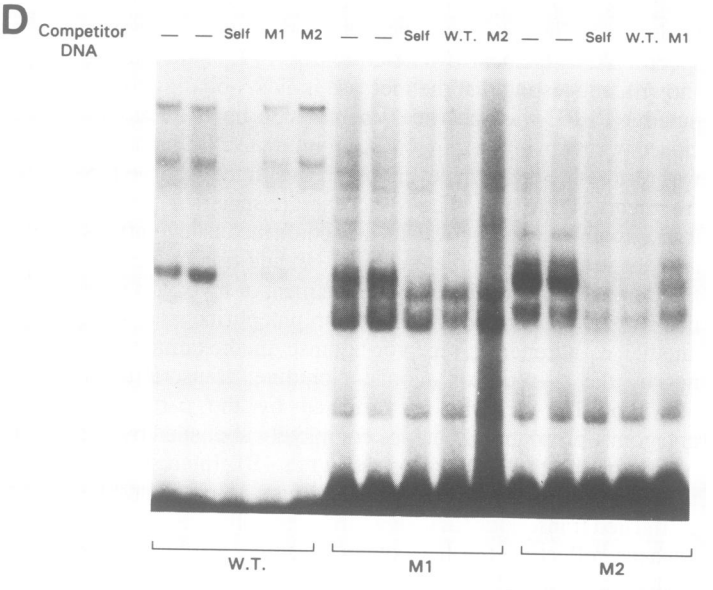
D
Competitor DNA
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W.T.
M1
M2

