The α-Adrenergic Stimulation of Atrial Natriuretic Factor Expression in Cardiac Myocytes Requires Calcium Influx, Protein Kinase C, and Calmodulin-regulated Pathways*

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It has been shown recently that α-adrenergic agonists can stimulate atrial natriuretic factor (ANF) expression in ventricular cardiac myocytes; however, little is known about the intracellular signals mediating this activation. The present study focused on the potential roles of calcium-regulated kinases and calcium influx in the α-adrenergic stimulation of ANF gene expression in ventricular myocaridal cell cultures. Myocardial cells maintained for 48 h in serum-free medium supplemented with phenylephrine (PE) possessed up to 15-fold higher levels of ANF peptide and ANF mRNA than control cells. The removal of PE, or the addition of nifedipine, resulted in a rapid decline in ANF expression, suggesting that the sustained elevation of some intracellular messenger (e.g., calcium and/or phospholipid hydrolysis products) was required for the adrenergic response. The calcium channel agonist BAY K 8644 was capable of increasing ANF expression in a nifedipine-sensitive manner; however, unlike PE, it did not stimulate phosphoinositide hydrolysis. The protein kinase C inhibitor, H7, caused an approximate 75% reduction in PE-stimulated ANF expression, but had no effect on BAY K-stimulated expression. W7, a calcium/calmodulin inhibitor, completely blocked the effects of both PE and BAY K 8644. The addition of either H7 or W7 24 h after the PE addition resulted in a decline of ANF expression.

These results indicate that α-adrenergic agonists augment ANF gene expression through at least two pathways, one that is H7-sensitive, perhaps involving the sustained activation of protein kinase C, and the other that is W7-sensitive, perhaps involving the sustained activation of calmodulin-regulated kinases. Further, it appears that BAY K 8644-mediated increases in ANF expression are independent of protein kinase C activation and dependent on calmodulin-regulated events.

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Although several studies have shown that a variety of hormones can alter ANF gene expression (Gardner et al., 1987; Argentin et al., 1987; Matsubara et al., 1987a, 1987b; LaPointe et al., 1988; Seidman et al., 1988; Fukuda et al., 1989; Wu et al., 1989; Shubeta et al., 1990), very little is known about the intracellular signaling mechanisms through which such hormonal regulation occurs. α-Adrenergic agonists, which are among the most effective stimulators of ANF gene expression (Knowlton et al., 1991), have been shown to activate phosphoinositide (PI) hydrolysis leading to the IP3-mediated mobilization of intracellular calcium and DAG/Ca2+-mediated activation of protein kinase C (Nishizuka, 1986). Although α1-adrenergic agonists stimulate PI hydrolysis in cardiac myocytes (Brown et al., 1985; Steinberg et al., 1989), the functions of IP3 and DAG in regulating myocyte function have never been clarified. Indeed, the classic role of IP3 in mediating the mobilization of intracellular calcium over relatively long periods of time (e.g. seconds to minutes) is not compatible with the rapid calcium transients (e.g. 0.2 s) that normally drive contractions in cardiac myocytes (Bals et al., 1990). Moreover, calcium transients in cardiac myocytes are not known to be dependent on the formation of inositol phosphates.

Only two published studies, both from Paul Simpson's laboratory (University of California, San Francisco), have addressed directly the activation of PKC cultured myocardial cells in response to α-agonists. In one study, it was shown that norepinephrine-mediated stimulation of α1-adrenergic receptors caused a transient translocation of PKC to the cytosol to the plasma membrane, with maximal translocation occurring after 1–2 min of agonist exposure and subsequent redistribution of the enzyme back to the cytosol within 10–15 min, even in the continued presence of agonist (Henrich and Simpson, 1988). Further, immunocytofluorescence analyses showed that α-agonists caused the transient translocation, and presumed activation, of PKC to not only plasma membrane, but also myofilament regions of the cell, where PKC...
could contribute to regulating contractility (Mochly-Rosen et al., 1990).

