Aldosterone-mediated Regulation of Na\(^{+},K^{+}\)-ATPase Gene Expression in Adult and Neonatal Rat Cardiocytes*

(Received for publication, December 26, 1990)

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By altering the Na\(^{+}/K^{+}\) electrochemical gradient, Na\(^{+},K^{+}\)-ATPase activity profoundly influences cardiac cell excitability and contractility. The recent finding of mineralocorticoid hormone receptors in the heart implies that Na\(^{+},K^{+}\)-ATPase gene expression, and hence cardiac function, is regulated by aldosterone, a corticosteroid hormone associated with certain forms of hypertension and classically involved in regulating Na\(^{+},K^{+}\)-ATPase gene expression and transepithelial Na\(^{+}\) transport in tissues such as the kidney. The regulation by aldosterone of the major cardiac Na\(^{+},K^{+}\)-ATPase isoform genes, \(\alpha-1\) and \(\beta-1\), were studied in adult and neonatal rat ventricular cardiocytes grown in defined serum-free media. In both cell types, aldosterone-induced a rapid and sustained 3-fold induction in \(\alpha-1\) mRNA accumulation within 6 h, \(\beta-1\) mRNA was similarly induced. \(\alpha-1\) mRNA induction occurred over the physiological range with an EC\(_{50}\) of 1–2 nM, consistent with binding of aldosterone to the high affinity mineralocorticoid hormone receptor. In adult cardiocytes, this was associated with a 36% increase in \(\alpha\) subunit protein accumulation and an increase in Na\(^{+},K^{+}\)-ATPase transport activity. Aldosterone did not alter the 3-h half-life of \(\alpha-1\) mRNA, indicating an induction of \(\alpha-1\) mRNA synthesis. Aldosterone-dependent \(\alpha-1\) mRNA accumulation was not blocked by the protein synthesis inhibitor cycloheximide, whereas amiloride inhibited both an aldosterone-dependent increase in intracellular Na\(^{+}\) ([Na\(^{+}\)]) and \(\alpha-1\) mRNA accumulation. This demonstrates that aldosterone directly stimulates Na\(^{+},K^{+}\)-ATPase \(\alpha-1\) subunit mRNA synthesis and protein accumulation in cardiac cells throughout development and suggests that the heart is a mineralocorticoid-responsive organ. An early increase in [Na\(^{+}\)], may be a proximal event in the mediation of the hormone effect.

The Na\(^{+},K^{+}\)-ATPase (EC 3.6.1.3) is a ubiquitous transmembrane heterodimeric protein complex consisting of a catalytic \(\alpha\) and glycosylated \(\beta\) subunit that couples the hydrolysis of ATP to the vectorial transport of Na\(^{+}\) outward and K\(^{+}\) inward across the cell plasma membrane. By generating and maintaining the Na\(^{+}/K^{+}\) electrochemical gradient, it represents the primary source of energy for unidirectional transepithelial Na\(^{+}\) transport found in the kidney and colon, restoration of the action potential of electrically excitable tissues such as nerve and muscle, and for a variety of Na\(^{+}\)-dependent ion and nutrient co- and countertransport pathways (for reviews see: Jorgensen, 1982; Sweadner, 1979). Additional roles for Na\(^{+},K^{+}\)-ATPase are suggested by its recent identification as a cell surface adhesion molecule involved in glial-neuronal cell interaction (Gloor et al., 1990). Contributing to these additional roles, at least three related but structurally distinct \(\alpha\) isoforms are expressed in a tissue-specific and developmentally regulated manner (Orlowski and Lingrel, 1988). Although the functional role for \(\beta\) is unknown, the identification of an additional \(\beta\) isoform adds to the complexity of Na\(^{+},K^{+}\)-ATPase heterogeneity (Martin-Vassalo et al., 1989).

Adrenocortical steroid hormones, such as the mineralocorticoid aldosterone, are important regulators of Na\(^{+},K^{+}\)-ATPase gene expression in renal tubule cells and other polarized epithelial cells involved in unidirectional Na\(^{+}\) ion transport (Petty et al., 1981; Doucet, 1988; Finkel and Aperia, 1986; Binder et al., 1989). In studies of the viral mouse mammary tumor virus promoter (Arriza et al., 1987; Cato and Weinmann, 1988), aldosterone regulates gene expression via its binding to, and activation of, a specific intracellular mineralocorticoid hormone receptor (MR). The activated MR induces transcription by binding to a DNA hormone response element on the mouse mammary tumor virus promoter. It is not known whether this viral mechanism is utilized in toto, or in part, in the regulation of aldosterone-responsive cellular genes. In renal and bladder epithelial cells, aldosterone induces Na\(^{+},K^{+}\)-ATPase \(\alpha\) and \(\beta\) gene expression at the transcriptional (Verrey et al., 1989) mRNA, protein, and functional transport levels (Verrey et al., 1987; Barlet-Bas et al., 1988). However, there is considerable controversy whether Na\(^{+},K^{+}\)-ATPase gene expression is dependent on changes in intracellular Na\(^{+}\) or additional protein synthesis. Tissue-specific and ionic factors and the interaction with thyroid hormone may also play important roles in modulating the regulatory response of Na\(^{+},K^{+}\)-ATPase to aldosterone.