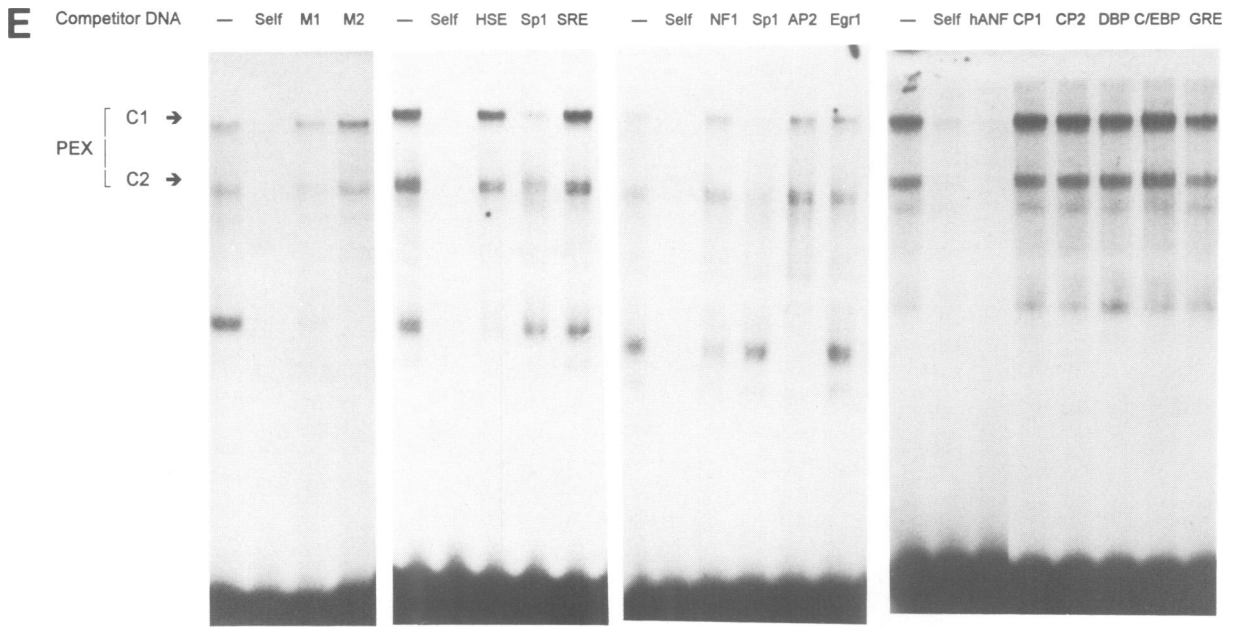

Fig. 4. (**A**) The response of the putative PERE to PE was tested in ventricular cardiocytes or HeLa cells by transfecting PERE constructs containing one copy of wild-type (PERE) or mutant (M1 and M2) elements fused to ANF-50/hGH. The sequence of the M1 and M2 mutants is shown in Table I. ANF promoter activity is expressed relative to ANF-135. The results are the mean ± SD of four (HeLa) and six to eight (cardiocytes) independent determinations. Addition of the PERE had no effect on basal or PE-stimulated transcription in fibroblast L cells and L6 skeletal myoblasts (data not shown). However, the PERE increased basal transcription 3-fold and mediated a 5-fold increase in promoter activity in response to PE treatment in atrial myocytes (data not shown). (**B**) α1 specificity of PERE induction by PE in ventricular cardiocytes. The ANF−PERE construct was treated with the various adrenergic agonists and antagonists for 48 h following transfection into ventricular myocytes. PE was added at 10^{-4} M; norepinephrine (NE), methoxamine (Met), prazosin (praz), clonidine (Clon) and propranolol (prop) were used at 10^{-5} M. As in all transfections, RSV-luciferase was used as an internal control. The results shown are the average of two separate experiments carried out in duplicate. (**C**) Methylation interference studies on the ANF−PERE. The techniques used for methylation interference are according to Argentin *et al.* (1991). F is free probe and C1 and C2 correspond to the upper and lower PE-induced complexes respectively. G residues interfering with binding are indicated by arrowheads. Identical results were obtained from two independent experiments. (**D**) Interaction of wild-type (W.T.) or mutant (M1 and M2) PERE probes with PE-treated cardiac extracts. A 50-fold molar excess of competitor oligonucleotides was used. Note that M1 and M2 are no longer able to form C1 and C2 complexes. (**E**) Characterization of PERE binding using competition experiments. A 50-fold molar excess of each competitor oligonucleotide was added to the binding reaction. Note that M1 and M2 oligonucleotides are unable to compete the PE-induced C1 and C2 complexes (PEX), although they are effective competitors of the lower complexes. HSE = heat shock element from the HSP70 promoter (Mosser *et al.*, 1990); Sp1 = site from TK promoter; SRE = c-*fos* serum response element; NF1 is from the adenovirus promoter; AP2 is from the hMTII promoter (Haslinger and Karin, 1985); Egr1 is the Egr1 binding site in the Egr1 promoter (Cao *et al.*, 1990); hANF corresponds to the putative PERE on the human promoter as determined by footprint experiments (not shown); CP1, CP2 and NF1 are described in Chodosh *et al.* (1988), DBP (site D on the albumin promoter) and C/EBP oligos are described in Howell *et al.* (1989) and the GRE oligonucleotide is the classic mouse mammary tumor virus (MTV) GRE.

to various CCAAT boxes, heat shock or serum response elements or to the binding sites of zinc-finger proteins like Egr1 and glucocorticoid receptor (Figure 4E). However, binding was effectively competed by the homologous site and the slowest migrating PE-inducible complex (C1) was competed by an oligonucleotide corresponding to the TK Sp1 site (Figure 4E).

These results prompted us to test whether the cardiac nuclear proteins that are induced by PE hypertrophy correspond to Sp1. Purified Sp1 protein and Sp1 antibodies were used to analyze further the relationship of PEX to Sp1. Purified Sp1 was able to bind both Sp1 and ANF−PERE probes and this binding was completely supershifted by the addition of the Sp1 antibody (Figure 5A). Similarly, when the Sp1 antibody was added to HeLa nuclear extracts, the majority of the binding over both Sp1 and ANF probes was supershifted, although some binding over the ANF probe could not be supershifted even with higher amounts of Sp1 antibody. In contrast, at least 50% of the binding to the Sp1 probe in cardiac extracts and >90% of the binding to the PERE was not affected by addition of the Sp1 antibody (Figure 5B). These results suggest that the PEX found in cardiac extracts are distinct from bona fide Sp1 and are consistent with the observation that the TK promoter which contains a high affinity Sp1 binding site is unresponsive to PE. Finally, because Sp1 is a zinc finger protein (Kadonaga *et al.*, 1987), the zinc dependence of PEX binding was tested. Addition of either 0.25−2.00 mM orthophenanthroline or 5 mM EDTA to the binding reaction inhibited PEX formation (Figure 6A); binding was restored by the addition of increasing amounts of $ZnCl_2$ but not by the addition of $MgCl_2$ or $CaCl_2$ (Figure 6B) A search of known DNA-binding proteins revealed a recently cloned zinc finger protein, designated MAZ, which binds an element on the c-*myc* promoter that contains a GAG motif (Bossone *et al.*, 1992). The MAZ cDNA clone was isolated from HeLa cells and it is ubiquitously expressed, including in the heart. While MAZ antibodies are not yet available to test the possibility that MAZ or a MAZ-related protein may be a PEX component, it is noteworthy that the ANF−PERE is completely inactive in HeLa cells in the presence or absence of PE (Figure 4A) and is not affected by cotransfection with a MAZ expression vector (data not shown). Together, these experiments suggest that the PERE interacts with novel zinc-dependent Sp1-related proteins whose activities are modulated by PE-induced hypertrophy.