If PKC translocation, and presumed activation, is all that is needed for the α₁-adrenergic induction of the ANF gene, then a short term treatment with the agonist phenylephrine (10 to 15 min) should be sufficient to lead to the maximum level of ANF expression. However, we found that the α₁-adrenergic agonist must be continually present in order for continued induction of the gene, indicating that the sustained elevation of intracellular messengers, along with the continual activation of effector enzymes, such as PKC, must be required. In pursuing this hypothesis we have shown in the present study that α₁-adrenergic agonists influence ANF expression through at least two pathways; one requires PI hydrolysis with the apparent sustained activation of PKC, and the other involves a calmodulin-regulated step, such as CaM kinase II activation.

RESULTS

Medium ANF as a Measure of ANF Gene Expression—Primary neonatal ventricular myocytes release ANF constitutively such that little of the peptide is stored within the cells (Bloch et al., 1986). Thus, changes in the level of ANF in the medium of ventricular myocyte cultures provide a reasonable estimate of changes in ANF mRNA levels. In many experiments discussed in this paper, the effects of a treatment on ANF expression were assessed initially by measuring changes in the levels of medium ANF. This was then followed with studies evaluating the effects of specific treatments on ANF mRNA and, in some cases, cellular levels of ANF. It was consistently found that a treatment that caused a change in medium ANF levels coordinately changed cellular ANF as well as ANF mRNA.

Time Course of PE Effects of ANF Production—Maintaining ventricular myocyte cultures in serum-free medium containing phenylephrine (PE) resulted in increased rates of ANF accumulation in the medium (Fig. 1A). Within 6 h of exposure to PE, the appearance of ANF in the medium increased by about 2- to 3-fold over control cultures. This differential increased steadily to a final value of about 15-fold over control cultures after 48 h of PE exposure. Northern analyses showed that ANF message levels had increased maximally (3- to 4-fold over control) after about 12 h and remained at these levels throughout 48 h of PE exposure (Fig. 1B). The α₁-agonist-mediated changes in message levels were shown to have a specific effect on ANF expression, since all ANF message values were normalized to levels of 28 S ribosomal RNA levels, as previously described (Shubeita et al., 1990).

When cultures maintained in PE for 24 h were washed free of the agonist medium, ANF began to drop so that within 6 h it was only about half that observed in cultures remaining in PE (Fig. 1A). This trend continued so that 12 h after PE removal, medium ANF levels had declined to nearly control values. Analyses of culture extracts indicated that cellular ANF levels had decreased by 66 and 80% within 6 h and 12 h of PE exposure, respectively, a time course similar to the decline in ANF mRNA and medium ANF. The finding that medium ANF levels reflected cellular levels indicated that PE removal affected total ANF production, and not simply secretion. This is consistent with previous findings that ventricular myocytes release ANF constitutively through a nonregulated pathway (Bloch et al., 1986). In accord with the decline in both cellular and medium ANF levels, the removal of PE also resulted in a decline in ANF mRNA (Fig. 1B). Interestingly, the kinetics of this decrease appeared to be slower than that for the peptide, which is perhaps consistent with a transcript
FIG. 1. Time course of the effects of phenylephrine on ANF message and peptide levels in ventricular myocardial cell cultures. Ventricular myocardial cells were dissociated, as described under "Materials and Methods," maintained on fibronectin-coated plastic wells for 4 h in 10% fetal bovine serum-containing medium, and then switched to serum-free medium with no additions. After 14 h, the medium on all cultures was replaced with fresh serum-free medium with no further additions (None) or with 50 μM phenylephrine (PE). At various times after this medium replacement, secretion experiments were carried out to determine the rate of ANF release into the medium; the secretion medium was identical with the medium with which the cultures had been incubated. The 2-h secretion period was followed by extraction of the cells for Northern analysis of RNA (panel A). Twenty-four h after the addition of PE, some of the cultures were washed free of the drug and incubated further with minimal serum-free medium. Each time point for each treatment was analyzed using three identical cultures; error bars represent the S.E. Statistical analyses for the data obtained at each time point were performed by one-way analysis of variance with Newman-Keuls post hoc analysis of variance (*, p < 0.05; **, p < 0.01 different from None).