The identification of mineralocorticoid hormone receptors in cardiac tissue (Pearce and Funder, 1987; Arriza et al., 1987) raises new questions on the role of aldosterone in cardiovas-
cular cell function and gene expression. As a nonclassical target tissue for aldosterone, the cardiovascular system provides an important new model to study tissue-specific factors modulating Na⁺,K⁺-ATPase gene expression in response to adrenocorticosteroid hormones. Cardiac cell excitability and contractility is profoundly influenced by alterations in Na⁺,K⁺-ATPase function and gene expression in response to thyroid hormone (Kim and Smith, 1984), extracellular Na⁺ (Ko and Smith, 1986), and extracellular K⁺ (Kim et al., 1984). The presence of mineralocorticoid receptors in the heart implies, but does not prove, that Na⁺,K⁺-ATPase gene expression is also regulated by aldosterone. If functional, these receptors may play a role in modulating cardiovascular Na⁺,K⁺-ATPase gene expression in response to changes in the renin-angiotensin-aldosterone hormonal axis in physiological and pathophysiological states such as hypertension (Herrera et al., 1988). To address this issue, two models of primary cultured rat neonatal and adult ventricular cardiocytes, grown in serum-free defined media, were utilized to demonstrate that aldosterone induces Na⁺,K⁺-ATPase α-1 and β-1 subunit gene expression and function through a cycloheximide-insensitive amiloride-dependent mechanism.

**EXPERIMENTAL PROCEDURES**

**Neonatal Rat Cardiocyte Cell Culture**—Primary cardiocytes from 1-day-old Sprague-Dawley rats were prepared using a modified method of Bloch et al. (1986). Briefly, cardiac suspensions after 0.4% trypsin dissociation were washed with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 7% fetal calf serum (FCS), plated on 100-mm culture dishes, and incubated for 2 h at 37 °C. During this interval, mesenchymal cells and a few myocytes attached to culture dishes, whereas most myocytes remained in the medium. The medium that contained myocytes was pooled and transferred to 60-mm dishes at a density of 4 × 10⁶ cells/60-mm dish in 7% FCS containing DMEM supplemented with thymidine (0.6 mg/ml), penicillin (20 units/ml), streptomycin (20 mg/ml), and gentamycin (20 mg/ml). After 48-h incubation, the medium was changed to serum-free DMEM supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml) (ITTS), and antibiotics. In all experiments, cells were incubated for 24 h in serum-free DMEM prior to the defined day 0 of the experiment.

**Adult Rat Heart Cell Culture**—As described previously (Ikeda et al., 1990), for each preparation, two male Sprague-Dawley rats (200–250 g) were euthanized by cervical dislocation and placed on a laminar flow hood. The hearts were then cut into segments of proximal aorta. Hearts were previously cannulated via the aorta and perfused retrogradely by the Langendorff technique. The perfusing solution was oxygenated with 95% O₂ + 5% CO₂ and warmed to 37 °C. Initially, the hearts were perfused with bicarbonate-based solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KHPO₄, 25 mM NaHCO₃, 15 mM glucose, pH 7.25) at a rate of 5 ml/min for approximately 5 min until the hearts were completely cleansed of blood. The perfusate was then changed to the same buffered solution without CaCl₂ for approximately 25 min at which time the hearts appeared pale and swollen. The ventricles were removed and cut into small pieces and placed in 20 ml of Ca²⁺-free solution containing 0.05% collagenase and 0.03% hyaluronidase were then added to Ca²⁺-free solution and perfused for 25 min at which time the hearts appeared pale and swollen. The ventricles were removed and cut into small pieces and placed in 20 ml of Ca²⁺-free solution containing 0.05% collagenase, 0.03% hyaluronidase, 0.002% trypsin, and 0.002% deoxyribonuclease. The tissue pieces were incubated at 37 °C in a shaking water bath (100 cycles/min) for 5 min and mechanical dissociation of the myocytes was accomplished by pipetting up and down through a 5-ml Pasteur pipette with a 3-mm tip diameter. The isolated myocytes were filtered through Nytex mesh and collected in a centrifuge tube containing Ca²⁺-free solution with 2.0% bovine serum albumin. This procedure was repeated several times until no more free myocytes were obtained. The collected cells were centrifuged at 1,000 × g for 5 min and washed twice in 10% FCS containing 50/50 (v/v) Ca²⁺-free solution, M199 medium. The pellet was resuspended in M199 culture medium containing 8% FCS, penicillin-streptomycin (100 units/ml + 100 μg/ml), fungizone (0.24 ng/ml), and fungizone (Ara-C) (5 μg/ml). The cells were then plated on laminin (60 μg)-coated 100-mm culture dishes at a density of 0.5 × 10⁶ cells/dish. Incubation was allowed to proceed for 5 days in a CO₂ incubator at 37 °C. At 5 days, unattached cells were washed away, and new M199 medium, without Ara-C, was added and replaced every 2–3 days thereafter. After 10–14 days incubation, the medium was transferred to serum-free M199 supplemented with ITS. The cells appeared healthy and continued to contract spontaneously for at least 3–4 days thereafter. The cells were used for experiments after 1-day incubation in serum-free medium. All dissection and perfusion were performed under sterile conditions in a laminar flow hood.