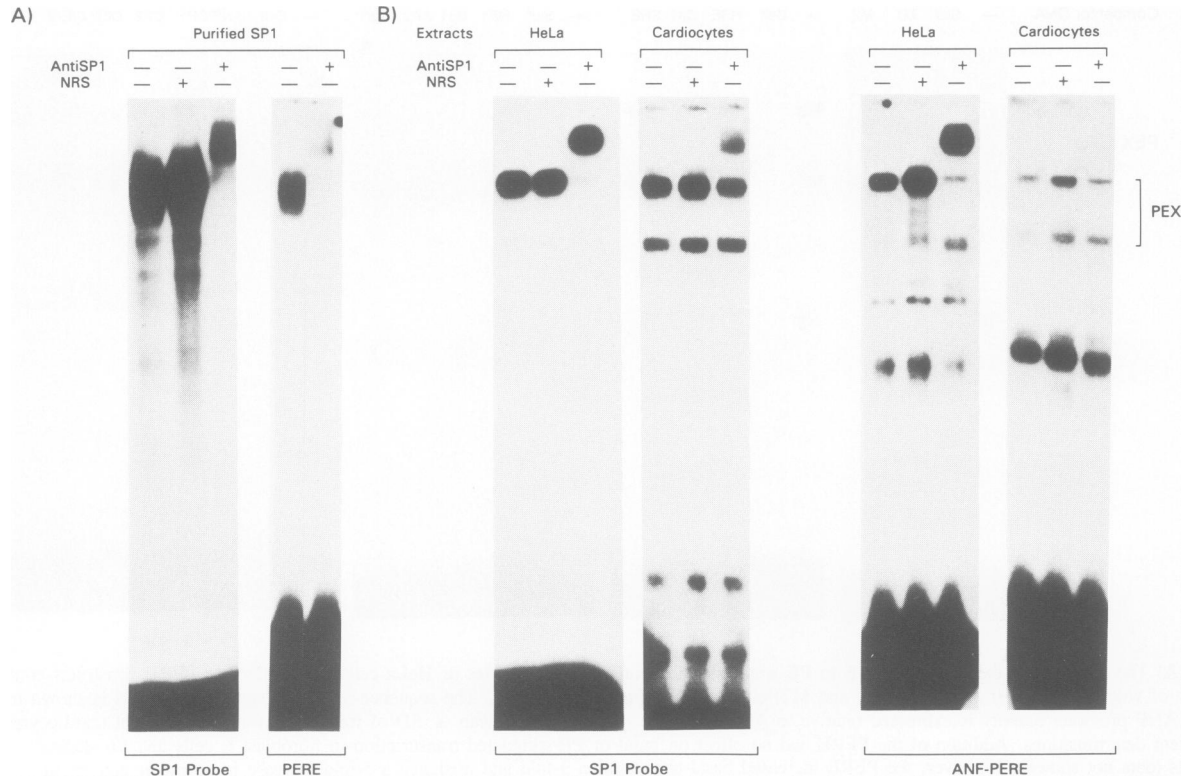

Fig. 5. Relationship of the PERE complexes to the Sp1 transcription factor. (**A**) Purified Sp1 protein was purchased from Promega and 1 FPU (footprinting unit) was used in binding reactions according to the manufacturer's specification. AntiSp1 was obtained from Santa Cruz Laboratories and is a rabbit affinity-purified polyclonal antibody corresponding to residues 520–538 of the Sp1 protein. NRS = non-immune rabbit serum. (**B**) AntiSp1 or an equivalent amount of NRS was added to 2 mg of HeLa or cardiocyte nuclear extracts. Binding reactions and gel conditions were as described in the legend to Figure 3. The addition of two times more Sp1 antibody to cardiocyte extracts did not result in further supershift of C1 or C2 complexes.

