Activation of Mitogen-activated Protein Kinase (Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase) Cascade by Aldosterone

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Aldosterone in some tissues increases expression of the mRNA encoding the small monomeric G protein Ki-RasA. Renal A6 epithelial cells were used to determine whether induction of Ki-*ras* leads to concomitant increases in the total as well as active levels of Ki-RasA and whether this then leads to subsequent activation of its effector mitogen-activated protein kinase (MAPK/extracellular signal-regulated kinase) cascade. The molecular basis and cellular consequences of this action were specifically investigated. We identified the intron 1-exon 1 region (rasI/E1) of the mouse Ki-*ras* gene as sufficient to reconstitute aldosterone responsiveness to a heterologous promoter. Aldosterone increased reporter gene activity containing rasI/E1 threefold. Aldosterone increased the absolute and GTP-bound levels of Ki-RasA by a similar extent, suggesting that activation resulted from mass action and not effects on GTP binding/hydrolysis rates. Aldosterone significantly increased Ki-RasA and MAPK activity as early as 15 min with activation peaking by 2 h and waning after 4 h. Inhibitors of transcription, translation, and a glucocorticoid receptor antagonist attenuated MAPK signaling. Similarly, rasI/E1-driven luciferase expression was sensitive to glucocorticoid receptor blockade. Overexpression of dominant-negative RasN17, addition of antisense Ki-*ras*A and inhibition of mitogen-activated protein kinase kinase also attenuated steroid-dependent increases in MAPK signaling. Thus, activation of MAPK by aldosterone is dependent, in part, on a genomic mechanism involving induction of Ki-*ras* transcription and subsequent activation of its downstream effectors. This genomic mechanism has a distinct time course from activation by traditional mitogens, such as serum, which affect the GTP-binding state and not absolute levels of Ras. The result of such a genomic mechanism is that peak activation of the MAPK cascade by adrenal corticosteroids is delayed but prolonged.

INTRODUCTION

Small, monomeric Ras GTP-binding proteins initiate pleiotropic signaling cascades to affect many aspects of cellular physiology. Ras signaling through the extracellular signal-regulated kinase (ERK) cascade mediated by mitogen-activated protein kinases (MAPKs) 1/2, for instance, is well documented to play a pivotal role in cellular growth and differentiation. Protein hormones, which target GTP exchange factors and GTPase-activating proteins via plasma membrane receptors, activate the MAPK cascade by increasing the GTP-bound state of Ras proteins and not the absolute levels of these proteins. Thus, the “classic” paradigm of Ras

→ MAPK signaling involves posttranslational control of Ras. Emerging evidence suggests that numerous steroids, including aldosterone, also affect Ras signaling (Spindler *et al.*, 1997; Mastroberardino *et al.*, 1998; Stockand *et al.*, 1999c). The molecular basis and end effect of this steroid action remain, for the most part, not well described. Because steroids control cell activity through receptors that function as *trans*-acting factors to modulate gene expression, it is possible that steroids act on the Ras signaling cascade via a “genomic” mechanism that is dependent on transcription and subsequent translation to increase Ras protein levels and thus, distinct from the classic mechanism. The current study, which investigated this possibility, identifies a novel paradigm by which corticosteroid activate the Ras → MAPK signaling cascade.

The adrenal cortical steroid hormone aldosterone is the major endocrine factor regulating Na⁺ and K⁺ homeostasis. Aldosterone, consequently, plays a central role in maintain-

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ing electrolyte and water balance (Verrey, 1995, 1999; Stockand, 2002). Aldosterone also plays a direct role in pathological remodeling of the heart, possibly by promoting fibrosis and cellular proliferation both of which are generally known to be impacted by Ras signaling via ERK cascades (Ramires *et al.*, 1998; Pitt *et al.*, 1999; Karlson *et al.*, 2000). Although the systemic effects and target tissues of aldosterone are well known, little is actually known about its cellular mechanisms of action.

Many integral membrane proteins involved in epithelial cell transport, such as the epithelial Na^+ channel (ENaC), apical membrane potassium channel, Na^+/Cl^- cotransporter, H^+/K^+ -ATPase, and Na^+/K^+ -ATPase are end effectors of aldosterone signaling (Verrey, 1995; Garty and Palmer, 1997; Binder *et al.*, 1999; Palmer, 1999; Rogerson and Fuller, 2000). Although aldosterone affects cell activity by modulating gene expression, the expression levels of these proteins involved in transport, however, are not themselves initially controlled by the steroid. This has led to the proposal that aldosterone must control expression of factors that initiate or impinge upon signal transduction.

Adrenal corticosteroids, including aldosterone, increase the levels of the small, monomeric GTP-binding protein Kirsten Ras (Ki-Ras; Shekhar and Miller, 1994; Spindler *et al.*, 1997; Spindler and Verrey, 1999; Stockand *et al.*, 1999c). Aldosterone preferentially increases expression in epithelia of the A splice variant of Ki-Ras via control of transcription with induction of Ki-ras mRNA being a primary response to steroid that is independent of de novo protein synthesis and begins within 30 min after steroid addition. Induction of Ki-RasA is necessary and sufficient for aldosterone action, in part, on Na^+ transport (Stockand *et al.*, 1999c). In addition, Ki-RasA activates ENaC when both proteins are overexpressed in a heterologous system (Mastroberardino *et al.*, 1998) and increases the open probability of this channel in native epithelia (Stockand *et al.*, 1999c; Al-Baldawi *et al.*, 2000). Consequently, Ki-RasA is a likely candidate in some instances to transduce information from the nucleus to final effectors in response to aldosterone.

