Atrial Natriuretic Peptide Inhibits Calcium-Induced Steroidogenic Acute Regulatory Protein Gene Transcription in Adrenal Glomerulosa Cells

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Atrial natriuretic peptide (ANP) is a potent inhibitor of mineralocorticoid synthesis induced in adrenal glomerulosa cells by physiological agonists activating the calcium messenger system, such as angiotensin II (Ang II) and potassium ion (K^+). While the role of calcium in mediating Ang II- and K^+-induced aldosterone production is clearly established, the mechanisms leading to blockade of this steroidogenic response by ANP remain obscure. We have used bovine adrenal zona glomerulosa cells in primary culture, in which an activation of the calcium messenger system was mimicked by a 2-h exposure to an intracellular high-calcium clamp. The effect of ANP was studied on the following parameters of the steroidogenic pathway: 1) pregnenolone and aldosterone production; 2) changes in cytosolic ($[Ca^{2+}]_c$) and mitochondrial ($[Ca^{2+}]_m$) Ca^{2+} concentrations, as assessed with targeted recombinant aequorin; 3) cholesterol content in outer mitochondrial membranes (OM), contact sites (CS), and inner membranes (IM); 4) steroidogenic acute regulatory (StAR) protein import into mitochondria by Western blot analysis; 5) StAR protein synthesis, as determined by [^{35}S]methionine incorporation, immunoprecipitation, and SDS-PAGE; 6) StAR mRNA levels by Northern blot analysis with a StAR cDNA; 7) *StAR* gene transcription by nuclear run-on analysis.

While clamping Ca^{2+} at 950 nM raised pregnenolone output 3.5-fold and aldosterone output 3-fold, ANP prevented these responses with an IC_{50} of 1 nM and a maximal effect of 90% inhibition at 10 nM. In contrast, ANP did not affect the $[Ca^{2+}]_c$ or $[Ca^{2+}]_m$ changes occurring under Ca^{2+} clamp or Ang II stimulation in glomerulosa cells. The accumulation of cholesterol content in CS (139.7 ± 10.7% of control) observed under high-Ca^{2+} clamp was prevented by 10 nM ANP (92.4 ± 4% of control). Similarly, while Ca^{2+} induced a marked accumulation of StAR protein in mitochondria of glomerulosa cells to 218 ± 44% (n = 3) of controls, the presence of ANP led to a blockade of StAR protein mitochondrial import (113.3 ± 15.0%). This effect was due to a complete suppression of the increased [^{35}S]methionine incorporation into StAR protein that occurred under Ca^{2+} clamp (94.5 ± 12.8% *vs.* 167.5 ± 17.3%, n = 3). Furthermore, while the high-Ca^{2+} clamp significantly increased StAR mRNA levels to 188.5 ± 8.4 of controls (n = 4), ANP completely prevented this response. Nuclear run-on analysis showed that increases in intracellular Ca^{2+} resulted in transcriptional induction of the *StAR* gene and that ANP inhibited this process.

These results demonstrate that Ca^{2+} exerts a transcriptional control on StAR protein expression and that ANP appears to elicit its inhibitory effect on aldosterone biosynthesis by acting as a negative physiological regulator of *StAR* gene expression. (Molecular Endocrinology 12: 962–972, 1998)

INTRODUCTION

In the zona glomerulosa cells of the adrenal cortex, the octapeptide hormone angiotensin II (Ang II) and po-

0888-8809/98/$3.00/0
Molecular Endocrinology

tassium (K^+) are the most powerful stimuli of aldosterone production. Mineralocorticoid biosynthesis and secretion are highly dependent upon the increase in intracellular calcium triggered by these two agonists through distinct mechanisms (1–5).

The rate-limiting step in the activation of steroidogenesis is the delivery of cholesterol from the mitochondrial outer membrane to the inner membrane, where the cytochrome P450 side-chain cleavage ($P450_{scc}$) enzyme is located (6–10). Earlier studies have shown that cycloheximide, an inhibitor of protein translation, blocked pregnenolone production elicited by either cAMP- or Ca^{2+}-mobilizing hormones (8, 9, 11). Indeed, the appearance of newly synthesized mitochondrial phosphoproteins, referred to as the 30 kDa-proteins, has been shown to correlate directly with steroid production in adrenal cells and MA-10 mouse Leydig tumor cells (12–15). Currently, the 30-kDa steroidogenic acute regulatory (StAR) protein is considered to be a key regulator of cholesterol delivery to the $P450_{scc}$ enzyme (16–19). The decisive demonstration came from an inherited disease that leads to a dramatic deficiency in all steroid hormones, congenital lipoid adrenal hyperplasia: mutations in the *StAR* gene have been shown to underlie this disorder (20, 21).

Atrial natriuretic peptide (ANP), a hormone originally identified in atrial cardiomyocytes, affects blood pressure through its concerted actions on various target organs, including vascular smooth muscle, kidney, and adrenal cortex. In adrenal glomerulosa cells, ANP strongly impairs aldosterone secretion stimulated by Ang II (4, 22–25), K^+ (26), or ACTH (27, 28). *In vitro* and *in vivo* studies indicate that ANP-induced inhibition of aldosterone synthesis is mediated by type A natriuretic peptide receptors endowed with intrinsic guanylyl cyclase activity (29, 30), and cGMP was initially thought to be the intracellular messenger mediating all effects of ANP (29, 31). However, a number of studies reported that the inhibition of aldosterone synthesis by ANP could not be mimicked with membrane-permeant, nonhydrolysable analogs of cGMP, such as 8-bromo-cGMP or dibutyryl-cGMP (4, 32, 33). The intracellular sites of the antisteroidogenic action of ANP remain, therefore, to be elucidated.

In theory, ANP could impede the generation of the cytosolic Ca^{2+} ($[Ca^{2+}]_c$) signal and the resulting rise in mitochondrial free Ca^{2+} concentration ($[Ca^{2+}]_m$) that are observed upon challenge with Ang II and K^+ (34). Alternatively, ANP could affect cholesterol supply to the mitochondria or intramitochondrial cholesterol transfer or interfere with either the expression or the action of the StAR protein (35, 36).

In the present study, we have examined whether ANP interferes with any of the above processes, using the Ca^{2+}-clamp technique to mimic Ang II or K^+ activation. We report that ANP inhibition is exerted on intramitochondrial cholesterol transfer and results from an inhibition of the Ca^{2+}-induced synthesis of StAR protein and therefore of StAR protein accumulation within mitochondria. We provide evidence that the increase in StAR protein synthesis elicited by Ca^{2+} is a consequence of an increase in the steady state level of StAR mRNA, suggesting that Ca^{2+} activates StAR protein gene transcription and that ANP modulates this activation.