Discussion

Although α_1-adrenergic agonists have potent biological effects, ranging from growth stimulation of myocytes (Majesky *et al.*, 1985; McBride *et al.*, 1993), hepatocytes (Cruise *et al.*, 1985) and smooth muscles (Majesky *et al.*, 1985) to simulation of gluconeogenesis (Garcia-Sainz and Hernandez-Sotomayor, 1985), the mechanisms of signal transduction for α_1 adrenoreceptors remain uncertain. In cardiac myocytes, α_1-adrenergic receptors are coupled to the transient stimulation of several immediate early growth response genes including nuclear proto-oncogenes c-*myc* (Starksen *et al.*, 1986), c-*fos* and c-*jun* (Iwaki *et al.*, 1990; McBride *et al.*, 1993). However, in recent studies (McBride *et al.*, 1993) we have found dissociation between *fos/jun* and PE effects at the level of ANF transcription because *fos/jun* repressed the ANF promoter. The finding that ANF promoter fragments containing −135 bp are still fully responsive to PE but are not affected by *fos/jun* expression (McBride *et al.*, 1993) is consistent with the presence of a *fos/jun*-independent α_1 signaling pathway. In addition to nuclear proto-oncogenes, the zinc finger protein Egr1 is also increased following α_1-adrenergic stimulation of cardiomyocytes (Iwaki *et al.*, 1990). However, the ANF–PERE is neither homologous nor competed by Egr1 binding sites (Figure 4), in agreement with transfection studies which show little effect of Egr1 on ANF promoter activity (McBride *et al.*, 1993).

DNA regulatory elements which mediate transcriptional response to activation of common intracellular mechanisms are usually highly conserved in target genes. Several DNA elements which mediate regulation of gene expression in response to the activation of kinase A and C signaling pathways have been identified on target genes. This has led to the isolation of transcription factors which translate extracellular stimulation into changes in gene expression (Angel and Karin, 1991; Brindle and Montminy, 1992). The PERE is not related to previously identified DNA elements that are targets for protein kinase A or C, including CREB, AP1 or AP2 sites or serum response elements, which suggests the involvement of novel pathways and transcription factors in mediating the α_1-adrenergic response. This is consistent with the work of Cotecchia *et al.* (1990, 1992) which showed that, unlike α_2- and β-adrenergic receptors, the α_1 adrenoreceptors are not coupled to adenylyl cyclase. In fact, the major coupling system of α_1 receptors appears to be the activation of phospholipase C via a pertussis toxin-insensitive G protein (Cotecchia *et al.*, 1990) whose precise identity has not yet been determined. However, the signaling pathway which is specifically activated by this G protein is not established; in the heart, and in other tissues, activation of the α_1-adrenergic receptors stimulates phosphoinositide hydrolysis which generates inositol trisphosphate (IP_3) and diacylglycerol, both of which can act as second messengers by activating intracellular calcium release or protein kinase C, respectively. At present the role of protein kinase C, calcium and calmodulin pathways in mediating α_1-adrenergic receptor activation is controversial (Brown *et al.*, 1985; Endou *et al.*, 1991; Fedida and Bouchard, 1992; Pucéat

Table I. Nucleotide sequence of the rat ANF PERE (rANF)

-85	A A G T G A C A G A A T G G G G A G G G T T C C A G C T C T	rANF
	A A G T G A C A G A A T G G G G A C C C T T C C A G C T C T	M1
	A A G T G A C A G A A T G C C C A G G G T T C C A G C T C T	M2
-85	A A G T G A C A G A A T G G G G A G G G T T C T G T C T C T	hANF
-85	A A G T G A C A G A A T G G G G A G G G T T C T A G C C C C	mANF
-85	A A G T G A C A G A A T G G G G A G G G T T C C G T C C C T	bANF
-130	C G G C C G A G G G A G G G G C T C T A	SKA
-125	A T A C G T G T G G A G G G G C C A G T T	βMHC
-316	A C C A G A A A G G G G G A G G G G T G G G C T G G C	G3 CarA

Open triangles indicate the pattern of methylation interference on the C1 and C2 complexes as determined in EMSA using cardiac nuclear extracts. The sequence of the two mutant PEREs (M1 and M2) is also shown. hANF, mANF and bANF correspond to the homologous region on the human, mouse and bovine ANF genes; G3 is a promoter element from the human cardiac actin gene (CarA) (Gustafson and Kedes, 1989); SKA is for human skeletal actin (Bishopric *et al.*, 1992) and the βMHC sequence is from the rat β-myosin heavy chain gene (Bouvagnet *et al.*, 1987). The conserved GAG motif is boxed.

et al., 1993). It is interesting that the ANF–PERE is not responsive to activators of protein kinase C, like phorbol esters (TPA) and serum (Ardati and Nemer, unpublished observations), suggesting that IP_3 rather than diacylglycerol might be the main effector mechanism for α_1-adrenergic receptors in the heart. Thus, the identification of a nuclear target sequence for α_1 stimulation may help elucidate the precise intracellular pathway of α_1-adrenergic receptors.