Induction of Ras expression by steroids may impact more than just ENaC and epithelial transport for glucocorticoids and estrogen increase Ras expression in mammary epithelia with enhanced expression possibly being associated with tumor formation and metastasis (Strawhecker *et al.*, 1989; Neades *et al.*, 1991; Shekhar and Miller, 1994; Pethe and Shekhar, 1999). Indeed, it has long been recognized that in cells that lack mutant Ras, elevated levels of normal Ras can lead to cell growth and/or transformation, presumably through inappropriate stimulation of Ras effector cascades (Schwab *et al.*, 1983; George *et al.*, 1986; Hoffman *et al.*, 1987). This suggests that through mass action, induction of Ras leads to activation of this protein and subsequent signaling.

The general consequences and in particular those associated with cell signaling of steroid-dependent induction of Ki-ras are not well understood. It also is not clear whether increases in Ki-RasA levels in response to aldosterone result from actions mediated by nuclear receptors and whether steroid-sensitive increases in Ki-RasA result in concomitant increases in functional GTP-bound Ki-RasA.

Similar to the other Ras proteins (Ha-Ras, N-Ras, and Ki-RasB), active Ki-RasA initiates many different intracellular signaling cascades, including the MAPK cascade. This

cascade is known to affect several aldosterone-target proteins, such as ENaC, Na^+/K^+ -ATPase, Na^+/H^+ exchanger, Na^+/Cl^- , and $\text{Na}^+/\text{bicarbonate}$ cotransport; and $\text{Na}^+/\text{Ca}^{2+}$ exchange proteins (Cho *et al.*, 1998; Zentner *et al.*, 1998; Lin *et al.*, 1999; Wang *et al.*, 1999; Pesce *et al.*, 2000; Turner *et al.*, 2000; Guerrero *et al.*, 2001; Robey *et al.*, 2001). Thus, the MAPK cascade may play a pivotal role in signaling aldosterone action secondarily to stimulation of Ki-RasA or may ultimately be involved in a negative feedback pathway initiated by this steroid.

The current work tested the hypothesis that aldosterone-stimulated Ki-RasA activates the MAPK cascade in renal epithelia. In addition, we asked whether Ki-RasA and the MAPK cascade are activated in response to aldosterone via nuclear steroid receptors, and whether increases in Ki-RasA expression in response to steroid result in increases in functional Ki-RasA:GTP levels. Through the course of this work, we also investigated possible molecular mechanisms by which aldosterone induces Ki-Ras expression and compared aldosterone effects on Ki-Ras $\rightarrow$ MAPK signaling with that of a traditional mitogen, such as serum.

EXPERIMENTAL PROCEDURES

Cell Culture

All experiments were performed with renal A6 epithelial cells (passages 75–81; American Type Culture Collection, Manassas, VA). Cells were cultured on polycarbonate supports (Transwell-Clear Inserts, pore size $0.4\ \mu\text{M}$, growth area $4.7\ \text{cm}^2$; Costar, Cambridge, MA) and allowed to form polar monolayers by using standard methods described previously (Stockand *et al.*, 1999a,b, c, 2000). In brief, cells were maintained at 26°C in 4% CO_2 with complete amphibian medium (3/10 Coon's F-12, 7/10 Leibovitz's L-15) supplemented with fetal bovine serum (10%). Basic medium was devoid of serum and aldosterone. High-resistance ($>2\ \text{K}\Omega$), polarized A6 cell monolayers were used for all experiments. To observe the full action of aldosterone, confluent cells were treated with basic media for 48 to 72 h before experimentation.

Molecular Biology

Plasmid Preparation and Isolation of Ki-ras Intron 1-Exon 1. The pMrasDN plasmid was a kind gift from Dr. G. Firestone (University of California at Berkeley, Berkeley, CA). In brief, this construct allows glucocorticoid-inducible expression of dominant-negative Ha-RasN17. Similar to that described previously by the Firestone laboratory for Con8 rat mammary epithelial cells (Woo *et al.*, 1999), this construct in conjunction with G418 selection was used to create clonal A6 cell lines stably expressing inducible dominant-negative RasN17.

The firefly luciferase reporter plasmid pGL2-TK was generated by subcloning the minimal herpes simplex virus thymidine kinase promoter from pRL-TK (Promega, Madison, WI) into pGL2 Basic Vector (Promega) with *Hind*III and *Bgl*II. (pGL2-TK was a kind gift from Dr. A. Firulli, University of Texas, San Antonio, TX.) The control pRL-CMV plasmid contains the cytomegalovirus promoter upstream of *Renilla* luciferase (Promega).

Mouse c-Ki-ras2 exon 1 plus its 5'-flanking region (intron 1-exon 1 region; nucleic acids –165–153 as labeled from the adenosine of the translation start codon ATG in exon 1; see GenBank accession numbers K01927, 52798, S39586, and M13294; George *et al.*, 1986; Hoffman *et al.*, 1987) were amplified with a standard polymerase chain reaction by using mouse whole tail genomic DNA and the 5'-ATGCGGTACCGACTTACAGGTTACTC (incorporating *Kpn*I site, underlined) and 5'-GCATCTCGAGCTGCCGTCTTTA-

CAAGCG (incorporating *Xho*I site, underlined) upstream and downstream primers, respectively. The 321-base pair product from this polymerase chain reaction was subcloned into pGL2-TK with *Kpn*I and *Xho*I to produce pGL2-TK-ras1/E1.

Luciferase Reporter Gene Assay. A quantitative assay with a *Renilla* luciferase internal control was used to measure the firefly luminescent signal in A6 cells overexpressing reporter genes. In brief, A6 cells plated at 80% confluence on 100- × 20-mm² culture dishes were transfected with 100 ng of pRL-CMV in addition to 3 μg of the firefly luciferase reporter plasmid (either pGL2-TK or pGL2-TK-ras1/E1) by using the LipofectAMINE Plus (Invitrogen, Carlsbad, CA) system per the manufacturer's instructions with the exception that cells were exposed to transfection reagents for ~8 h. Twenty-four hours after transfection and 24 h before performing assay, cells were replated in a 96-well culture plate. Luciferase activity then was measured with the Dual-Luciferase Reporter assay system (Promega) per the manufacturer's instructions directly following the experimental treatment period (i.e., exposure to 1.5 μM aldosterone for 4 h) and 1-h extract preparation period required with passive lysis buffer (see Dual-Luciferase Reporter instructions). An MLX microtiter plate luminometer (Dynatech Labs, Chantilly, VA) was used to record luminescent signal. For these experiments, all firefly luciferase activity data are normalized to the internal *Renilla* luciferase control.