**Fluorescent Flow Cytometry**—Cells cultured on plates were fixed in 2 ml of 1% paraformaldehyde in PBS and 0.1% Triton X-100 for 10 min at 4 °C. After washing with PBS once, cells were incubated in 2 ml of PBS solution containing 1% bovine serum albumin and either antiserum (α-1 or β-1) and monoclonal IgG1, Schenk et al., 1984) or a mouse monoclonal Pan-MHC antibody (Amersham Corp.). After incubation for 1 h at room temperature, cells were washed twice with PBS, and then incubated in 2 ml of biotinylated anti-mouse IgG1 (Amersham Corp., 1:100 in PBS) for 1 h at room temperature, followed by addition of 2 ml of fluorescein-streptavidin (Amersham Corp., 1:100 in PBS) for 1 h at room temperature. After washing with PBS twice, cells were detached from the plates with 0.2 ml of Versene solution (1:5000) containing 0.1% Triton X-100. The fluorescein-labeled cells (5–10 × 10⁶) were analyzed with a flow cytometer (Coulter Epic S) by logarithmic integral green fluorescence after gating on forward angle light scatter. As all patterns approached normal curves, the peak fluorescence was taken as the fluorescence channel with the highest number of cells. Peak fluorescence was then converted from channel units of logarithmic fluorescence to linear units and used to derive percentage of maximal peak fluorescence.

**Northern Blot Analysis**—RNA was prepared by the guanidinium thiocyanate-cesium chloride method (Chargrin et al., 1979). Equal amounts of total RNA (15 μg) were size-fractionated by electrophoresis on denaturing 1.2% agarose, formaldehyde gels and transferred to nitrocellulose filters. Hybridizations were performed with an excess of [³²P]dCTP-labeled rat α-1 (2.2-kilobase NcoI-BclII restriction fragment) and β-1 (0.9-kilobase PstI restriction fragment) cDNA probes (specific activity > 1 × 10⁸ cpm/μg DNA). The filters were washed twice at high stringency (30 min each in 0.2 × SSC with 0.2% sodium dodecyl sulfate at 65 °C). Autoradiography was performed at ~70 °C and quantitated by densitometric scanning (Bromma 2202 Ultrascan).

**Slot Blot Analysis**—RNA samples were denatured by heating at 65 °C for 5 min and cooled on ice. Aliquots containing 5 μg of RNA in 10 × SSC were spotted directly onto nitrocellulose filters using Bethesda Research Laboratories slot blot apparatus. After washing with 20 × SSC, filters were baked at 80 °C for 2 h and hybridized. Washing and autoradiography were performed as mentioned in Northern blot analysis.

**Sodium Content**—The cell monolayers were washed three times (within 15 s) with ice-cold 100 mM MgCl₂. The washed cells were air-dried and extracted overnight in 1.5 ml of 3 N nitric acid. The HNO₃ extract (500 ml) was diluted to 2 ml with Na⁺- and K⁺-free solution (10 mM NaCl, 10 mM KCl, 20 mM NaF, and 0.01% ascorbic acid) and analyzed for sodium content using an atomic absorption spectrophotometer (Perkin-Elmer, model 460). Na⁺ in the standard solutions used for calibration ranged from 20 to 120 mM. After removing the residual HNO₃ solution from the culture plates, 2 ml of 0.2 N NaOH was added to dissolve the cells. Protein content was assayed by the method of Lowry et al. (1951). The Na⁺ content was calculated and expressed as nanomoles/mg of protein.

**Miscellaneous**—Statistical analysis was performed using a Student’s t test. p values less than 0.05 were considered to indicate a statistically significant difference. Chemicals were of the highest grade commercially available. [³²P]dCTP was from Du Pont-New England Nuclear.

**RESULTS**

**Cultured Rat Neonatal Cardiocytes (RNC) Are a homogeneous Cell Population in Vitro**—To study the effects of aldosterone on cardiocyte gene expression and function, an in vitro primary rat neonatal cardiocyte tissue culture model was developed and characterized. Due to its potential effects on gene expression and myogenesis (Tapscott et al., 1989; Lin et al., 1989), and in contrast to other cultured neonatal cardiocyte systems (e.g. Orlovski and Lingrel, 1990), we determined whether a homogeneous cardiocyte cell population could...
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be achieved in the absence of bromodeoxyuridine (BrDU), a thymidine analogue often used to inhibit non-cardiocyte cell proliferation. After transfer into serum-free defined media (DME-ITS), the cardiocyte population was characterized regarding cell function and population homogeneity. One day following transfer to DME-ITS (defined as day 0, see “Experimental Procedures”), microscopic inspection of many fields revealed close to 100% spontaneously beating cardiocytes. This high fraction of functional myocardial cells was observed for up to 2 weeks. The non-cardiocyte component of the cell population was also directly measured. Cells containing high levels of myosin heavy chain protein were identified by an indirect immunofluorescent assay of a mouse myosin heavy chain (MHC) antibody binding to permeabilized cells. By fluorescence microscopy, only cells with well defined myofibrillar striations strongly bound the antibody. Nonstriated cells either did not bind or bound very weakly (data not shown).

To determine the proportion of cardiocytes and non-cardiocytes in the cell population, MHC antibody-treated cells were analyzed by fluorescent flow cytometry (Fig. 1). Simultaneous analysis of cell size (x axis) and myosin associated fluorescence (y axis) demonstrated that approximately 95% of the cells expressed large amounts of MHC (Fig. 1, quadrant 2), whereas non-MHC-containing cells represented less than 5% of the total cell population (Fig. 1, quadrant 4). Cellular fragments and debris constitute quadrants 1 and 3. These studies suggest that this in vitro population is highly homogeneous for functional cardiocytes with a minimal non-cardiocyte cell component even in the absence of bromodeoxyuridine. In addition, the cardiocyte population can be maintained for extended periods in defined media.