RESULTS

ANP Inhibits Ca^{2+}-Induced Pregnenolone and Aldosterone Biosynthesis

In Ca^{2+}-clamped bovine glomerulosa cells, pregnenolone production was stimulated in a concentration-dependent manner by $[Ca^{2+}]_c$ as previously reported (37). This activation was inhibited in a concentration-dependent manner by both Thr-Ala-Pro-Arg-human (h)ANP(1–28) (urodilatin, an analog of ANP originally isolated from human urine) and hANP(4–28) (Fig. 1). Similar results were obtained for aldosterone production (data not shown). Both peptides were equipotent in preventing pregnenolone production in cells submitted to an intracellular calcium clamp of 600 nM, with an IC_{50} of approximately 1 nM and a maximal inhibition of 90% (Fig. 1B). They were therefore used indifferently in subsequent experiments. Since the Ca^{2+} signal generated by the high-Ca^{2+} clamp is, by definition, constant, these results globally suggested to us that the target for the inhibitory mechanism of ANP resides downstream of the production of the calcium signal.

The membrane-permeant analogs of cGMP, 8-Br-cGMP and dibutyryl-cGMP (10 μM), failed to mimick the antisteroidogenic action of ANP, as shown in Fig. 2. Ten-fold higher concentrations (100 μM) of the analogs were equally uneffective (data not shown).

ANP Does Not Affect Ang II-Induced $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ Responses

The lack of an effect of ANP on Ca^{2+} responses was confirmed in bovine glomerulosa cells transfected with targeted aequorin. In cells that had been pretreated with 100 nM hANP for 3–30 min, the $[Ca^{2+}]_c$ response to Ang II in glomerulosa cells transfected with nontargeted-aequorin was not affected by the presence of hANP, and the $[Ca^{2+}]_c$ values established under Ca^{2+} clamp were not altered (not shown). Since the mitochondrion is a known target for the Ca^{2+} signal, we also examined $[Ca^{2+}]_m$ changes. When adrenal glomerulosa cells were challenged with Ang II (10 nM), a biphasic $[Ca^{2+}]_m$ response was observed, as previously reported (34). In the presence of hANP, this response was superimposable to that recorded in the absence of hANP (Fig. 3). When added after Ang II challenge, hANP did not alter the plateau $[Ca^{2+}]_m$ phase. Clearly, therefore, the generation of the calcium signal was unaffected by hANP.

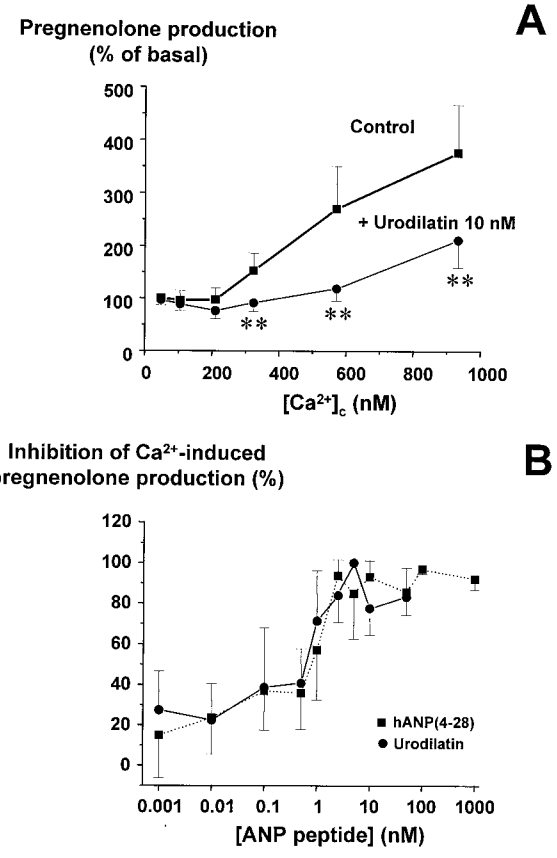

Fig. 1. Effect of ANP on Ca^{2+}-Induced Pregnenolone Formation in Bovine Adrenal Glomerulosa Cells

A, Glomerulosa cells were stimulated with a cytosolic Ca^{2+}-clamp for 2 h, in the absence or in the presence of 10 nM urodilatin, as described in *Materials and Methods*. Pregnenolone production is expressed as a percentage of the production determined in control cells incubated in nominally Ca^{2+}-free medium ($[Ca^{2+}]_c < 100$ nM). **, Significantly different ($P < 0.01$, n = 6) from the respective control without urodilatin. B, Concentration dependence of the inhibitory effect of hANP(4–28) (■) and urodilatin (●) on pregnenolone production in high-Ca^{2+} (600 nM) clamped cells. Percent inhibition was calculated by comparing pregnenolone production above zero-Ca^{2+} control in the presence of ANP peptides to that measured in the absence of peptide. Each point is the mean ± SEM of two to nine separate experiments. Pregnenolone production: Control, 0.102 ± 0.02 nmol/mg prot/2 h; high-Ca^{2+}, 0.319 ± 0.135; control + hANP (10 nM), 0.092 ± 0.037; high-Ca^{2+} + hANP, 0.120 ± 0.054.

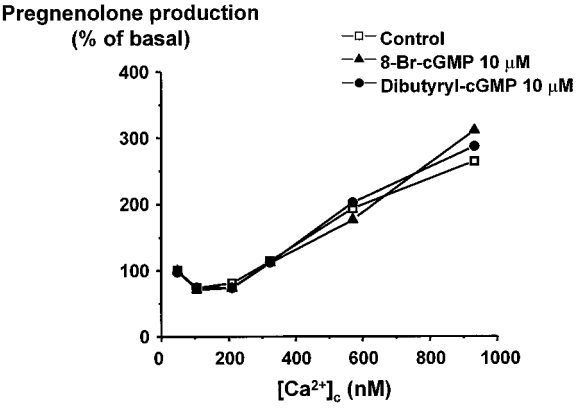

Fig. 2. Lack of Effect of cGMP Analogs on Ca^{2+}-Induced Pregnenolone Production

Glomerulosa cells were stimulated with a cytosolic Ca^{2+}-clamp for 2 h, in the absence or in the presence of 8-Br-cGMP (▲) or dibutyryl-cGMP (●) (10 μM), as described in *Materials and Methods*. Pregnenolone production is expressed as a percentage of the production determined in control cells incubated in nominally Ca^{2+}-free medium ($[Ca^{2+}]_c < 100$ nM). Each point is the mean value of triplicate samples from two separate experiments.