Biochemistry

Western Blot Analysis. Whole A6 cell lysate was extracted after three washes with Tris-buffered saline by using standard procedures (Stockand *et al.*, 1999c). Cells were scraped and then maintained for 1–2 h at 4°C in gentle lysis buffer (GLB) (76 mM NaCl, 50 mM HCl-Tris, 2 mM EGTA plus 1% Nonidet P-40, and 10% glycerol, pH 7.4) and protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, tosylphenylalanyl chloromethyl ketone, and 1-chloro-3-tosylamido-7-amino-2-heptanone). For Western blot analysis of phosphorylated proteins, GLB was supplemented with 0.1 mM NaPP_i, 0.5 mM NaF, 0.1 mM Na₂MoO₄, 0.1 mM ZnCl₂, and 0.04 mM Na₃VO₄ prepared fresh from 1000× stocks. After clearing cellular debris, standardizing total protein concentration, and addition of Laemmli sample buffer (0.005% bromophenol blue, 10% glycerol, 3% SDS, 1 mM EDTA, 77 mM HCl-Tris, and 20 mM dithiothreitol), lysates were heated to 85°C for 10 min. Proteins were then separated by standard SDS-PAGE and subsequently electrophoretically transferred to nitrocellulose (0.2 μM). Western blot analysis was performed using standard techniques and appropriate antibodies (Stockand *et al.*, 1999a,b,c, 2000; see below; primary and secondary antibodies were used at 1/1000 and 1/20000, respectively). Tween 20 (0.1%) and 5% dried milk (Carnation) were used as blocking reagents. Band intensity was quantified with densitometric scanning using Sigmagel (Jandel Scientific, Costa Madre, CA). When possible, the flood configuration with the highest practical threshold was used to measure band density.

Western blots were often stripped of primary and secondary antibody to subsequently reprobe with a control antibody. All Western blots were stripped in 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.7, and 2% SDS for 30 min at 55°C with constant agitation. After removal of antibodies, nonspecific interactions were reblocked by incubating in TBS-Tween, 5% milk for 2 h before reprobing with primary antibody.

Ras:GTP Assay. Raf-1 RBD agarose was from Upstate Biotechnology (Lake placid, NY). This immobilized fusion protein corresponds to the human Raf-1 Ras binding domain (RBD) (residues 1–149). Raf-1 RBD binds Ras complexed with GTP. Pull-down experiments were performed in 400 μl (0.4 mg of total protein) of whole A6 cell lysate isolated with GLB. Lysates were incubated with 30 μl of Raf-1 RBD agarose overnight (at 4°C and with constant agitation); pellets were washed five times with 2 volumes of fresh GLB each time for

a total wash time of 2 h; and after resuspending in sample buffer and heating, Raf-1 RBD agarose precipitated proteins were separated by SDS-PAGE and Ki-RasA:GTP identified by immunoblotting.

MAPK Assay. MAPK activity in lysates prepared from cells treated with and without aldosterone was assayed by quantifying phosphorylation of exogenous myelin basic protein (MBP). MAPK activity was measured in whole A6 cell lysate (2 mg/ml) extracted in the presence of phosphatase inhibitors as described above. MAPK activity was measured for 30 min at 30°C in the following assay dilution buffer (ADB; Upstate Biotechnology): 20 mM MOPS pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 0.4 mM MnCl₂, 0.4 mM CaCl₂, 1 mM sodium orthovanadate, and 1 mM dithiothreitol. The final reaction contained 10 μl of substrate cocktail (from stock of 2 mg/ml dephosphorylated MBP in ADB), 10 μl of inhibitor cocktail (from stock of 20 μM PKC inhibitor peptide, 2 μM PKA inhibitor peptide, and 20 μM compound R24571 in ADB), 10 μl of A6 cell extract, and 10 μl of Mg²⁺/ATP cocktail (from a stock of 75 mM MgCl₂, 500 μM ATP in ADB). Reactions were initiated with Mg²⁺/ATP and terminated with Laemmli sample buffer (described above). Phosphorylation of MBP (in 10 μl of final reaction) was assessed after SDS-PAGE by immunoblot analysis with a specific anti-phospho-MBP antibody.

Electrophysiology

Trans epithelial Na⁺ current was calculated as described previously (Stockand *et al.*, 1999a,b,c, 2000), from Ohm's law as the ratio of transepithelial voltage to transepithelial resistance under open circuit conditions by using a Millicel Electrical Resistance System with dual Ag/AgCl pellet electrodes (Millipore, Bedford, MA) to measure voltage and resistance.

Materials

All reagents unless indicated otherwise were from either BIOMOL Research Laboratories (Plymouth Meeting, PA), Calbiochem (San Diego, CA), Invitrogen, or Sigma-Aldrich (St. Louis, MO). Phosphorothiate oligonucleotides were synthesized by the Emory University Microchemical Facility and stored frozen as 10 mM (in water) stocks. Aldosterone, dexamethasone, and mifepristone (RU486) were stored frozen as 1.5, 0.1, and 1.0 mM (in dimethyl sulfoxide [DMSO]) stocks. Cycloheximide (in MeOH) and emetine (in H₂O) were stored frozen as 1.0-mg/ml stocks. Actinomycin D was stored at 4°C as a 1.0-ng/ml (in MeOH) stock. PD-98059 and U-0126 were prepared fresh (in DMSO) before each experiment at stock concentrations of 10 and 5 mM, respectively. All reagents used for Western blot analysis unless noted otherwise were from Bio-Rad (Hercules, CA) and Pierce Chemical (Rockford, IL). For each lysate, protein concentration was determined with the bicinchoninic acid protein assay. Kodak BioMax Light-1 film and Chemiluminescence Reagents Plus (PerkinElmer Life Sciences, Boston, MA) were used to develop Western blots.