Aldosterone Regulates Both \(\alpha\)-1 and \(\beta\)-1 Gene Expression—
The effect of aldosterone on Na\(^+\), K\(^+\)-ATPase \(\alpha\)-1 and \(\beta\)-1 gene expression in ventricular cardiocytes was determined in the absence of serum growth factors and hormones. As described under “Experimental Procedures,” cardiocytes were placed in serum-free media for at least 24 h prior to the addition of aldosterone. Aldosterone was added directly to the media to a final concentration of 1 \(\mu\)M. At subsequent time points, total cardiocyte RNA was isolated, size-fractionated by denaturing gel electrophoresis, and transferred to nitrocellulose filters. The relative accumulation of \(\alpha\)-1 and \(\beta\)-1 mRNA was determined by hybridization with an \(^{32}\)P-labeled \(\alpha\)-1- or \(\beta\)-1-specific cDNA probe. After 6 h, aldosterone induced an approximately 3-fold increase in 27 S \(\alpha\)-1 mRNA accumulation (Fig. 2, panel A) that was maintained for over 24 h. Neither \(\beta\)-actin mRNA levels in the presence of aldosterone nor \(\alpha\)-1 mRNA levels in the absence of aldosterone showed significant changes in expression (data not shown). Na\(^+\), K\(^+\)-ATPase \(\beta\)-1 mRNA levels showed a similar 4–5-fold induction in response to aldosterone (Fig. 2, panel B), although with a somewhat delayed time course, reaching steady-state levels in approximately 12 h. These results suggest that aldosterone is mediating a rapid and specific induction of both \(\alpha\)-1 and \(\beta\)-1 gene expression in rat neonatal cardiocytes.

The dependence of \(\alpha\)-1 gene expression on the continued presence of aldosterone was determined. Based on the above time course of induction, cardiocyte \(\alpha\)-1 mRNA levels have already reached steady state in the presence of aldosterone after 6 h. These cells were then switched to aldosterone-free DME-ITS media. Within 6 h of removal of aldosterone, \(\alpha\)-1 mRNA accumulation decreased to nearly basal levels (Fig. 3, lane 3). By 24 h, \(\alpha\)-1 mRNA levels were at basal levels (Fig. 2, lane 4). No change in \(\beta\)-actin mRNA levels were detected (data not shown), suggesting that aldosterone withdrawal results in the specific de-induction of \(\alpha\)-1 gene expression. These results demonstrate that the \(\alpha\)-1 gene expression is

**Fig. 1.** Fluorescent MHC antibody flow cytometric analysis of cultured RNC. Cells were permeabilized and treated with a monoclonal MHC antibody as described in the text. The x axis represents a logarithmic scale of increasing cell size. The y axis represents a logarithmic scale of increasing cell fluorescence. The intensity of the dots corresponds to number of cells at each x, y coordinate. Quadrants 1–4 correspond to cell size and cell fluorescent determinations: quadrant 1, fluorescent debris; quadrant 2, fluorescent large cells (cardiocytes); quadrant 3, nonfluorescent debris; quadrant 4, nonfluorescent large cells (non-cardiocytes). Ten thousand cells were analyzed.

**Fig. 2.** Kinetics of induction of RNC Na\(^+\), K\(^+\)-ATPase \(\alpha\)-1 and \(\beta\)-1 gene expression by aldosterone. RNC cells were exposed to 1 \(\mu\)M aldosterone for the indicated times and assayed for \(\alpha\)-1 (panel A) and \(\beta\)-1 (panel B) mRNA accumulation. Total RNA (15 \(\mu\)g) was size-fractionated by 1.2% agarose-formaldehyde gel electrophoresis, transferred to nitrocellulose filter papers, and hybridized to excess \(^{32}\)P-labeled \(\alpha\)-1- or \(\beta\)-1-specific cDNA probe. The final wash stringency was 65 °C, 0.2 x SSC. Autoradiography was performed within the linear exposure range of the film for 24–48 h at ∼70 °C with one intensifying screen.

**Fig. 3.** Deinduction of RNC \(\alpha\)-1 mRNA accumulation by aldosterone withdrawal in RNC. 15 \(\mu\)g of total RNA was prepared from RNC for analysis of \(\alpha\)-1 mRNA accumulation by Northern filter hybridization as described in Fig. 2. RNC cells were exposed to 1 \(\mu\)M aldosterone for 6 h (lane 2). Parallel dishes were then incubated in serum-free medium without aldosterone for 6 (lane 3) and 24 h (lane 4). RNC cells not exposed to aldosterone (lane 1) was used as the control.
induced by aldosterone and that the continued presence of aldosterone is necessary for the maintenance of induced α-1 mRNA levels. Withdrawal of the hormone results in the rapid down-regulation of the gene.

**Physiologic Concentrations of Aldosterone Regulate RNC α-1 Gene Expression**—To determine whether aldosterone regulation of Na⁺,K⁺-ATPase α-1 gene expression occurred within the physiologic range of the hormone, a dose-response relationship was established. As shown in Fig. 4 (panel A), α-1 mRNA levels were determined by slot blot analysis of total RNA from cardiocytes cultured in DME-ITS for 24 h in the presence of increasing concentrations of aldosterone. An increase in α-1 levels was first detected at 1 nM with maximal induction occurring at 10 nM aldosterone. A quantitative dose-response curve was derived by laser densitometry of multiple experiments (Fig. 4, panel B). A steep dose-response curve with an EC₅₀ of 2 nM aldosterone was calculated. This value is very close to the binding affinity of aldosterone for the type I mineralocorticoid receptor found in rat ventricle (Kd = 2.1 nM, Peace and Funder, 1987). These results demonstrate that aldosterone regulates cardiac α-1 gene expression at physiologically relevant nanomolar concentrations, likely through high-affinity binding to the mineralocorticoid receptor.