ANP Prevents Ca^{2+}-Induced Stimulation of Intramitochondrial Cholesterol Transfer

We have previously shown that the stimulation of ionomycin-treated bovine adrenal glomerulosa cells with Ca^{2+} markedly decreased cholesterol content in outer mitochondrial membranes (OM) and concomitantly increased cholesterol content in contact sites (CS) and inner membranes (IM), reflecting a stimulation of intramitochondrial cholesterol transfer (35, 36). Since ANP almost entirely prevented Ca^{2+}-supported pregnenolone formation, the first enzymatic step after cholesterol supply to $P450_{scc}$, we therefore examined the effect of ANP on Ca^{2+}-induced intramitochondrial cholesterol distribution. As we have previously shown, stimulation of glomerulosa cells with Ca^{2+} led to a significant increase of cholesterol content in CS and IM to 139.7 ± 10.7% and 131.5 ± 7% of the respective controls ($P < 0.05$, n = 3) and to a concomitant significant decrease of cholesterol content in OM, to 70.4% ± 2.1% of controls ($P < 0.001$, n = 3) (Fig. 4). hANP (10 nM) completely prevented this Ca^{2+}-induced transfer of cholesterol to CS and IM (92.4 ± 4% and 103.1 ± 16.1% of the respective controls, n = 3). Moreover, no significant change was observed in cholesterol content in OM when glomerulosa cells were simultaneously treated with Ca^{2+} and hANP (88.2 ± 6.5% of controls, n = 3). hANP alone had no effect on cholesterol content in OM, CS, and IM (105.6 ± 2.9%, 95.5 ± 9.8%, and 104.2 ± 1.4% of the respective controls, n = 3) (Fig. 4).

ANP Prevents Ca^{2+}-Induced Accumulation of StAR Protein within Mitochondria

We showed recently that Ca^{2+}-activated intramitochondrial cholesterol transfer is accompanied in bovine glomerulosa cells by an increase in StAR protein within mitochondria, a finding consistent with a role for StAR protein in cholesterol transport (36). To determine whether ANP affects the StAR protein accumulation induced by Ca^{2+}, we performed immunoblot analysis on mitochondrial proteins from glomerulosa cells that had been calcium-clamped in the presence or in the absence of hANP. As shown in Fig. 5, while Ca^{2+} induced the expected marked increase of StAR

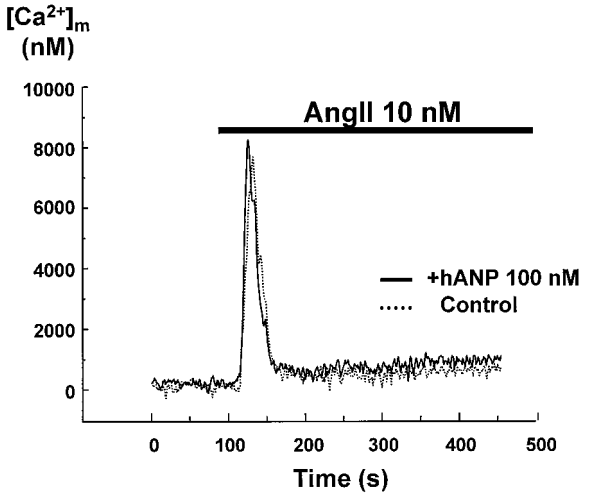

Fig. 3. Lack of Effect of ANP on the $[Ca^{2+}]_m$ Response to Ang II in Bovine Glomerulosa Cells

Cells were transfected with targeted aequorin as described in *Materials and Methods*. Aequorin luminescence was recorded under stimulation with Ang II (10 nM) in control cells (*dotted trace*) or in cells that had been pretreated with hANP (100 nM) (*solid trace*) for 30 min. This trace is representative of four similar experiments.

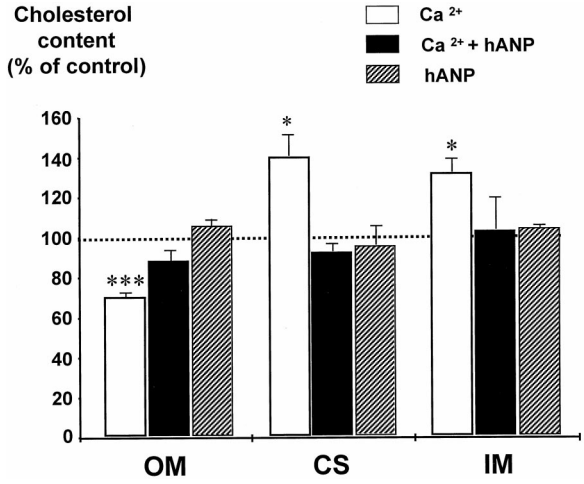

Fig. 4. Effect of ANP on Ca^{2+}-Induced Intramitochondrial Cholesterol Transfer in Bovine Glomerulosa Cells

Cells were stimulated with a cytosolic low- (<100 nM, control cells) or high- (600 nM) Ca^{2+} clamp for 2 h, in the absence or in the presence of 10 nM hANP(4–28) as described in *Materials and Methods*. After submitochondrial fractionation, the cholesterol content of OM, CS, and IM in Ca^{2+} clamped cells was determined and expressed as a percentage of that measured in the respective submitochondrial fractions of control low-Ca^{2+} clamped cells (mean ± SEM, n = 3). In a typical experiment, mass unit values for cholesterol in OM, CS, and IM were, respectively, 10.9, 3.4, and 1.6 μg/mg protein for controls. (* and ***, significantly different from the respective control with $P < 0.05$ and $P < 0.001$, respectively).

protein content in mitochondria (to 218.2 ± 44.4% of controls, n = 3, $P < 0.01$), hANP (10 nM) completely prevented this Ca^{2+}-induced increase in StAR (94.5 ± 12.8% of controls). hANP had no significant effect in itself.

ANP Prevents Ca^{2+}-Induced Stimulation of StAR Protein Synthesis

The Ca^{2+}-induced accumulation of StAR protein content in mitochondria could result from increased StAR protein synthesis. This hypothesis was tested in glomerulosa cells radiolabeled with [^{35}S]methionine/cysteine during Ca^{2+} stimulation in the presence or in the absence of hANP. StAR protein was then immunoprecipitated from total cellular extracts as described in *Materials and Methods*. Upon SDS-PAGE analysis and autoradiography of StAR immunoprecipitates, we observed that, in high Ca^{2+}-clamped cells, the labeling of StAR protein was significantly stimulated (167.5 ± 17.3% of controls, n = 3, $P < 0.01$) (Fig. 6), reflecting increased StAR protein synthesis. The addition of hANP (10 nM) simultaneously with Ca^{2+} completely prevented this increase in StAR protein labeling.