Half-life of about 17–24 h, as previously determined (Gardner et al., 1987). Although it is clear that both peptide and mRNA levels decrease upon agonist removal, the more rapid decline in peptide levels suggests that ANF production may be regulated at both the translational and transcriptional levels.

These results indicated that PE-mediated elevation of an intracellular messenger, such as IP₃ and/or calcium, was important for maintaining ANF expression. The effects of hormones on intracellular calcium in cardiac myocytes have been studied very little, perhaps because of the rapid calcium transients that continually take place in these cells during the contractile cycle. Since it has been inferred from two previous studies that α-adrenergic agonists can affect slight increases in intracellular calcium in cardiac myocytes (Endoh and Blinks, 1988; Fedida et al., 1989), and since it has recently been shown that ANF mRNA and peptide levels in primary myocardial cultures decrease when extracellular calcium is lowered (LaPointe et al., 1990), it was of interest to evaluate whether the mechanism of PE-stimulated ANF expression, at least in part, was dependent on calcium influx.

Effects of BAY K 8644—Treatment of cultures with the calcium channel agonist BAY K 8644 alone resulted in an increase in ANF production of approximately 10-fold over control cultures (Fig. 2A, see 0 μM PE). A BAY K 8644 dose-response experiment demonstrated a half-maximal dose of about 0.1 μM with the maximum achieved at 1 μM and maintaining at that level to 10 μM (not shown). This is in accord with the effects of BAY K 8644 on other peptide systems and its affinity for L-type calcium channels (Enyeart and Hinkle, 1984; Heisler, 1985; Stojilkovic et al., 1988). The maximum response produced by BAY K 8644 amounted to about 20–25% of the response obtained with maximal levels of PE. Interestingly, BAY K 8644 augmented PE-stimulated ANF production at all levels of the α-adrenergic agonist (Fig. 2A).

Since either PE or BAY K 8644 caused increases in ANF production, and PE has been shown to increase IP hydrolysis in myocardial cultures (Brown et al., 1985; Steinberg et al., 1989), it was of interest to determine whether BAY K 8644 could stimulate IP hydrolysis. As expected, PE increased PI hydrolysis by 5–10-fold, with a maximal effect occurring at 10 μM PE (Fig. 2B). In contrast, BAY K 8644 had no effect on PI hydrolysis, nor did it augment the PI hydrolysis observed in response to PE. In fact, a slight, although statistically insignificant, decrease in PI hydrolysis was observed when BAY K 8644 was added to PE. Thus, the increases in ANF levels in response to BAY K 8644 occurred in the absence of any detectable changes in the rate of PI hydrolysis or DAG formation (not shown) over control. This suggested that BAY K 8644 could augment ANF expression through increases in calcium influx. However, the lower efficacy of BAY K 8644 compared to PE indicated that an α-agonist-
mediated event, perhaps related to increased PI hydrolysis, was required for maximal augmentation of ANF expression.

**Effects of Nifedipine**—Due to the known effects of BAY K 8644 on calcium channel activity, it was of interest to evaluate the effects of nifedipine, a calcium channel antagonist, on ANF expression. As expected, nifedipine inhibited BAY K 8644-dependent increases in ANF peptide and mRNA levels in the cultures (Fig. 3, A and C). Nifedipine also inhibited PE-induced increases in ANF peptide (Fig. 3A) and mRNA (Fig. 3C); however, it did not affect α-adrenergic agonist-stimulated PI hydrolysis (Fig. 3B). Dose-response experiments showed that the effects of nifedipine on each of these functions was half-maximal at about 0.05 μM (not shown), in accord with its effects on other functions regulated by the activity of L-type calcium channels (Wolfe and Brostrom, 1986; Stojilkovic et al., 1988). Thus, it appeared as though the ability of PE to influence ANF expression was primarily dependent on calcium influx.