**Aldosterone Regulates RNC α-1 Gene Expression by Inducing α-1 mRNA Synthesis**—Regulation of α-1 mRNA accumulation may be due to changes in α-1 mRNA synthesis or its rate of degradation or both. To determine whether aldosterone primarily increased α-1 mRNA synthesis or decreased its rate of degradation, the stability of α-1 mRNA in the presence and absence of aldosterone was determined. Cardiocytes were exposed to 1 μM aldosterone for 24 h to reach steady-state-induced levels of α-1 mRNA. Both aldosterone treated and control cardiocytes were then “chased” with actinomycin D at 5 μg/ml. At this concentration, total RNA synthesis is over 95% inhibited within 15–20 min (data not shown). At varying time points, total RNA was isolated and α-1 mRNA levels determined by Northern filter hybridization. As quantitated in Fig. 5, the rate of decrease in α-1 mRNA signal strength during the actinomycin D chase, relative to total RNA, was unchanged between control and aldosterone-treated cardiocytes, with a calculated half-life (t½) of 2–3 h. Thus, aldosterone does not alter the stability of α-1 mRNA. These results suggest that aldosterone regulates α-1 mRNA accumulation by regulating its rate of synthesis.

**Aldosterone Regulates RNC α-1 Gene Expression by a Cycloheximide-independent, Amiloride-sensitive Pathway**—We have presented data suggesting that α-1 mRNA synthesis, and likely gene transcription, is induced by aldosterone via its binding to a high-affinity receptor. However, this does not distinguish between direct and indirect effects of aldosterone on α-1 gene expression. Indirect effects of aldosterone may involve the synthesis of an intermediate regulatory protein or alterations in the ionic milieu of the cell. The latter is especially important given the proposed linkage between Na⁺,K⁺-ATPase gene expression and intracellular Na⁺ concentration ([Na⁺]). To distinguish between these two possibilities, cardiocytes were treated with the protein synthesis inhibitor cycloheximide (20 μg/ml) 30 min prior to and then throughout exposure to 1 μM aldosterone. This resulted in the inhibition of over 90% of protein synthesis (data not shown). As shown in Fig. 6, cycloheximide did not inhibit the aldosterone-mediated induction of α-1 mRNA accumulation. Thus, aldosterone does not regulate α-1 gene expression via the synthesis of an intermediate protein product.

To determine whether aldosterone mediated changes in the intracellular ionic milieu of cardiocytes, cells were incubated with 1 μM aldosterone from 4 to 48 h and [Na⁺], was measured using atomic absorption spectrophotometry. Corrected for protein content, [Na⁺], began to rise 4 h after exposure to aldosterone, peaking at 6 h, and returning to the control levels by 24 h (Fig. 7). Thus, there is a transient increase in net cardiocyte Na⁺ influx that closely correlates with the increase in α-1 mRNA synthesis. Amiloride was used as an initial approach to uncouple aldosterone from its ionic effects. The addition of 100 μM amiloride completely inhibited aldosterone-dependent [Na⁺], elevation (Fig. 8A), presumably by
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Fig. 6. Effect of protein synthesis inhibition on the aldosterone-mediated induction of RNC α-1 gene expression. RNC cells were exposed for 6 h to 1 μM aldosterone either in the presence or absence of cycloheximide (20 μg/ml). Total RNA was isolated and α-1 mRNA levels assayed by slot blot analysis as described in Fig. 4. Each point is the mean ± S.E. of four separate experiments.

Fig. 7. Aldosterone induces a transient increase in cardiocyte Na\textsuperscript{+} levels. RNC cells were exposed to 1 μM aldosterone and at the indicated time points total cellular Na\textsuperscript{+} content was determined using an atomic absorption spectrophotometer (see "Experimental Procedures"). The intracellular Na\textsuperscript{+} content was calculated and expressed as nanomoles of Na\textsuperscript{+}/mg of protein. Each point is the mean ± S.E. of three experiments. *, p < 0.05 versus control.

Fig. 8. Aldosterone induces RNC α-1 gene expression via an amiloride-sensitive mechanism. Panel A, RNC cells were exposed to aldosterone (1 μM) in the presence or absence of amiloride (100 μM) for 6 h. Total cellular Na\textsuperscript{+} content was determined using an atomic absorption spectrophotometer. The Na\textsuperscript{+} content was calculated and expressed as nanomoles/mg of protein. Each point is the mean ± S.E. of three experiments. *, p < 0.05 versus control. Panel B, parallel cultures of the above RNC cells were assayed for α-1 mRNA levels as described in Fig. 4. Each point is the mean ± S.E. of four separate experiments.

Fig. 9. Induction of α-1 mRNA accumulation by aldosterone in long-term cultured RAC. Adult rat cardiocytes were prepared as described under "Experimental Procedures" and exposed to 1 μM aldosterone from 6 to 48 h. Total RNA was isolated and α-1 mRNA accumulation assayed as in Fig. 2. Quantitation of α-1 mRNA levels was performed by laser densitometry. The percent increase was related to the α-1 mRNA level of control samples. The tabular data are the means ± S.E. of three separate experiments.
The presence of the indicated concentrations of aldosterone and assayed for expression to aldosterone. RAC cells were incubated for 24 h in the presence of the indicated concentrations of aldosterone and assayed for α-1 mRNA accumulation by slot blot analysis as described in Fig. 4. The data are expressed as the percent increase in α-1 mRNA accumulation compared with the maximal response.