ANP Inhibits Ca^{2+}-Induced Increase in StAR mRNA Levels

The above results prompted us to examine whether ANP could exert its antisteroidogenic effect by acting directly on StAR mRNA production and/or stability. Upon Northern blot analysis of StAR mRNA, we observed consistently two, sometimes three, transcripts that hybridized with StAR cDNA. Two major bands migrated at approximately 2.6 and 1.6 kb (Fig. 7A), and a third one migrated at 0.9 kb. These transcripts showed coordinate induction. We therefore quantified only the most abundant 2.6-kb transcript. The quantification of StAR 2.6 kb mRNA from four independent experiments indicated that Ca^{2+} provoked a significant accumulation of this transcript (189 ± 14.5% of controls, $P < 0.001$) and that hANP (10 nM) prevented the Ca^{2+}-induced increase in StAR mRNA (116 ± 13% of controls) (Fig. 7B).

The Ca^{2+}-induced accumulation of StAR mRNA may result from changes in transcription rate and/or in mRNA turnover. To determine whether Ca^{2+} or hANP affected StAR mRNA stability, glomerulosa cells were submitted to a high-calcium clamp (600–700 nM) for 2 h. The incubation buffer was then removed and replaced with fresh buffer containing ionomycin and actinomycin D. Incubation was then continued for 6 h in the presence or in the absence of Ca^{2+} and/or hANP (10 nM). After a 6 h-incubation period in Krebs buffer, StAR mRNA levels decayed to 69.6 ± 6.6% (n = 3) of the zero time value, whereas in the presence of Ca^{2+}, StAR mRNA decayed to 52 ± 15% of the zero time value (n = 3). These differences were not statistically significant. The addition of hANP did not elicit any significant effect on StAR mRNA stability, as mea-

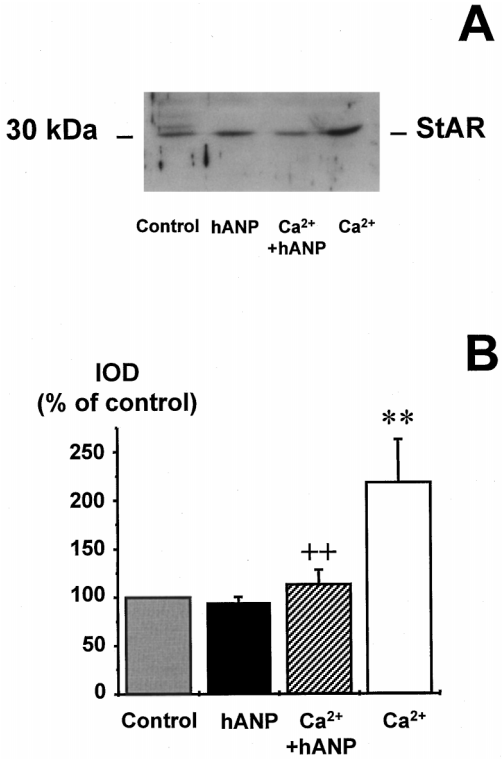

Fig. 5. Effect of ANP on the Ca^{2+}-Induced Increase in StAR Protein Content in Mitochondria of Bovine Glomerulosa Cells

A, Mitochondria were isolated from low- or high-Ca^{2+} clamped cells incubated in the presence or in the absence of hANP (10 nM). Shown is a Western blot from one representative experiment. For each sample, 10 μg of mitochondrial proteins were analyzed by SDS-PAGE and immunoblotting as described in *Materials and Methods.* B, The immunospecific bands for StAR protein were quantitated by densitometry in three independent experiments. Control, low-Ca^{2+}-clamp; Ca^{2+}, high-Ca^{2+} clamp (600 nM); hANP, low-Ca^{2+} clamp in the presence of hANP (10 nM); Ca^{2+} + hANP, high-Ca^{2+} clamp in the presence of hANP. Integrated optical density (IOD) values are expressed as a percentage of that measured in mitochondria from control cells. **, Significantly different from control with $P < 0.01$, ++, significantly different from Ca^{2+} stimulation with $P < 0.01$.

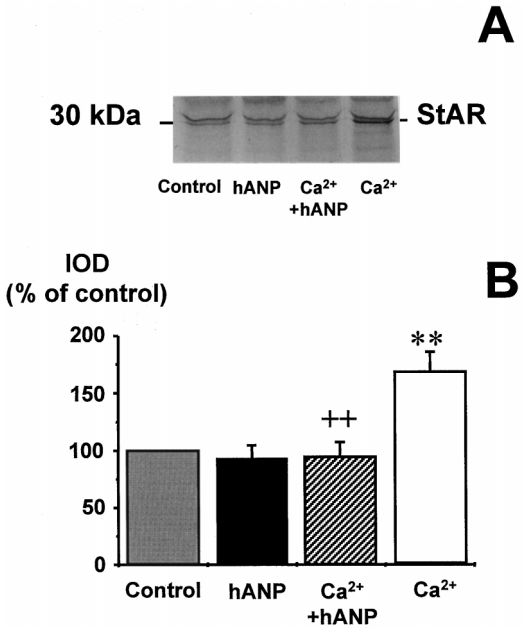

Fig. 6. Effect of ANP on the Ca^{2+}-Induced Increase in StAR Protein Radiolabeling

A, Immunoprecipitation of StAR protein from [^{35}S]methionine/cysteine-labeled bovine glomerulosa cells treated as described in the legend of Fig. 5. StAR protein was immunoprecipitated from equivalent amounts of radioactivity for each treatment as described in *Materials and Methods*, and the antibody-StAR protein complexes were analyzed by SDS-PAGE and autoradiography. B, Immunoprecipitated labeled StAR protein was quantitated by densitometry in three independent experiments. Integrated optical density (IOD) values are expressed as a percentage of that measured in control cells. **, Significantly different from control with $P < 0.01$; ++, significantly different from Ca^{2+} stimulation with $P < 0.01$.

sured 6 h after the addition of actinomycin D (63.6 ± 4.7% of zero time value).