**Effects of H7, W7, and HA-1004**—In order to begin to address the potential functions of protein kinases, studies utilizing membrane-permeable enzyme inhibitors were performed. Three inhibitors were chosen based on their enzyme selectivities. The membrane-permeable isoquinoline derivative, H7, which inhibits PKC and protein kinase A with similar potencies, and HA-1004, which is about 20-fold more selective for protein kinase A than for PKC inhibition, were used (Hidaka et al., 1984). In addition to inhibitors of PKC and protein kinase A, a compound that would inhibit calmodulin-regulated kinases was also employed. W7, a relatively selective inhibitor of calmodulin-regulated reactions such as myosin light chain kinase and CaM/kinase II (K_i = 30–60 μM) is ineffective as an inhibitor of PKC or protein kinase A (e.g., K_iPKC > 100 μM; Tanaka and Hidaka, 1980; Ito et al., 1986). Each of these compounds was tested for the ability to inhibit rat ventricular tissue PKC enzyme activity in vitro. In these analyses, H7 produced half-maximal inhibition at 25 μM and up to 80% inhibition at 100 μM, while HA-1004 at up to 100 μM caused only a 29% decrease in PKC enzyme activity (Table I). Like HA-1004, W7 was a poor PKC inhibitor, resulting in a loss of only 3% of enzyme activity at 100 μM. However, based on previous studies (Ito et al., 1986; Davis and Wilson, 1989; Nguyen et al., 1990), it was expected that W7 could completely inhibit calmodulin-dependent reactions when used at approximately 10 to 30 μM.

In dose-response studies, it was shown that HA-1004 inhibited PE-mediated ANF expression by only 38% at levels of 50 μM or more (Fig. 4A), as expected if protein kinase A activation has little involvement in α-adrenergic-stimulated ANF expression. However, H7 inhibited PE-stimulated ANF expression at a half-maximal concentration of between 10 and 20 μM, with a maximal effect of about 80% inhibition at 50 μM (Fig. 4B). The dose-response range for H7 inhibition of PE-stimulated ANF expression correlated well with that observed for inhibition of PKC (see Table I). Although H7 inhibited PE-stimulated ANF expression, it had no inhibitory effect on PE-stimulated PI hydrolysis or DAG formation (Table II). Thus, H7-mediated inhibition of the PE effect appeared to be distal to α-adrenergic receptor activation of phospholipase C, most likely at the level of a protein kinase such as PKC. In contrast to H7, W7 was more potent at inhibiting PE-stimulated ANF expression, displaying a half-maximal concentration of about 2 μM and a maximal effect of greater than 95% inhibition at 10 μM (Fig. 4C). H7 (100 μM) and W7 (10 μM) also diminished ANF message levels (ANF mRNA/28 S ratios for PE = 4.75 ± 0.35; PE + H7 =

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**Table I**

**Effects of H7, HA-1004, and W7 on cardiac PKC activity**

| [Inhibitor] | H7 | HA | W7 |
|------------|----|----|----|
| μM         | %  |    |    |
| 0          | 100| 100| 100|
| 1          | 98 | 100| 67 |
| 5          | 90 | 96 | 77 |
| 10         | 80 | 85 | 92 |
| 25         | 54 | 79 | 79 |
| 100        | 17 | 71 | 97 |