Antibodies

The rabbit polyclonal anti-MAPK 1/2 (Erk 1/2-CT) antibody was from Upstate Biotechnology. The mouse monoclonal anti-c-Raf-1 antibody was from Transduction Laboratories (Lexington, KY). The rabbit polyclonal anti-MKP-1 (V-15; MAPK phosphatase), anti-Fra-2 (L-15), and anti-K-Ras2A antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). This latter antibody recognizes only the Ki-RasA isoform of Ras proteins. The mouse monoclonal anti-v-Ha-Ras antibody was from Oncogene Science (Cambridge, MA). This antibody recognizes all isoforms of Ras protein, including Ha-Ras, Ki-RasA, Ki-RasB, and N-Ras. All phospho-specific antibodies were from Cell Signaling Technologies (Beverly, MA). All secondary horseradish peroxidase-conjugated antibodies were from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

Figure 1. Aldosterone increases active, GTP-bound Ki-RasA levels. (A) Western blot analysis of total Ras (top), Ki-RasA (middle), and Ki-RasA: GTP (bottom) in A6 cells treated without (CON) and with ALDO for 3 h. The representative blot probed for Ki-RasA: GTP (bottom) contained the precipitant from whole cell lysate by using Raf-RBD agarose, which specifically binds GTP-bound Ras. Blot probed with anti-Ki-RasA antibody to detect Ki-RasA: GTP. (B) Summary graph showing the relative changes in the levels of Ras, Ki-RasA and Ki-RasA: GTP in response to aldosterone treatment for 3–4 h. * indicates significantly greater relative increases for Ki-RasA and Ki-RasA: GTP vs. Ras.

Statistics

All values reported as mean $\pm$ SEM. Statistical significance ($p \leq 0.05$) was determined using the t test for differences in mean values, and a one-way analysis of variance in conjunction with the Student-Newman-Keuls test for multiple comparisons.

RESULTS

Aldosterone Increases Absolute and Active GTP-bound Levels of Ki-RasA

Aldosterone via transcriptional control increases Ki-rasA mRNA (Spindler *et al.*, 1997) and Ki-RasA protein (Stockand *et al.*, 1999c) levels in renal A6 epithelial cells. Experiments in Figure 1 tested the hypothesis that aldosterone in these cells also increases the amount of active Ki-RasA. Activated Ras bound by GTP interacts with the RBD of Raf (de Rooij and Bos, 1997; Foschi *et al.*, 1997). The representative Western blots of Figure 1A show that the addition of aldosterone for 3 h to A6 cell monolayers markedly increased Ki-RasA (middle) and Ki-RasA:GTP levels (bottom), but had little effect on total Ras levels (top). It is known that Ki-RasA is expressed at levels much lower than other Ras isoforms (Hoffman *et al.*, 1987; Pells *et al.*, 1997). Thus, the lack of a marked change in total Ras was not unexpected. For these experiments, Ras:GTP was isolated using GST-RBD agarose from whole cell lysates from cells treated with vehicle (CON; 0.1% DMSO) and aldosterone (ALDO; 1.5 μ M) for 3 h. After isolating total cellular Ras:GTP, Ki-RasA:GTP was identified with anti-K-Ras2A antibody, which reacts only with Ki-RasA (Pells *et al.*, 1997). Total Ras protein was identified with the anti-v-Ha-Ras antibody, which is reactive with Ki-RasA and B, Ha-Ras, and N-Ras isoforms. The summary graph in Figure 1B shows the relative change in response to aldosterone for the levels of Ras (1.6 ± 0.2 , $n = 11$), Ki-RasA (3.1 ± 0.4 , $n = 8$), and Ki-RasA:GTP (2.8 ± 0.4 , $n = 8$). Aldosterone, compared with vehicle, significantly increased Ki-RasA, and Ki-RasA:

Figure 2. Aldosterone increases MAPK activity. (A and B) MAPK activity was assessed by measuring phosphorylation of exogenous MBP. Exogenous MBP was added to equal amounts of A6 cell lysate from cells treated with aldosterone for the indicated times (in minutes, A; and hours, B). These typical Western blots were probed with anti-phospho-MBP antibody. (C) Summary graph of such experiments. * $p \leq 0.05$ vs. time 0.

GTP levels ($p < 0.005$ for both). Although aldosterone also significantly increased total Ras levels ($p = 0.03$) at a similar time point, as reported previously by our laboratory (Al-Baldawi *et al.*, 2000), the relative increase in total Ras was significantly less than that of Ki-RasA ($p = 0.002$) and Ki-RasA:GTP ($p = 0.009$). Importantly, the relative changes in Ki-RasA vs. Ki-RasA:GTP levels were not different ($p = 0.60$).