These results suggested that the effect of Ca^{2+} on the steady state levels of StAR mRNA must reflect transcriptional activation. To assess this possibility, glomerulosa cells were treated simultaneously with Ca^{2+} and actinomycin D (5 μg/ml), in the absence or in the presence of hANP (10 nM). Densitometric analysis of Northern blots from three independent experiments clearly indicated that actinomycin D completely prevented the Ca^{2+}-induced StAR mRNA accumulation (108 ± 5% of controls) (Fig. 7C). In separate experiments, we observed that actinomycin D inhibited Ca^{2+}-induced pregnenolone production by 69.8 ± 11.8% (n = 4). Since ANP had no effect on StAR mRNA stability, it is likely that ANP exerts its inhibitory effect on Ca^{2+}-induced StAR mRNA accumulation by preventing *StAR* gene transcription.

Ca^{2+} Stimulates ANP-Sensitive Transcription of the StAR Gene

The above hypothesis was confirmed by the results of nuclear run-on experiments performed in bovine glomerulosa cells. As shown in Fig. 8, after 2 h of a high-Ca^{2+} clamp, transcriptional activity of the *StAR* gene was 171 ± 19% ($P < 0.01$, n = 3) of that measured in control (low-Ca^{2+} clamp) cells. Once again, hANP (10 nM) prevented this Ca^{2+}-induced activation of *StAR* gene transcription. Transcription of the β-actin gene was not significantly affected by Ca^{2+} or hANP, and no signal was detected with the empty vector (Fig. 8A).

DISCUSSION

ANP exerts one of its major physiological antihypertensive effects by preventing the stimulation of aldo-

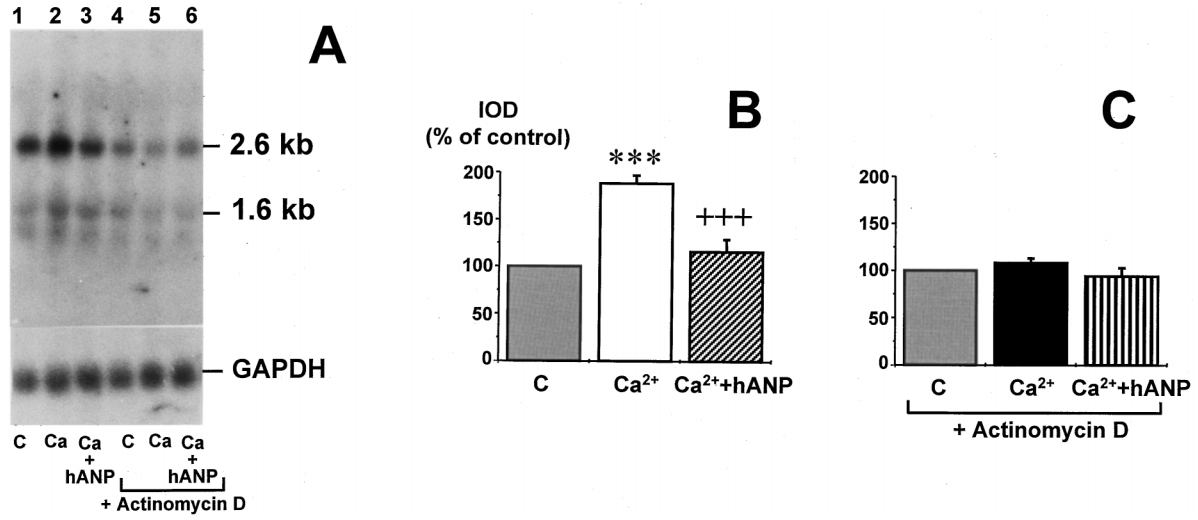

Fig. 7. Effect of ANP on the Ca^{2+}-Induced Increase in Steady State StAR mRNA Levels in Bovine Glomerulosa Cells

A, Northern blot analysis of StAR mRNA in control (C) and high-Ca^{2+} clamped cells, in the absence or in the presence of 10 nM hANP (lanes 1–3). In lanes 4–6, the same experiment was performed in the presence of actinomycin D (5 μg/ml). Total RNA was isolated as described in *Materials and Methods* and 15 μg of each sample were analyzed by agarose gel electrophoresis. StAR specific transcripts were detected by hybridization with a mouse full-length StAR cDNA. The blot was then stripped and rehybridized with a cDNA probe specific of the mouse GAPDH to normalize for equivalent loading of RNA. B, Quantification of StAR mRNA levels in four independent experiments (panel A, lanes 1–3). The major 2.6-kb transcript was quantitated and normalized to GAPDH mRNA levels. The corrected values for the IOD are expressed as a percentage of the IOD of StAR mRNA levels in control cells. ***, Significantly different from controls with $P < 0.001$; ++, significantly different from Ca^{2+} stimulation with $P < 0.01$ (n = 3). C, Quantification of StAR mRNA in control and high Ca^{2+}-clamped cells incubated in the presence of actinomycin D, with or without hANP (10 nM) (panel A, lanes 4–6) (n = 3).

sterone secretion elicited in adrenal glomerulosa cells by agonists such as Ang II and K^+ (4, 26, 38). Although ANP is known to stimulate cGMP formation in glomerulosa cells (29, 31), the intracellular mechanism of action of ANP as well as its molecular targets in adrenocortical cells are still poorly defined, due in part to the failure of membrane-permeant, nonhydrolysable analogs of cGMP to mimic the biological effect of ANP (4, 32, 33). This lack of effect of cGMP analogs was also observed in this work.

The present study was therefore undertaken in an attempt to identify intracellular targets for the inhibitory action of ANP on aldosterone biosynthesis. In a previous work on the mechanisms of activation of aldosterone biosynthesis by Ang II and Ca^{2+} in bovine adrenal glomerulosa cells, we have shown that physiological rises in $[Ca^{2+}]_c$ acutely stimulate the rate-limiting step in this process, namely cholesterol translocation from the mitochondrial outer membrane to intermembrane contact sites and inner membrane (35). Concomitantly, Ca^{2+} induced a specific increase in StAR protein in the inner mitochondrial membrane, confirming the well documented role of StAR in intramitochondrial cholesterol redistribution (36). In addition, both Ca^{2+}-stimulated intramitochondrial cholesterol transfer and StAR accumulation in the inner membrane were cycloheximide-sensitive, indicating the requirement for ongoing protein synthesis in the acute activation of aldosterone production. Using the ionomycin-mediated calcium clamp technique as a tool to mimic agonist-induced physiological rises of $[Ca^{2+}]_c$ (37), we have investigated whether ANP affects any of these Ca^{2+}-sensitive events.