Maximal enzyme activity

*Fig. 3. Effects of nifedipine on ANF peptide and message levels and the rate of phosphoinositide hydrolysis. Ventricular myocardial cells were treated as described in Fig. 2, except they were incubated in either minimal medium (Min), 10 μM BAY K 8644 (BAY K), or 50 μM PE (PE) with or without 1 μM nifedipine. After 24 h of incubation with these treatments, the ANF secretion rate was determined and then the culture extracts were processed for ANF message analysis. Each treatment was analyzed using three identical cultures; error bars represent the S.E. Statistical analyses were performed by one-way analysis of variance with Newman-Keuls post hoc analysis of variance (*, p < 0.05; **, and ††, p < 0.01 different from all other values; and ‡, p < 0.01 different from Min).*
with PE-containing serum-free medium with either no additions (0) or various concentrations of HA-1004, H7, or W7 (100 μM), 100 μM PE and 100 μM H7 (PE/H7). Following a 60-min incubation, the cells were harvested, and the extracts were analyzed for DAG and labeled inositol phosphates, as described under “Materials and Methods.” Each point for each treatment was analyzed using four identical cultures; errors represent the S.E. Statistical analyses were performed by one-way analysis of variance with Newman-Keuls post hoc analysis of variance (n = 4 cultures for each treatment; **, p < 0.01 different from 0).

**TABLE II**

Effects of H7 on PE-stimulated PI hydrolysis and DAG formation

Cultures were labeled with [3H]inositol, as described in the legend to Fig. 2B. At this time, the cultures were washed and the medium was replaced with serum-free medium containing either no further additions (Min), 100 μM phenylephrine (PE), or 100 μM PE and 100 μM H7 (PE/H7). Following a 60-min incubation, the cells were harvested, and the extracts were analyzed for DAG and labeled inositol phosphates, as described under “Materials and Methods.” Each point for each treatment was analyzed using four identical cultures; errors represent the S.E. Statistical analyses were performed by one-way analysis of variance with Newman-Keuls post hoc analysis of variance (n = 4 cultures for each treatment; **, p < 0.01 different from 0).

| Treatment | DAG pmol/10⁶ cells plated | [3H]inositol phosphate cpm |
|-----------|---------------------------|---------------------------|
| Min       | 541 ± 42                  | 2,498 ± 180               |
| PE        | 889 ± 36*                 | 15,903 ± 756             |
| PE/H7     | 920 ± 67*                 | 18,316 ± 1320*           |

**DISCUSSION**

In the present study, we have investigated the potential roles of calcium influx, protein kinases, and calmodulin in α-adrenergic-mediated increases of ANF expression in primary neonatal rat ventricular myocardial cell cultures. We found that the continual occupation of α-adrenergic receptors with an agonist is required for maximal activation of ANF expression in ventricular myocardial cultures, indicating that the presence or activities of some intracellular signal(s) (either messengers and/or kinases) must be sustained in order to achieve the maximal response. It appears as though the stimulation of ANF expression requires calcium influx and a CaM-regulated step(s) (Fig. 6, Required Pathway), while PKC activation may play a secondary role by augmenting the calcium influx/CaM-dependent pathway (Fig. 6, Enhancement Pathway). In support of this model is the finding that PE produced an increase in the rate of PIP₂ hydrolysis and DAG formation in myocardial cells in the presence or absence of calcium influx; however, blocking calcium influx abolished any effects of α-adrenergic agonists on ANF production. Moreover, when calcium influx was stimulated with BAY K 8644, there was no increase in PIP₂ hydrolysis, but there was a significant increase in ANF expression. Interestingly, PE was consistently more efficacious than BAY K at stimulating ANF expression. Therefore, it seems probable that the enhanced

**FIG. 4. Effects of HA-1004, H7, or W7 on ANF production.** Ventricular myocardial cell cultures were prepared and treated for the first 18 h as described in Fig. 1. The medium was then replaced with serum-free medium containing either no further additions (0) or various concentrations of HA-1004, H7, or W7, as indicated. After 24 h, the medium was replaced with identical medium and 2 h later it was sampled for ANF RIA. Statistical analyses were performed by one-way analysis of variance with Newman-Keuls post hoc analysis of variance (n = 4 cultures for each treatment; **, p < 0.01 different from 0).