Aldosterone Increases MAPK Activity

One potential signaling pathway activated by Ki-RasA is the MAPK cascade. Aldosterone stimulation of MAPK activity in A6 cells was measured in an *in vitro* reaction by following phosphorylation of exogenous MBP. The typical Western blots in Figure 2, A and B, showing the time course of aldosterone stimulation of MAPK activity, were probed with anti-phospho-MBP antibody. For these blots, each lane contained 3.5 μ g of MBP processed for 30 min at 30°C by A6 whole cell lysate (equal concentrations of total cellular protein; supplemented with MBP, Mg^{2+} /ATP, and protein kinase C, protein kinase A, and calcium-calmodulin-dependent protein kinase II, and phosphatase inhibitors) from cells treated with aldosterone for the indicated times (in minutes for Figure 2A and hours for Figure 2B). The bar graph in Figure 2C summarizes such experiments. Aldosterone significantly increased MAPK activity 6.6 ± 1.2 -, 10.5 ± 1.8 -, 11.5 ± 2.5 -, 12.0 ± 4.2 -, 7.1 ± 2.8 -, and 3.1 ± 0.9 -fold at the 15 and 30 min, and 1-, 2-, 4-, and 6-h time points ($n \geq 4$). At 1 and 5 min, MAPK activity was 1.4 ± 0.2 - and $3.3 \pm$

Figure 3. Aldosterone compared with serum activates MAPK signaling in a delayed manner. (A) Western blots of A6 whole cell lysate from cells treated with serum (10% FBS, top), aldosterone (1.5 μ M), and aldosterone plus RU486 (0.1 μ M) for the indicated time in minutes. All blots probed with anti-phospho-MAPK antibody. (B) Blots in A stripped and reprobbed with anti-MAPK antibody. (C) Western blot of MBP processed by A6 whole cell lysates from cells treated with serum (10% FBS) for the indicated times in minutes. Blot probed with anti-phospho-MBP antibody.

0.8-fold higher ($n = 2$), respectively. MAPK activity in response to aldosterone increased steadily peaking between 0.5 and 2 h and waning after 4 h.

Time Course of Aldosterone Activation of MAPK Is Distinct from That of Classic Mitogens

Results in Figure 2 suggested that compared with classic mitogens, aldosterone activates MAPK signaling in a distinct manner, taking longer (up to 1–2 h) to reach maximal activity and having a persistent signal (up to 4 h). Such a time course would be consistent with aldosterone-dependent activation of the MAPK requiring a latent period necessary for transcription and translation of Ki-RasA. Experiments in Figure 3 were performed to determine whether the time course of aldosterone-dependent activation (phosphorylation) of MAPK in A6 cells was indeed distinct from activation by the traditional mitogen, serum. For these experiments, determining phospho-MAPK levels assessed activation of MAPK. The Western blots in Figure 3A were probed with anti-phospho-MAPK antibody. These blots contained equal amounts of whole cell lysate from A6 cells treated with serum (10% FBS; top) and aldosterone (1.5 μ M) in the absence (middle) and presence (bottom) of the corticosteroid receptor antagonist RU486 (mifepristone; 0.1 μ M) for the indicated times in minutes. Western blots in Figure 3A were stripped and subsequently reprobbed with anti-MAPK antibody (Figure 3B, same order). Serum and aldosterone clearly have temporally distinct effects on activation

Figure 4. Aldosterone stimulates MAPK signaling in A6 cells through induction of gene expression mediated by the glucocorticoid receptor. (A) Western blot containing A6 whole cell lysate from cells treated with aldosterone (1.5 μ M), CON, actinomycin D (1 μ g/ml), cycloheximide (1 μ g/ml), and aldosterone plus actinomycin D or cycloheximide for 2 h. This blot was probed with anti-phospho-MAPK antibody (top), stripped, and then reprobbed with anti-MAPK antibody (bottom). (B) This typical Western blot shows phosphorylation of exogenous MBP added to A6 cell lysate from cells treated with aldosterone, and steroid plus RU486 (0.1 μ M), emetine (1 μ g/ml), cycloheximide (1 μ g/ml), PD-98059 (10 μ M), and U-0126 (0.5 μ M) for 2 h. This typical blot was probed with anti-phospho-MBP antibody. (C) Below is a summary graph of five such experiments showing relative MAPK activity.

of MAPK with the effects of serum peaking in the first 15–30 min and waning thereafter, whereas those of aldosterone rising from 30 min onwards. The actions of aldosterone on MAPK were completely reversed by RU486, suggesting that this steroid stimulates MAPK signaling via its genomic actions. As shown in Figure 3C, similar results were observed when MAPK activity was assessed in an in vitro reaction by following phosphorylation of exogenous MBP. This blot was probed with anti-phospho-MBP antibody and each lane contained 3.5 μ g of MBP processed by A6 whole cell lysate (equal concentrations of total cellular protein) from cells treated with serum (10% FBS) for the indicated times (in minutes).

Persistent Stimulation of MAPK by Aldosterone Is Mediated by Nuclear Steroid Receptors

Experiments in Figure 4 were performed to further characterize the relation of aldosterone-stimulated MAPK at the 2-h time point with the genomic effects of this steroid. Both the effects of aldosterone on activation (phosphorylation) of MAPK in the presence of inhibitors of transcription and translation (Figure 4A), and the effects of aldosterone on

MAPK activity in the presence of inhibitors of nuclear corticosteroid receptors, translation and mitogen-activated protein kinase kinase (MEK) (Figures 4, B and C) were determined.

The typical Western blot of Figure 4A was probed with anti-phospho-MAPK antibody (top), stripped, and then re-probed with anti-MAPK antibody (bottom). This blot contained equal amounts of A6 whole cell lysate from cells treated with ALDO (1.5 μ M), CON (vehicle), actinomycin D (1.0 μ g/ml), cycloheximide (1.0 μ g/ml), and aldosterone plus actinomycin D or cycloheximide for 2 h. Although actinomycin D and cycloheximide had no overt effect on activation of MAPK when added alone, when added simultaneously with aldosterone, they abolished steroid-dependent activation of MAPK, demonstrating that transcription and translation are necessary for aldosterone to activate MAPK signaling.