Fig. 10. Concentration-effect relation of RAC α-1 gene expression to aldosterone. RAC cells were incubated for 24 h in the presence of the indicated concentrations of aldosterone and assayed for α-1 mRNA accumulation by slot blot analysis as described in Fig. 4. The data are expressed as the percent increase in α-1 mRNA accumulation compared with the maximal response.

Fig. 11. Immunofluorescence analysis of aldosterone-mediated induction of RAC α subunit gene expression. RAC cells were exposed to 1 μM aldosterone from 12 to 72 h. The cells were then permeabilized and incubated for 1 h at 4 °C with a 1:100 dilution of Na+K+-ATPase α subunit-specific monoclonal antibody 9-A5. The cells were washed with PBS and then exposed to a 1:100 dilution of biotinylated anti-mouse IgG1 followed by fluorescein-streptavidin conjugate. The data are expressed as the percent increase in α-1 mRNA expression in control samples (no aldosterone). Each bar represents the mean ± S.E. of six separate experiments. *, p < 0.05 versus control. **, p < 0.01 versus control.

The intensity distribution of anti-α sites in permeabilized RACs using a monoclonal antibody to the α subunit 9-A5 (Schenk et al., 1984). In cells exposed to 1 μM aldosterone for 48 and 72 h, the peak fluorescence was enhanced by 18% (p < 0.05, n = 5) and 36% (p < 0.01, n = 5), respectively, compared with control samples (Fig. 11). A significant change in cell volume or protein content was not observed (data not shown). These results suggest that the aldosterone-mediated increase in Na+K+-ATPase α gene expression leads to an increased Na+K+-ATPase α protein content per cell.

To determine whether the aldosterone-mediated induction of Na+K+-ATPase α gene expression correlated with an increase in Na+K+-ATPase ion-transport function, an acute aldosterone withdrawal experiment was performed. Adult rat cardiocytes were grown for 72 h in the presence or absence of 1 μM aldosterone. Total sodium content was not significantly different between control and aldosterone-exposed cells (181 nmol/mg protein ± 14 versus 193 ± 21; mean ± S.E., n = 6) (Fig. 12). This is consistent with our finding of only a transient induction of net Na+ influx in neonatal cardiocytes (see Fig. 7). However, total cellular sodium content is a steady-state value dependent on two dynamic parameters including active Na+ influx by Na+K+-ATPase and passive Na+ influx driven by the transmembrane Na+ electrochemical gradient. Thus, the same measured [Na+]i, may reflect equivalent inductions in both Na+K+-ATPase activity and Na+ influx.

A measurement of short-term changes in total sodium content following acute withdrawal of aldosterone (i.e. an acute decrease in Na+ permeability) from aldosterone-exposed cells would allow for a determination of the relative contribution of Na+K+-ATPase-mediated Na+ efflux and Na+ influx. After 72 h, aldosterone-exposed and control RACs were washed and further incubated in serum-free M199 medium for 60 min. As shown in Fig. 12, the sodium content was significantly lower by 18% (p < 0.05) in aldosterone-exposed cells compared with control cells (138 ± 8 versus 162 ± 5 nmol/mg protein, n = 6). These results suggest that acute aldosterone withdrawal "uncovers" a higher level of Na+K+-ATPase ion-transport activity in aldosterone-exposed versus control cells. As aldosterone withdrawal should result in a rapid decrease in Na+ permeability, this result is consistent with a higher basal level of Na+K+-ATPase activity in aldosterone-treated cells. This result is supported by treatment of the same cells for 10 min with 1 mM ouabain following the 60-min aldosterone-free period. The cellular sodium content was indistinguishable between aldosterone-exposed and control cells (Fig. 12).

**DISCUSSION**

Both mineralocorticoid (MR) and glucocorticoid hormone receptors are expressed in the rat heart, each exhibiting differential tissue-specific expression between the atria and ventricle and during cardiac development (Funder et al., 1973; Pearce and Funder, 1987; Arriza et al., 1987). The physiologic role of each receptor, or their interactions, in cardiac development and function is not known. We have established for the first time that physiologic concentrations of the mineralocorticoid hormone aldosterone can regulate the expression
of the major cardiac Na+,K+-ATPase isofrom gene in both neonatal and adult cardiocytes. As both adult and neonatal cardiocytes were grown in defined serum-free media, we have established that aldosterone requires no other hormonal or serum growth factors to induce Na+,K+-ATPase α-1 or β-1 gene expression, with the possible exception of insulin. Our results demonstrate at both the protein and mRNA level that a cardiac gene, and by extrapolation the heart itself, is a specific target for mineralocorticoid hormone-mediated regulation. As such, it supports earlier observations linking aldosterone with regulation of cardiac Na+,K+-ATPase activity in intact rat (Hegyvary, 1977). As the α-1 subunit encodes the major Na+,K+-ATPase isofrom expressed in the heart throughout development, this result implies a potentially direct regulation of cardiac gene expression and, by extrapolation, cardiac function by the renin-angiotensin-aldosterone axis.