**FIG. 5. Effects of H7 and W7 on BAY K 8644- and PE-augmented ANF peptide levels.** Ventricular myocardial cell cultures were prepared and treated for the first 18 h in culture as described in Fig. 1. At the appropriate time, the serum-free medium was supplemented with either no additions (Min), 100 μM PE, 100 μM H7, or W7 (10 μM). After 24 h, fresh medium containing these same additions was applied to the cultures and 2 h later sampled for ANF RIA. Statistical analyses were performed by one-way analysis of variance with Newman-Keuls post hoc analysis of variance (**, p < 0.01 different from all other treatments; †, p < 0.01 different from all other treatments).
the binding to and regulation of certain AP-1-containing transcriptional regulatory proteins. For example, the CRE binding protein, CREB, is phosphorylated in response to either increases in calcium or calcium influx through L-type calcium channels in response to either depolarization or BAY K 8644 (Morgan and Curran, 1986). As with prolactin and proenkephalin gene transcription, it has been proposed that since the calcium-dependent augmentation of c-fos expression occurs in a W7-sensitive manner, it is also likely to involve the calmodulin-mediated activation of a kinase which phosphorylates a transcription factor and then stimulates expression (Morgan and Curran, 1986).

The calcium response element in the c-fos promoter lies about 60 bases upstream of the start site and is distinct from the regulatory elements for polypeptide growth factors, serum, and phorbol esters (Sheng et al., 1988). Since only a few calcium-response elements have been characterized, a consensus sequence has not yet been established. However, in several cases, it has been shown that the calcium-response elements are indistinguishable from cyclic AMP response elements (CRE) (Sheng et al., 1988; Nguyen et al., 1990). Further, it has been shown that the CRE binding protein, CREB, is phosphorylated in response to either increases in calcium or cAMP in such a way that it activates transcription (Sheng et al., 1990, 1991). Interestingly, between -603 and -596 bp in the rat ANF promoter is a TGACTTCA sequence (Seidman et al., 1988) that is similar to the CRE consensus (TGACGTCGA; Montminy et al., 1986). Although extensive studies have not yet been carried out, preliminary experiments in our laboratory have indicated that in primary cardiac myocytes, cAMP does not stimulate ANF expression. Perhaps the CRE sequence in the ANF promoter serves as a calcium-response element.

In summary, calcium is an important determinant of ANF expression in ventricular myocytes. The nifedipine sensitivity to either depolarization or BAY K 8644 (Morgan and Curran, 1986). As with prolactin and proenkephalin gene transcription, it has been proposed that since the calcium-dependent augmentation of c-fos expression occurs in a W7-sensitive manner, it is also likely to involve the calmodulin-mediated activation of a kinase which phosphorylates a transcription factor and then stimulates expression (Morgan and Curran, 1986).

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In summary, calcium is an important determinant of ANF expression in ventricular myocytes. The nifedipine sensitivity

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of PE- and BAY K 8644-stimulated ANF expression implies that the optimal effects of these compounds depend on calcium influx through L-type calcium channels, indicating that calcium-sensitive kinases may ultimately regulate transcriptional activity. The partial H7 sensitivity of PE-stimulated ANF expression suggests that PKC activation plays an important signaling role. Additionally, since BAY K 8644-enhanced ANF expression is insensitive to H7, but sensitive to W7, it is apparent that calmodulin is also involved. Preliminary studies from our laboratory have shown that the CaM kinase II-specific inhibitor, KN-62 (a gift from H. Hidaka, Nagoya University School of Medicine, Showa-ku Nagoya, Japan), is a potent blocker of PE-stimulated ANF expression, thus describing at least one role for calmodulin in this process. Future studies evaluating further the potential roles of PKC and calmodulin-regulated kinases, as well as the regulation of nuclear transcription factor levels by calcium influx and the mapping of calcium-sensitive promoter regions, will be of interest in unraveling the mechanisms of hormone-regulated ANF production in cardiac myocytes.

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