The typical Western blot of Figure 4B shows phosphorylation of exogenous MBP added to A6 cell lysate from cells treated with aldosterone for 2 h in the absence and presence of the corticosteroid receptor inhibitor RU486 (mifepristone; 0.1 μ M) and inhibitors of translation (cycloheximide and emetine 1.0 μ g/ml) and MEK (PD-98059 and U-0126 at 10 and 0.5 μ M, respectively). For these experiments, inhibitors were added simultaneously with aldosterone. This Western blot was probed with anti-phospho-MBP antibody and contained equal amounts of exogenous MBP processed by the respective A6 cell lysate (equal concentration of total protein). As shown in the summary graph of Figure 4C, RU486 significantly decreased relative aldosterone-stimulated MAPK activity to 0.2 ± 0.04 ($n = 5$). Similarly, relative aldosterone-induced MAPK activity was decreased to 0.2 ± 0.1 ($n = 5$) and 0.3 ± 0.2 ($n = 4$) by emetine and cycloheximide, respectively. Moreover, PD-98059 and U-0126 decreased relative aldosterone-sensitive MAPK activity to 0.2 ± 0.1 ($n = 5$) and 0.2 ± 0.1 ($n = 5$), respectively. At this time point (2 h), the negative control for U-0126, U-0124, did not affect MAPK signaling and Ki-RasA levels were 53, 61, 62, 96, and 108% of (aldosterone-treated) control in the RU486, emetine, cycloheximide, PD-98059, and U-0126 groups, respectively (our unpublished data).

Ki-ras Gene Contains a Functional Steroid Response Element That Confers Aldosterone Responsiveness to a Heterologous Promotor

The results described above and those reported previously by us (Stockand *et al.*, 1999c) and others (Spindler *et al.*, 1997; Spindler and Verrey, 1999) suggest that aldosterone via steroid receptors directly affects *Ki-ras* expression and that this then impinges upon MAPK signaling. However, the molecular basis of this regulation has not been studied. Glucocorticoids and aldosterone ultimately target similar *cis*-acting elements through either the glucocorticoid or mineralocorticoid receptor (reviewed in Stockand, 2002). The human and rat *Ki-ras* genes contain partially characterized *cis*-acting elements within the intron 1-exon 1 region that are *trans*-activated by the glucocorticoid-steroid receptor complex (Shekhar and Miller, 1994). Conserved elements have similarly been identified in the *Ha-ras* gene as responsive to glucocorticoids (Strawhecker *et al.*, 1989; Neades *et al.*, 1991; Shekhar and Miller, 1994; Pethe and Shekhar, 1999). Using

Figure 5. *rasI/E1* region of *c-Ki-ras2* is sufficient for steroid responsiveness. A6 cells were transiently transfected with pRL-CMV plus either control reporter plasmid (pGL2-TK), which contained firefly luciferase expression driven by the minimal thymidine kinase promoter, or reporter plasmid that contained the *rasI/E1* region (pGL2-TK-*rasI/E1*). Luciferase activity in cells treated with vehicle, aldosterone, and aldosterone plus RU486 was quantified 48 h after transfection. * $p < 0.05$ vs. pGL2-TK treated with aldosterone and vehicle, and pGL2-TK-*rasI/E1* treated with vehicle.

this paradigm, we prepared a reporter plasmid containing the mouse *c-Ki-ras2* intron 1-exon 1 region (–165–153 from adenosine of the translation start codon within exon 1; pGL2-TK-*rasI/E1*), which contains several putative steroid response elements, and as shown in Figure 5, tested whether this region conferred functional aldosterone responsiveness to a heterologous promoter in A6 epithelial cells. Luciferase activity in cells transfected with pGL2-TK-*rasI/E1* in the presence of vehicle and aldosterone (1.5 μ M, 4 h) was 1.5 ± 0.3 and 4.6 ± 0.9 ($n = 6$), respectively. Thus, aldosterone significantly increased (threefold) luciferase expression driven by the pGL2-TK-*rasI/E1* chimeric reporter plasmid. Simultaneous addition of RU486 (0.1 μ M, $n = 3$) with aldosterone markedly decreased luciferase activity 50% to 2.3 ± 0.9 in cells transfected with pGL2-TK-*rasI/E1*. Dexamethasone (DEX) (0.1 μ M, 4 h) had a similar effect as aldosterone increasing luciferase activity vs. vehicle 2.5-fold to 3.7 ± 0.9 ($n = 3$; $p = 0.06$; our unpublished data). Luciferase activity in the presence of either steroid in cells transfected with pGL2-TK-*rasI/E1* was significantly greater ($p > 0.05$) than that in cells transfected with pGL2-TK and treated similarly (ALDO = 1.0 ± 0.2 , $n = 6$; DEX = 0.7 ± 0.2 , $n = 3$). In contrast, luciferase activity in the presence of vehicle was not different ($p = 0.3$) between pGL2-TK (0.8 ± 0.3 , $n = 4$) and pGL2-TK-*rasI/E1*-transfected cells. Reporter gene activity in cells transfected with the minimal promoter thymidine kinase luciferase plasmid (pGL2-TK) or pRL-CMV control plasmid alone was unaffected by steroid treatment (our unpublished data).

Activation of MAPK Cascade by Aldosterone Is Dependent on Ki-RasA Expression

Although all of the above-reported results support the idea that aldosterone activates *Ki-RasA* via transcriptional control mediated by nuclear steroid receptors, it is unclear

Figure 6. Aldosterone activates the MAPK cascade through induction of Ki-RasA. (A) These typical Western blots show the effects of aldosterone on Ki-RasA (top), phospho-Raf (top middle), active (phospho)-MAPK (bottom middle), and absolute MAPK (bottom) levels in A6 cells treated with steroid alone (vehicle) and in the presence of sense (SENSE) and antisense (ANTI) Ki-ras oligonucleotide. (B) Typical Western blot probed with anti-Ras antibody containing equal amounts of whole cell lysate extracted from cells stably expressing inducible DNRasN17 treated with vehicle (CON) and aldosterone for 2 h. (C) Typical Western blot probed with anti-phospho-MAPK (top) and then stripped and reprobed with anti-MAPK (bottom) antibodies containing equal amounts of whole cell lysate extracted from control cells and cells stably expressing inducible DNRasN17 treated with vehicle (–) and aldosterone (+) for 2 h.