Several criteria are met demonstrating that the cardiac MR type I receptor is mediating the induction of α-1 gene expression. In serum-free media, physiological concentrations of aldosterone alone were sufficient to induce the α-1 gene. The concentration-effect curve was steep suggesting binding to a single high-affinity receptor site with an EC50 of 1–2 nM in both neonatal and adult cardiocytes. This correlates well with the reported Kd values of aldosterone binding to MR ranging from 0.5 to 3 nM (Marver, 1985; Fanestil and Park, 1981). It is unlikely that this effect is mediated by the type II glucocorticoid receptors also expressed in the heart. Aldosterone does bind to the type II glucocorticoid receptor, although with a much higher Kd of 25–65 nM (Marver, 1985; Fanestil and Park, 1981). The functional consequence of this is that saturating concentrations for the MR type I receptor, aldosterone does not activate type II GR receptor-mediated transcription of the hormonally responsive mouse mammary tumor virus promoter (Arriza et al., 1987). Activation of the GR type II receptors by exposure of cardiocytes to the potent synthetic glucocorticoid dexamethasone was without effect on either α-1 or β-1 gene expression (Orlowski and Lingrel, 1990). Thus, cardiac Na+,K+-ATPase α-1 gene expression appears to be regulated by a type I, and not a type II, receptor-mediated process. This extends the observations of aldosterone-mediated Na+,K+-ATPase gene expression from polarized transport epithelial cells to a new class of mineralocorticoid receptor-containing cells.

An important finding of these studies is that aldosterone, in the absence of other hormones or growth factors, is alone sufficient to induce α-1 and β-1 gene expression in cardiocytes. Previous studies have focused on models of highly specialized polarized epithelial cells involved in the unidirectional transport of Na+ and K+. Cultured toad bladder epithelial cells induce the expression of Na+,K+-ATPase α and β mRNA, protein, and transport activity in response to aldosterone (Verrey et al., 1987). Although, these experiments were done in serum containing medium and thus could not definitively eliminate other hormonal or growth factor effects. The interaction of aldosterone with other hormones and growth factors may regulate Na+,K+-ATPase gene expression in a tissue-specific manner and thus explain some conflicting data in the literature. For example, in mammalian renal tubule cells, thyroid hormone has a synergistic permissive effect on aldosterone-mediated induction of Na+,K+-ATPase gene expression (Barlett-Bas et al., 1988), whereas in toad bladder epithelium the effect is the opposite, with thyroid hormone inhibiting aldosterone-mediated Na+,K+-ATPase activity (Pratt and Johnson, 1984). We have presented data that in rat cardiocytes, aldosterone can function in the absence of thyroid hormone. Although the interaction of aldosterone with thyroid hormone in regulating cardiac Na+,K+-ATPase α-1 isofrom gene expression has not been directly studied, thyroid hormone has been reported to play a role in modulating the response of the Na+,K+-ATPase α-2 and α-3 isofroms to the synthetic glucocorticoid, dexamethasone, in cultured rat neonatal cardiocytes (Orlowski and Lingrel, 1990). Recently, tissue-specific positive and negative transcriptional regulatory elements have been identified in the upstream regulatory region of the rat α-1 gene promoter (Medford and Olliff, 1990; Allen, manuscript in preparation). Although additional mechanisms may be functioning, these transcriptional elements may play a role by interacting with thyroid and steroid hormone responsive elements of the α-1 promoter to effect the tissue-specific regulatory effects observed.

Our studies in cardiocytes demonstrate that regulation of α-1 mRNA synthesis is a major determinant in regulating Na+,K+-ATPase α-1 mRNA and, in adult cardiocytes, protein accumulation, as well as function. Various approaches have been employed to quantitate Na+,K+-ATPase α subunit binding sites. Although [1H]ouabain binding is often used, this approach is difficult in neonatal rat cardiac tissue because of the relatively low ouabain binding affinity of the rat α-1 subunit. Antibody-labeled flow cytometric analysis allows for an accurate measurement of changes in total Na+,K+-ATPase α subunit protein content per cell. As the monoclonal antibody used 9-A5 (Schenk et al., 1984) is specific for the α-2 subunit, we have demonstrated an aldosterone-mediated induction in α-2 protein accumulation per cardiocyte. After 72 h, a 36% increase in cellular α-2 protein content was measured. It is likely that this represents a specific increase in the expression of Na+,K+-ATPase α-1 protein in the cardiocyte.

We have determined for the first time the surprisingly short half-life of α-1 mRNA in post-mitotic cardiac cells. By actinomycin D chase, a 2–3-h half-life was measured. It has been shown that the potent RNA polymerase inhibitor actinomycin D can be used to determine mRNA turnover without grossly affecting post-transcriptional steps leading to mRNA degradation (Dani et al., 1984; Medford et al., 1983). In parallel experiments, β-actin mRNA half-life was measured at the expected 18–25 h, suggesting no dramatic changes in general mRNA turnover mechanisms (data not shown). Our studies suggest that alteration in α-1 mRNA stability is not a major mechanism mediating the aldosterone effect. The short half-life of α-1 mRNA suggests two roles: 1) the ability to rapidly up- and down-regulate in response to appropriate regulatory signals and 2) the ability to maintain a constant intracellular concentration despite fluctuations in cellular growth rate (see Medford et al., 1983). This short mRNA half-life is in contrast to measured turnover rates of Na+,K+-ATPase protein in the rat kidney cortex with a calculated half-life of 3–4 days (Lo and Edelman, 1976). Although these studies may not be directly applicable to cardiac cells, it is likely that the half-life of the cardiac Na+,K+-ATPase membrane protein is at least 24 h. Thus, it is not surprising that a change in mRNA levels (3-fold) over the period of observation (48–72 h) exceeds the change in cellular Na+,K+-ATPase α-2 protein content (1.4-fold).