Finally, transcription of other cardiac genes, including skeletal and cardiac actin and β-myosin heavy chain (Long *et al.*, 1989; Waspe *et al.*, 1990), is also induced by α_1-adrenergic stimulation. A search for consensus DNA sequences on the promoters of these target genes revealed the presence, within the first 300 bp of upstream sequences, of several putative PEREs containing perfectly conserved GAG motifs (Table I). Furthermore, consistent with a conserved α_1-adrenergic regulatory mechanism at the level of the ANF gene (Manning *et al.*, 1985; Uehlinger *et al.*, 1986), the sequence and position of the PERE are perfectly conserved in bovine (Vlasuk *et al.*, 1986), rat (Argentin *et al.*, 1985), mouse (Seidman *et al.*, 1984) and human (Nemer *et al.*, 1984) ANF genes (Table I).

In conclusion, this study identified for the first time an α_1-adrenergic response element which is present within the regulatory region of several α_1 responsive cardiac genes. The identification of the α_1 response element represents an important step towards elucidating the intracellular effectors of α_1-adrenergic receptors.

Materials and methods

Cell cultures and transfections

Primary cardiocyte cultures were prepared from 1 day-old Sprague–Dawley rats and kept in serum-free medium as previously described (Argentin *et al.*, 1991). HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Transfections were carried out using the calcium phosphate precipitation technique 24 h after plating. RSV-luciferase (2 μg per dish) was included as an internal control to normalize for transfection efficiencies. Cells were harvested and the cell media were collected 48 h after transfection. Luciferase activity was assayed using an LKB luminometer and human growth hormone (hGH) was measured in cell media using radioimmunoassay as previously described (Argentin *et al.*, 1991). The results reported were obtained from at least four independent experiments each performed in duplicate using at least two different DNA preparations for each plasmid. For α_1-adrenergic stimulation, 10^{-4} M PE were added to the cells for 48 h. As previously described (McBride *et al.*, 1993), PE treatment resulted in changes in the spontaneous beating of the cardiocytes and produced a significant enlargement of myocytes. RNA extractions and Northern blot hybridization were as described by Argentin *et al.* (1991).

Plasmids

The hGH plasmids containing various rat ANF promoter fragments were previously described in (Argentin *et al.*, 1991) and (McBride *et al.*, 1993). 5′ deletions were generated using appropriate restriction enzyme sites or by PCR amplification. All PCR-generated mutants were confirmed by sequencing. The heterologous ANF promoter constructs were obtained by inserting synthetic oligonucleotides upstream of the −50 bp minimal ANF promoter.

DNA binding assays

Extracts were prepared from cardiac myocyte cultures according to the procedure of Schöler *et al.* (1989). The sequence of oligonucleotides used