whether the aldosterone-sensitive MAPK signaling reported in Figures 2–4 is in fact dependent on induction of Ki-RasA. Experiments in Figure 6 directly test the link between aldosterone-dependent induction of Ki-RasA and steroid-dependent activation of MAPK signaling. The typical Western blots in Figure 6A are of lysates extracted from cells treated with aldosterone (1.5 μ M; control) and aldosterone after pretreatment (24 h, 10 μ M) with sense and antisense Ki-rasA oligonucleotides. Use of these oligonucleotides in A6 cells has been described previously (Stockand *et al.*, 1999c). The top blot in Figure 6A was probed with anti-K-Ras2A antibody and demonstrates the efficacy of the antisense oligonucleotide to decrease Ki-RasA levels. Ki-RasA levels in the antisense group were ~40% of those in the control and sense groups ($n = 6$). In response to Ras signaling, Raf and MAPK become phosphorylated. The top middle blot in Figure 6A was probed with anti-phospho-Raf antibody and demonstrates that the Ki-ras antisense oligonucleotide attenuates aldosterone-sensitive phosphorylation of Raf. Phospho-Raf levels in the antisense group were <20% of those in the control and sense groups ($n = 3$). Similarly, as shown by the bottom middle blot, antisense inhibited aldosterone-dependent activation of MAPK with phospho-MAPK levels in the antisense group being <25% of those in the control and sense groups ($n = 3$). In contrast, as shown by the bottom blot, antisense had no effect on total MAPK levels with all three groups having similar levels of MAPK.

The representative Western blot in Figure 6B shows the effects of aldosterone on two distinct clonal A6 cell lines (DNRas1 and DNRas2) stably expressing corticosteroid-inducible dominant-negative RasN17. For such experiments ($n = 2$), confluent cells were treated with vehicle (CON) or aldosterone (ALDO) for 2 h. This blot, which contains equal amounts of total protein for each lysate, was probed with anti-Ras antibody and demonstrates that aldosterone increases total Ras levels in these clonal lines. Because aldosterone has little effect on total Ras expression in untransfected A6 cells (Figure 1A), these results demonstrate that these clonal lines stably express corticosteroid-inducible DNRasN17. The effects of aldosterone on activation of MAPK in these two clonal lines were determined next (Figure 6C). This typical Western blot ($n = 2$), which contained

lysates with equal amounts of total protein from control cells and cells stably expressing inducible DNRasN17 treated with vehicle (–) and aldosterone (+) for 2 h, was probed with anti-phospho-MAPK antibody (top). This blot was subsequently stripped and reprobed with anti-MAPK antibody (bottom). In A6 cells stably expressing inducible DNRasN17, aldosterone had less of an effect on activation of MAPK compared with untransfected cells. Interestingly, stable cells had elevated levels of phospho-MAPK in the absence of aldosterone with steroid actually decreasing these levels. This may reflect a feedback response to chronically depressed MAPK signaling due to DNRasN17 leak. Nonetheless, these results show that for aldosterone to stimulate MAPK signaling, Raf kinase must be available to Ki-RasA, which it is not in the presence of DNRasN17. These results also are consistent with those in Figure 6A and together suggest that Ki-RasA transduces the aldosterone signal onto the MAPK cascade.

Peak Activation of MAP Kinase Cascade by Aldosterone-stimulated Ki-RasA Is Delayed and Prolonged

The experiments in Figure 7 temporally map the actions of aldosterone on MAPK signaling at several discrete levels within the transduction cascade. The representative Western blot of Figure 7A shows that whereas aldosterone increases the amount of active (phosphorylated) MAPK, it does not affect the total cellular pool of MAPK. For these blots, each lane contained 50 μ g of total cellular protein, and the aldosterone (1.5 μ M) treatment time is indicated in hours. For this set of experiments, cells representing time 0 were washed with vehicle 2 h before extraction. No difference was observed between time zero and wash for these and all other experiments. The top blot in this experiment was stripped after being probed with anti-phospho-MAPK (ERK 1/2) antibody and subsequently reprobed with anti-MAPK 1/2 antibody (bottom blot).

The blots in Figure 7B show that similar to MAPK, aldosterone increases the levels of phospho-Raf compared with total cellular Raf. These blots contain lysate from cells treated with aldosterone for the indicate times (in hours).

Figure 7. Aldosterone activates the MAPK cascade in a delayed but prolonged manner. (A) Western blot analysis of aldosterone effects on MAPK and active (phosphorylated) MAPK levels in A6 cells. Top blot probed with anti-phospho-MAP kinase antibody. This blot was subsequently stripped and reprobed with anti-MAPK antibody (bottom). (B) Western blot analysis testing the effects of aldosterone on c-Raf and phosphorylated c-Raf. Top and bottom blots probed with anti-phospho-Raf and anti-Raf antibody, respectively. (C) Western blot analysis testing the effects of aldosterone on the MAPK cascade effectors, RSK (top), Fra-2 (middle), and MKP-1 (bottom). Top blot probed with anti-phospho-p90^{RSK} antibody; middle blot probed with anti-Fra-2 antibody; and bottom blot probed with anti-MKP-1 antibody. Each lane for blots in A–C contained equal amounts of whole A6 cell lysate extracted from cells treated with aldosterone for the indicated time in hours. (D and E) Summary graphs showing the time course of the relative (vs. time 0) actions of aldosterone on Ki-RasA, phospho-MAPK, MAPK, phospho-Raf, Raf, MKP-1, phospho-RSK, Fra-2, and Na⁺ transport.

Top and bottom blots were probed with anti-phospho-(Ser259)-Raf and anti-Raf antibody, respectively. Each lane contained ~50 μ g of total protein.