The role of protein synthesis during steroid induction has been investigated in a number of different experimental systems. For example, the induction of mouse mammary tumor virus by glucocorticoid occurs as a result of a 10-fold accumulation of mouse mammary tumor virus RNA, an effect that is not blocked by protein synthesis inhibitor (Ringoeld et al., 1985).
1977), suggesting a direct transcriptional control by the glucocorticoid receptor complex. In contrast, the induction of ovalbumin and conalbumin mRNA by estrogen and progesterone measured in chick oviduct explant cultures is fully blocked by cycloheximide or puromycin (McKnight, 1979). In this case, the induction of an intermediate (regulatory) protein by the steroid hormone has been proposed. In the present study, the aldosterone-dependent α-1 mRNA accumulation was shown not to be sensitive to cycloheximide. At the cycloheximide concentrations used, over 90% of cardiocyte protein synthesis measured by [35S]methionine uptake was inhibited (data not shown). These results suggest that aldosterone regulates directly α-1 mRNA synthesis and does not require an intermediate regulatory protein.

We have established that in cultured cardiocytes, aldosterone induces a transient increase in intracellular Na⁺ content and a concurrent sustained increase in Na⁺,K⁺-ATPase α-1 mRNA synthesis, both sensitive to amiloride. Considerable controversy surrounds the role of intracellular Na⁺ in the regulation of Na⁺,K⁺-ATPase gene expression by aldosterone. In several studies of amphibian and mammalian transport epithelial cells (Weigt et al., 1987; Stokes et al., 1981; Laskie and Kurtzman, 1983), the induction of Na⁺,K⁺-ATPase activity occurs in two distinct phases: a short-term early induction (<3 h) associated with increased apical Na⁺ entry and K⁺ permeability and a long-term late induction (>3 h) associated with an increase in basolateral Na⁺,K⁺-ATPase activity and pump sites. The early induction is sensitive to amiloride, suggesting that increased Na⁺,K⁺-ATPase activity is secondary to increased Na⁺ entry, which in turn is mediated via a Na⁺/H⁺ exchange mechanism. Consistent with this, early induction is not sensitive to either the protein synthesis inhibitor cycloheximide or the RNA polymerase inhibitor actinomycin D. In contrast, the late induction of Na⁺,K⁺-ATPase is sensitive to both cycloheximide and actinomycin D demonstrating a dependence on de novo mRNA and protein synthesis. However, both amiloride sensitivity (Verrey et al., 1987) and amiloride insensitivity (Barlet-Bas et al., 1988) have been reported for the late induction. Thus, there is no consensus on the dependence of the late response to changes in intracellular Na⁺.

The mechanism(s) by which the aldosterone-mediated transient increase in intracellular Na⁺ induces Na⁺,K⁺-ATPase α-1 mRNA synthesis are not known but could be mediated by a direct effect of Na⁺ on cell metabolism, changes in intracellular pH via Na⁺/H⁺ exchange, an incremental change in cell Ca²⁺ content through the Na⁺/Ca²⁺ exchanger, and/or other factors such as cyclic nucleotide metabolism (Walsh-Reitz et al., 1984). However, aldosterone maintained a 3-fold induction of α-1 mRNA levels for at least 48 h, even when intracellular Na⁺ returned to the control levels after 24 h. Given the short half-life of α-1 mRNA (τ₁/₂ = 3 h), the prolonged α-1 subunit gene induction suggests the possibility that α-1 mRNA expression at late stages (>24 h) is effected via a direct hormonal activation of transcription by aldosterone rather than an ionic effect.

Consistent with our results, an early study in intact rat models suggested that mineralocorticoids induce cardiac Na⁺,K⁺-ATPase activity (Hegyvary, 1977). However, after 8 weeks of treatment with the mineralocorticoid deoxycorticosterone (DOC), Herrera et al. (1988) found no changes in α-1 mRNA levels in left ventricular myocardium in nonadrenalectomized adult rats. Possible reasons why they could not detect an effect of DOC on α-1 subunit expression in intact rats are: 1) the effect of aldosterone occurs over the physiological range and other factors including thyroid hormone and growth factors also influence Na⁺,K⁺-ATPase gene expression in cardiocytes (Ikeda et al., 1988), therefore it may be difficult to demonstrate additive effects of DOC in rats without adrenalectomy. 2) In our study, removal of aldosterone from the culture medium resulted in a rapid de-induction of α-1 mRNA accumulation to the basal level within 6 h, therefore subcaneous biweekly injections of DOC to rats might not maintain sufficiently high plasma levels of DOC resulting in only a transient increase in α-1 mRNA accumulation.

Our data demonstrate that aldosterone modulates Na⁺,K⁺-ATPase gene expression by increasing the rate of α-1 mRNA synthesis, and likely gene transcription, in cultured cardiac cells. An aldosterone-mediated increase in Na⁺ influx via an amiloride-sensitive Na⁺/H⁺ exchange mechanism may provide an early signal for this effect. How these signals are transduced to the nucleus to alter gene expression are unknown. One intriguing possibility is recent preliminary data suggesting that the upstream transcriptional regulatory elements of the rat α-1 promoter contains interacting tissue-specific steroid hormone-responsive and Na⁺-sensitive elements (Medford and Ollif, 1990). The identification and characterization of these mineralocorticoid and Na⁺-responsive transcriptional regulatory proteins may provide important insights into the mechanisms mediating transmembrane signaling by adrenal corticoids hormones resulting in the regulation of the intracellular ion content of cardiac cells, and hence, cardiac excitability, contractility, and cellular volume.

Acknowledgments—We would like to thank Dr. K. Sweadner for generous use of antibody probes and expert advice, Dr. H. Leffert for the 9A-5 antibody, Dr. Margaret K. Offermann for patient and critical reading of the manuscript, and Mary O'Neil for excellent technical assistance.

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