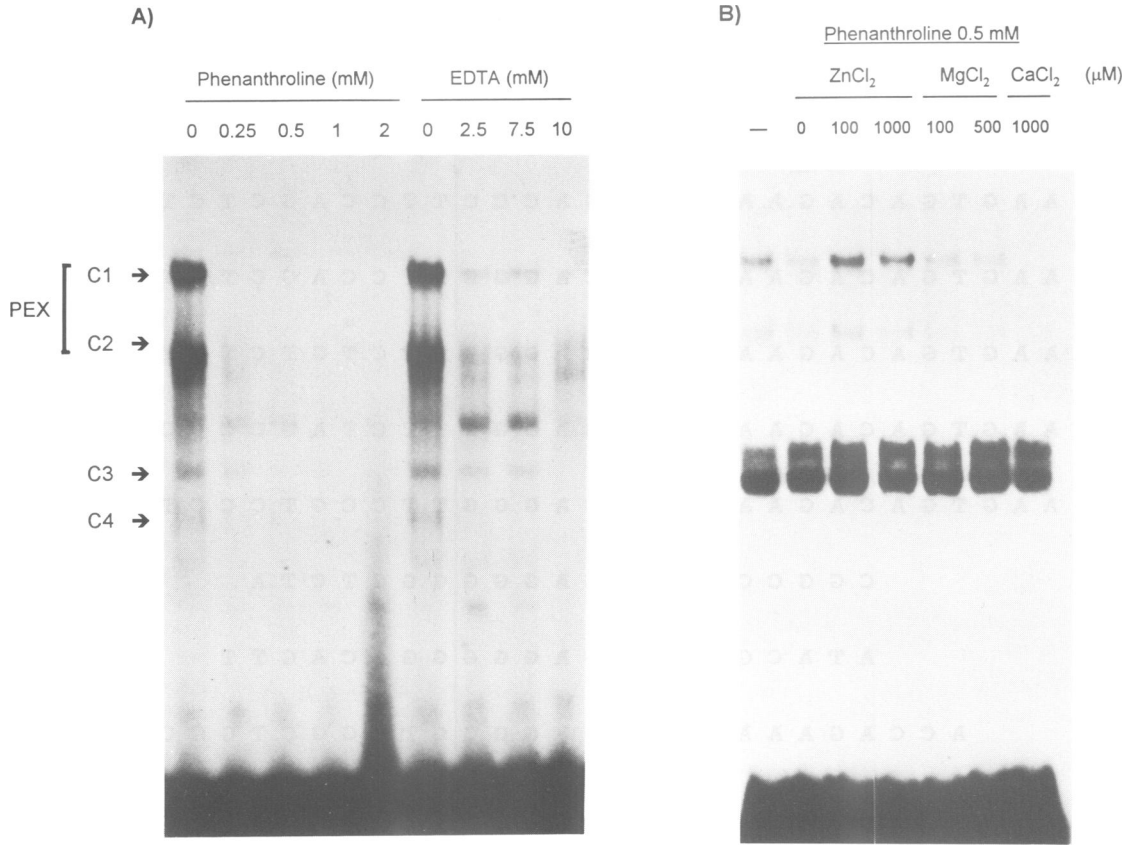

Fig. 6. Formation of PEX requires divalent zinc ions. **(A)** Effect of phenanthroline and EDTA on PERE–protein binding. Increasing amounts of 1,10-phenanthroline (Aldrich Chemical Company) or EDTA was added to 2 μg of PE-treated cardiocyte extracts and incubated with ANF–PERE probe using the conditions described in the legend to Figure 3. Note that C1 and C2 exhibited a greater sensitivity to the chelating agents than C3 and C4. **(B)** Addition of $ZnCl_2$ at 100 or 1000 μM final concentration, but not $MgCl_2$ or $CaCl_2$, to extracts incubated with 0.5 mM phenanthroline restores C1 and C2 complexes. Extracts used in (A) and (B) were obtained from separate PE-treated cardiomyocyte cultures.

in gel retardation assays is shown in Table I. The conditions at which binding reactions were carried out are detailed in the legend to Figure 3.

Nuclear extracts were also prepared from cardiac tissues; cardiac organs were dissected from Sprague–Dawley rats and finely minced, then homogenized at 4°C in buffer A (50 mM NaCl, 0.5 M sucrose, 10 mM Tris–HCl pH 7.5, 0.5 mM spermidine, 0.15 mM spermine, 1 mM EDTA pH 8.0, 0.25 mM EGTA pH 8, 10 mM 2-mercaptoethanol). The homogenate was centrifuged at 2500 r.p.m. for 10 min and the pellet was resuspended in buffer A with 0.5% Triton X-100 and centrifuged at 2500 r.p.m. for 10 min. Nuclear proteins were eluted using 0.3 M NaCl, precipitated with ammonium sulfate (45% saturation) and centrifuged at 10 000 r.p.m. for 20 min. The pellet consisting of nuclear proteins was resuspended and dialyzed twice against buffer B (10 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA pH 8, 1 mM DTT, 20% glycerol). Protein concentration was determined using a Bradford assay. For footprinting experiments, DNA fragments were 5′ end-labeled using T4 kinase (USB). Binding reactions were carried out at 20°C for 30 min in the presence of 1 μg poly(dI/dC) in 10 mM Tris–HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA pH 8 and 10% glycerol in a final volume of 20 μl. DNase I was added and the reaction was stopped after 30 s by the addition of 130 μl of 100 mM Tris–HCl pH 8, 20 mM EDTA pH 8, 0.5% SDS and 5 mg/ml of salmon sperm DNA at 100°C for 5 min. After phenol extraction, the DNA was loaded on 6% denaturing PAG. The position of the footprint was determined by simultaneously running a Maxam–Gilbert sequencing reaction on the same end-labeled fragments.

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