Two major conclusions can be drawn from the results of the present work: 1) Ca^{2+} regulates *StAR* gene expression at the transcriptional level and 2) ANP inhibits aldosterone production by preventing Ca^{2+}-induced StAR mRNA accumulation.

While the role of cAMP in mediating the activation of *StAR* gene transcription via steroidogenic factor 1 (SF-1), an orphan nuclear receptor, has been extensively investigated (39–43), the potential participation of Ca^{2+} in regulating *StAR* gene expression has received little attention to date. We first examined the effect of Ca^{2+} stimulation on StAR mRNA levels. In agreement with previous data on bovine *StAR* gene expression (43), Northern blot analysis of StAR mRNA showed that two major StAR mRNA transcripts of 2.6 and 1.6 kb were present in bovine adrenal cells. Importantly, Ca^{2+} induced a marked and significant increase in StAR mRNA levels within 2 h of exposure of glomerulosa cells to a high Ca^{2+}-clamp. This observation raised the question of whether Ca^{2+} could increase StAR mRNA half-life. Our results show that StAR mRNA decayed to the same extent in low- and high-Ca^{2+} clamped cells for up to 6 h after addition of actinomycin D, thus excluding a stabilizing effect as the mechanism leading to Ca^{2+}-induced acute accumulation of StAR mRNA.

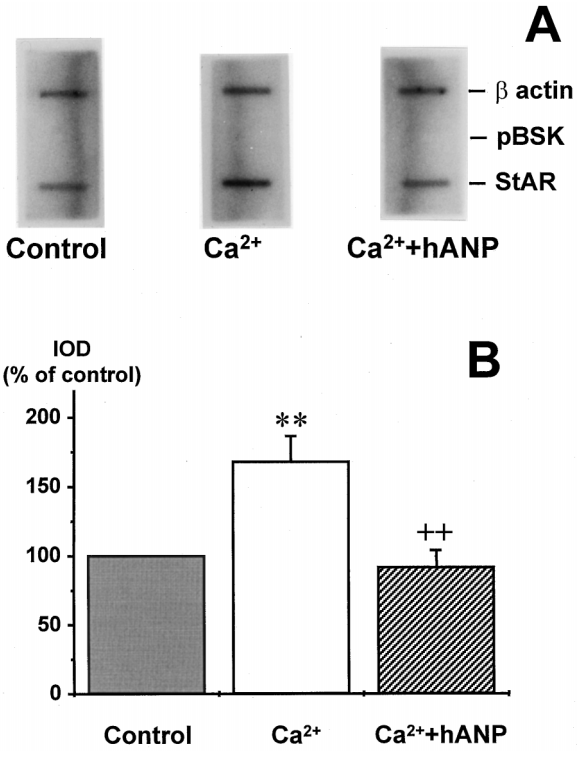

Fig. 8. Nuclear Run-on Transcriptional Analysis of the *StAR* Gene

A, Bovine glomerulosa cells were submitted to a low- (control) or a high-Ca^{2+} (600 nM) clamp in the presence or in the absence of hANP (10 nM), as described in *Materials and Methods*. The transcription of the *StAR* gene was assessed in isolated nuclei incubated with [^{32}P]UTP to generate radiolabeled RNA transcripts. RNA was hybridized to membranes on which StAR cDNA cloned in a Bluescript plasmid, empty Bluescript plasmid (pBSK, negative control), and mouse β-actin cDNA cloned in Bluescript (invariant internal control) had been immobilized. B, Quantification of nuclear run-on analysis from three independent experiments. Results are expressed as a percentage of controls.

Rather, the clear-cut inhibitory effect of actinomycin D on the Ca^{2+}-induced increase in StAR mRNA steady state levels suggested a direct relationship between *StAR* gene transcription and Ca^{2+}-stimulated pregnenolone production. However, data from the literature on the requirement of transcription for the acute steroidogenic response are conflicting. Indeed, actinomycin D exerted little or no effect on ACTH-induced RNA synthesis and corticosteroid production in rat adrenal quarters or dispersed cells (44, 45), while it blocked to a variable extent RNA synthesis and steroid production in bovine adrenal slices (44), in a mouse testicular interstitial cell line (46), in rat testicular interstitial cells in primary culture, and in Leydig cells (47, 48). More recently, Clark *et al*. (49) demonstrated that actinomycin D potently inhibits StAR mRNA accumulation and steroid production in MA-10 mouse Leydig tumor cells. Our results with bovine glomerulosa cells are consistent with the studies on testicular cells, MA-10 cells, and, more importantly, bovine adrenal slices and strongly speak in favor of a transcriptional activation exerted by Ca^{2+}.

Increased $[Ca^{2+}]_c$ up-regulates eukaryotic gene and, in particular, immediate-early response gene transcription in a variety of cell types (50). In rat aortic smooth muscle cells (51), as well as in bovine adrenal glomerulosa (52) and fasciculata (53) cells, Ang II was found to induce the expression of the early response genes c-*jun*, c-*fos* and/or *Jun B*. Whether these protooncogenes could be involved in the control of *StAR* gene transcription remains to be determined. However, at the present time, no activator protein-1 (AP-1)-responsive elements have been found in the 1.3 kb of DNA upstream of the transcription start site (39, 41). Interestingly, Sugawara *et al*. (39) reported the presence within the StAR promoter of two Sp1-responsive elements. This transcription factor has recently been shown to mediate Ca^{2+}-regulated gene expression in various cell types (50, 54). Alternatively, *StAR* gene transcription could be promoted by a Ca^{2+}-dependent relief of a blockade to elongation in intragenic sites, as has been proposed for c-*fos* transcription (50). Whatever the mechanism, our results clearly demonstrate a dual site of action for the calcium messenger in the acute activation of mineralocorticoid biosynthesis: in addition to its previously described intramitochondrial effects (35–37, 55), Ca^{2+} exerts clear-cut rapid genomic actions that result in *de novo* synthesis of StAR protein.

The second important finding of this work relates to the antisteroidogenic action of ANP. While ANP did not affect the $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ changes elicited by either Ang II or a Ca^{2+} clamp, it markedly reduced the Ca^{2+}-supported increase in pregnenolone formation, indicating that the inhibition occurred downstream of the generation of the Ca^{2+} signal. By contrast, ANP did not significantly affect pregnenolone synthesis in control nonstimulated cells. These results are consistent with previous studies suggesting that ANP may interfere with the early steps of steroidogenesis (22–24, 56). Elliott and Goodfriend (23) reported that the inhibitory effect of ANP on Ang II-elicited aldosterone synthesis was less effective at high extracellular Ca^{2+} concentrations, suggesting that ANP may impair a process that is stimulated by Ca^{2+}. Our data demonstrate for the first time that ANP decreases the availability of endogenous cholesterol to the $P450_{scc}$ enzyme, by preventing Ca^{2+}-stimulated intramitochondrial cholesterol transfer. Earlier studies on the hormonal regulation of cholesterol mobilization in steroidogenic cells had demonstrated that ongoing synthesis of StAR protein is required to facilitate cholesterol translocation to the inner mitochondrial membrane. Our results show that ANP completely abolished both the accumulation of mature StAR protein within mitochondria and the increase in [^{35}S]methionine labeling of StAR protein in high Ca^{2+}-clamped glomerulosa cells. This finding indicates that ANP inhibits the Ca^{2+}-induced *de novo* synthesis of StAR

protein and is consistent with previous data reported by Elliott *et al.* (57). Using two-dimensional gel electrophoresis, these authors have shown that Ang II induces in bovine adrenal glomerulosa cells the rapid appearance of a family of 28- to 30-kDa proteins, subsequently assumed to be StAR proteins (58), and that cotreatment with ANP and Ang II markedly reduced the amount of these proteins in mitochondria.

Interestingly, ANP abolished the Ca^{2+}-elicited increase in StAR mRNA levels. We therefore examined whether this inhibitory effect of ANP was due to blockade of *StAR* gene transcription and/or to a decrease in StAR mRNA stability. RNA decay experiments performed in the presence of actinomycin D showed that ANP did not alter StAR mRNA stability. Hence, it was likely that ANP counteracted the positive effect of Ca^{2+} on *StAR* transcription. Further evidence for the transcriptional control of the *StAR* gene by Ca^{2+} and ANP was obtained using nuclear run-on assays. To our knowledge, this is the first report relating increases in intracellular Ca^{2+} to transcriptional induction of *StAR* gene as well as an inhibitory effect of ANP on this process. The process by which ANP exerts its negative control through non-cGMP-mediated mechanisms remains to be elucidated. Interestingly, DAX-1, a member of the nuclear-receptor superfamily of transcription factors, has been recently shown to repress *StAR* gene expression and steroidogenesis (59), and one could speculate that ANP might induce DAX-1 expression in adrenal glomerulosa cells.

In summary, our results directly show that Ca^{2+} ion is a potent physiological regulator of *StAR* gene expression in bovine adrenal glomerulosa cells and that ANP impedes this transcriptional activation, thus exerting its physiological inhibitory action on aldosterone biosynthesis.

MATERIALS AND METHODS

Chemicals

Ionomycin was purchased from Calbiochem (Lucerne, Switzerland). Human ANP [hANP(4–28)] and urodilatin [Thr-Ala-Pro-Arg-hANP(1–28)] were obtained from Bachem (Bubendorf, Switzerland). [^{3}H]pregnenolone was purchased from Amersham (Zurich, Switzerland), [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{32}P]UTP (800 Ci/mmol) from Hartmann Analytic (Braunschweig, Germany). [^{35}S]methionine/cysteine labeling mix was purchased from ICN Biomedicals GmbH (Eschwege, Germany). Antipregnenolone antiserum was obtained from Biogenesis Ltd (Poole, UK). Win 19758 was purchased from Farillon (London, UK). All other chemicals used were purchased from Sigma (St. Louis, MO) or from Fluka (Buchs, Switzerland).

Bovine Adrenal Zona Glomerulosa Cell Culture and Treatments

Bovine adrenal glands were obtained from a local slaughterhouse. Zona glomerulosa cells were prepared by enzymatic dispersion with dispase and purified on Percoll density gradients (60). Primary cultures of purified glomerulosa cells were established as described in detail elsewhere (60) and kept in serum-free medium for 1 day before experiments, which were performed on the third day of culture.

Cells cultured in 10 cm plastic Petri dishes (10^7 cells per dish) or in 24-well plates (500,000 cells per well) were washed twice with a modified Krebs-Ringer (NaCl, 136 mM; $NaHCO_3$, 5 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; KCl, 1.8 mM; EGTA, 0.2 mM; D-glucose, 5.5 mM; HEPES, 20 mM; pH 7.4) and preincubated at 37 C for 30 min in the same buffer. Ca^{2+}-clamping was performed at 37 C for 2 h, in the presence of 2 μM ionomycin, 1 mM total extracellular Ca^{2+}, and 0.2 mM EGTA, to achieve a $[Ca^{2+}]_c$ of 600–700 nM (high-Ca^{2+} clamp), as described elsewhere (37). Control cells were Ca^{2+}-clamped in Krebs-Ringer buffer lacking Ca^{2+}, in the presence of 0.2 mM EGTA (low-Ca^{2+} clamp, $[Ca^{2+}]_c < 100$ nM). Thr-Ala-Pro-Arg-hANP(1–28), ANP(4–28), or membrane-permeant cGMP analogs were added to the incubation medium during stimulation with Ca^{2+} as indicated. In experiments designed to measure cholesterol content in submitochondrial membranes, aminogluthetimide (500 μM) was included in the incubation medium to inhibit cholesterol side-chain cleavage. At the end of the incubation period, the cells were scraped and sedimented at 200 $\times$ *g* for 15 min before subcellular fractionation or processing for total RNA isolation as described hereafter.

Steroid Measurement

Aldosterone content in incubation media was measured by direct RIA, using a commercially available kit (Diagnostic Systems Laboratories, Webster, TX). For the assessment of pregnenolone production, WIN 19758 (5 μM) was included in the incubation medium to prevent conversion of pregnenolone into progesterone. At the end of the incubation period, pregnenolone was determined directly in the medium by RIA. Steroid production was normalized and expressed per milligram cellular protein.

Measurement of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$

$[Ca^{2+}]_m$ was measured in bovine glomerulosa cells transfected with a plasmid coding for mitochondrial matrix-targeted aequorin as previously described in detail (34). $[Ca^{2+}]_c$ was determined in cells transfected with the same plasmid lacking the targeting mitochondrial presequence cDNA. Cells were transfected by the phosphate calcium precipitation method using the CellPhect Transfection Kit (Pharmacia Biotech, Zürich, Switzerland), according to the specifications of the manufacturer.

Preparation of Submitochondrial Fractions and Cholesterol Measurements

Glomerulosa cells were homogenized with a Potter-Elvejhem homogenizer (1200 rpm, 35 strokes), in a 5 mM Tris-HCl buffer, pH 7.4, containing 275 mM sucrose. The homogenate was centrifuged at 200 $\times$ *g* for 15 min to remove large debris and nuclei. Further centrifugation of the supernatant at 10,000 $\times$ *g* for 10 min yielded the mitochondria. The mitochondrial pellet was washed twice at 8,000 $\times$ *g* with the same buffer. Osmotically shocked adrenocortical mitochondria were fractionated on a sucrose density gradient into OM, CS, and IM as previously described (35). Protein was quantified using the Bio-Rad protein microassay (Bio-Rad Laboratories, Richmond, CA) and BSA as a standard. The cholesterol content of submitochondrial fractions (15–20 μg protein for OM, 30–40 μg protein for CS, and 80–100 μg for IM) was determined by a coupled cholesterol oxidase-peroxidase assay as reported elsewhere (35).

SDS-PAGE

SDS-PAGE was performed according to Laemmli (61). Mitochondrial proteins (10 μg/lane) were solubilized in sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) and loaded onto a 12% SDS-PAGE minigel (Mini Protean II System, Bio-Rad). Electrophoresis was performed at 150 V for 1 h.

Blotting Procedure and Immunodetection

SDS-PAGE-resolved proteins were electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schuell, Feldbach, Switzerland) according to Towbin *et al.* (62). After transfer, the membrane was incubated in a blocking buffer (PBS buffer containing 0.4% Tween 20 and 5% nonfat dry milk) for 1 h at room temperature, and then incubated with an antiserum generated by CovalAb (Lyon, France) against a peptide fragment (amino acids 88–98) of the 30- kDa StAR for 1 h in PBS/Tween 20 buffer (36). The membrane was thoroughly washed with the same buffer (3 × 10 min), and then incubated for 1 h with horseradish peroxidase-labeled goat antirabbit IgG (CovalAb). The nitrocellulose sheet was then washed four times for 15 min, and the antigen-antibody complex was revealed by enhanced chemiluminescence, using the Western blotting detection kit and Hyper-ECL film from Amersham.

Radiolabeling of Glomerulosa Cells and Immunoprecipitation of StAR

For radiolabeling of cellular proteins, 3×10^6 glomerulosa cells, plated in 6-cm petri dishes, were calcium-clamped for 2 h at 37 C, in the presence or in the absence of ANP. The incubation medium (3 ml) contained [^{35}S]methionine-cysteine (300 μCi). At the end of the labeling period, cells were washed three times with ice-cold PBS and lysed in RIPA buffer (10 mM sodium phosphate, pH 7.4, containing 150 mM NaCl, 1% Triton, 1% sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). The lysate was cleared by centrifugation for 5 min at 12,000 × *g* at 4 C. The StAR antiserum was added at a 1:100 dilution to aliquots of the supernatant (200 μl, ~100 μg protein), which were then gently rocked for 2 h at 4 C, before being incubated for 30 min with Protein-A Sepharose beads (Pharmacia Biotech AG, Dubendorf, Switzerland). Immunoprecipitates were pelleted, washed four times with RIPA buffer and analyzed by SDS-PAGE and autoradiography.

RNA Isolation and Northern Blot Analysis

Glomerulosa cell total RNA was extracted using the RNAgents kit (Promega, Zurich, Switzerland) according to the instructions of the manufacturer. This system consistently yields 50–80 μg total RNA/10^7 cells. For Northern blot analysis, 15–20 μg RNA were size-fractionated on a 1% formaldehyde agarose gel, vacuum-transferred onto Nytran membranes (Schleicher & Schuell) and fixed by UV cross-linking. The integrity of the 18 S and 28 S RNA was checked by ethidium bromide staining of the gel. Hybridization was performed using the previously cloned 1.5-kb mouse StAR cDNA (16). The cDNA was labeled with [α^{32}P]dCTP using the Rediprime random primer labeling kit from Amersham. Northern blots were prehybridized in Rapid Hybridization Buffer (Amersham) at 65 C for 30 min. The α^{32}P-labeled probe (specific activity: 2×10^6 cpm/ng DNA) was then added and the incubation was continued for 2 h at 65 C. Blots were washed for 5 min and 15 min successively at room temperature in 2 × saline sodium citrate (SSC), 0.1% SDS, and then for 15 min in 1 × SSC, 0.1% SDS. The final wash was performed at 65 C for 15 min in 1 × SSC, 0.1% SDS. RNA-cDNA hybrids were visualized on Hyperfilms (Amersham) after a 12- to 24-h exposure period. Blots were stripped and reprobed with mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Ambion, Lugano, Switzerland) to assess RNA loading.

Nuclear Run-on Assays

Nuclear run-on was performed according to described protocols (63). Bovine glomerulosa cells (5×10^7 cells per treatment) were subjected to a low- or high-calcium clamp in the presence or in the absence of hANP for 2 h. Transcription reactions were then carried out on isolated nuclei using 150 μCi ^{32}P-labeled UTP and 1 mM ATP, CTP, and GTP (Pharmacia Biotech AG, Dübendorf, Switzerland) in reaction buffer (5 mM Tris-HCl, pH 8, 2.5 mM $MgCl_2$, 150 mM KCl) at 30 C for 30 min. Nuclear RNA was isolated and hybridized for 36 h at 65 C to Hybond-N^+-charged nylon membranes (Amersham) on which 5 μg of a Bluescript plasmid containing a 1.5-kb StAR cDNA insert, 5 μg of a Bluescript plasmid containing a 250-bp mouse β-actin insert (Ambion), or 5 μg of empty Bluescript plasmid had been immobilized. Membranes were washed and exposed to hyperfilms for visualization.

Analysis of Data

Results are expressed as means ± SEM. The mean values were compared by ANOVA using Fisher's test. A value of $P < 0.05$ was considered as statistically significant. Quantification of immunoblots and autoradiograms was performed using a Molecular Dynamics (Sunnyvale, CA) Computing Densitometer.

Acknowledgments

The authors are grateful to Liliane Bockhorn, Walda Dimeck, and Gisèle Dorenter for their excellent technical assistance.

Received January 16, 1998. Revision received March 11, 1998. Accepted March 17, 1998.

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This work was supported in part by Swiss National Science Foundation Grants 31.42178–94 (to A.M.C.) and 32.49297.96 (to M.F.R.), by NIH Grant HD-17841 (to D.M.S.) and by grants from the Ciba-Geigy Jubiläumsstiftung and the Sandoz Foundation. M.F.R. is a recipient of a grant from the Professor Max Cloëtta Foundation.

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