The actions of aldosterone on downstream effectors of the MAPK cascade are shown in Figure 7C. These Western blots are of the same lysates (similar to that for Raf and phospho-Raf in 7B) from the indicated time points after aldosterone treatment. Each lane contained 50 μ g of total protein. Top, middle, and bottom blots were probed with anti-phospho-RSK-1 (p90 ribosomal S6 kinase; also referred to as MAPKAP kinase-1), Fra-2, and MKP-1 antibody, respectively. Clearly, all three of these MAPK effector proteins are either activated/phosphorylated, as for RSK-1, or induced, for Fra-2 and MKP-1, in response to aldosterone.

The summary plots of Figure 7D show relative changes in Ki-RasA (inverted triangles), phospho-MAPK (diamonds), and MAPK (squares) levels in response to aldosterone at 0-, 0.5-, 1-, 2-, 4-, and 6-h time points. Also shown in this graph are the effects of aldosterone on Na⁺ transport (current, circles) across A6 cell monolayers at each time point. Figure 7E shows diary plots of relative changes in phospho-Raf (circles) and total Raf (gray triangles) in response to aldosterone. Also shown in this graph are the temporal actions of aldosterone on expression of the MAPK effectors Fra-2 (inverted triangles) and MKP-1 (diamonds). Included, in addition, are the effects of aldosterone on phosphorylation of the MAPK effector RSK-1 (squares). An expanded time course for aldosterone actions of effectors and effectors of MAPK is included in Table 1.

DISCUSSION

The present results support a novel mechanism whereby aldosterone induces Ki-RasA expression at the level of transcription and then through stoichiometric increases in the levels of active, GTP-complexed Ki-RasA stimulates the MAPK cascade. Figure 8 compares this genomic mechanism with the classic mechanism initiated by traditional mitogens. Traditional mitogens, such as serum, in contrast, stimulate MAPK signaling via a mechanism involving posttranslational control of active, GTP-complexed Ras levels without effects on absolute Ras levels. Compared with the classic mechanism, the distinct but possibly complimentary genomic mechanism activated by aldosterone leads to delayed but prolonged MAPK signaling. It is speculated that the delayed but prolonged MAPK signaling in response to steroids will differentially affect cellular activity compared with traditional mitogens.

Aldosterone to Ki-RasA

Aldosterone increases both Ki-RasA mRNA and protein levels in amphibian renal epithelial cells (Spindler *et al.*, 1997; Spindler and Verrey, 1999; Stockand *et al.*, 1999c). The current results in Figure 1 are consistent with this finding. The analogous findings in mammals, however, have been more controversial (reviewed in Loffing *et al.*, 2001; Stockand, 2002) with corticosteroids increasing Ki-ras and Ki-RasA expression in mammalian colonic and mammary epithelial cells (Strawhecker *et al.*, 1989; Neades *et al.*, 1991; Shekhar and Miller, 1994; Pethe and Shekhar, 1999; Fuller, personal

Table 1. Temporal effects of aldosterone on MAPK signaling and current

	Relative level after aldosterone addition at each time point (in h)*							
	0.5	1	2	3	4	6	8	24
Ki-RasA	2.3 ± 0.7	3.0 ± 0.8	3.9 ± 1.3	3.1 ± 0.4	3.2 ± 0.5	2.8 ± 0.7		
Raf	0.9	1.0	1.0		1.4	1.1		1.3
phospho-Raf	1.3 ± 0.3	2.1 ± 0.4	3.8 ± 1.0	4.8 ± 1.0	4.0 ± 1.2	5.1 ± 0.6		1.1 ± 0.07
phospho-MEK	3.2	3.7			4.1 ± 0.4			
MAPK	1.09 ± 0.04	0.9 ± 0.02	0.9 ± 0.01	0.9 ± 0.01	1.0 ± 0.1	1.0 ± 0.2	1.1	0.7
phospho-MAPK	1.6 ± 0.3	1.8 ± 0.2	2.1 ± 0.2	2.0 ± 0.2	1.6 ± 0.2	1.5 ± 0.2	1.5	1.1
phospho-RSK	1.6	2.9 ± 0.9	2.7 ± 0.8		3.0 ± 0.5	1.8 ± 0.4	1.3	1.4 ± 0.4
MKP-1	1.3	1.8	2.9		5	3.1		0.6
Fra-2	1.8 ± 0.4	2.8 ± 0.6	3.5 ± 0.05		2.5 ± 0.6	1.6 ± 0.3	0.9	0.7

* Relative to time 0, or wash. SEM included for measurements with n ≥ 3.

communication) and cardiac fibroblasts (Stockand and Meszaros, 2002), but not kidney epithelia (Ramage *et al.*, 2000; Verrey, personal communication). The underlying molecular and cellular basis for these apparent discrepancies are currently unclear, but may be related to similarly undetermined mechanisms resulting in tissue-selective aldosterone induction of other proteins, such as ENaC and serum and glucocorticoid-inducible kinase (reviewed in Stockand, 2002). Nevertheless, in A6 cells and in the heterologous *Xenopus laevis* oocyte expression system, Ki-RasA activates ENaC, which is one final effector of aldosterone signaling in epithelial cells (Mastroberardino *et al.*, 1998; Stockand *et al.*, 1999c; Al-Baldawi *et al.*, 2000).

The molecular basis whereby aldosterone induces Ki-RasA has not been described. Similarly, it also is unclear whether merely increasing absolute Ki-RasA levels in response to aldosterone is sufficient to activate Ki-RasA leading to dependent stimulation of its effector MAPK cascades. Results in Figure 1 demonstrate that aldosterone increases active, GTP-bound Ki-RasA levels proportionately with absolute Ki-RasA levels. This is consistent with a mechanism where through mass action, aldosterone-induced Ki-RasA leads to concomitant increases in the active pool of Ki-RasA. Results in Figures 2, 4, 5, and 6 are consistent with this aldosterone-increased active pool of Ki-RasA then subsequently stimulating effector MAPK signaling.

The results in Figure 5 identify a putative *cis*-acting element/region (−165–153 for mouse *c-Ki-ras2*) within the *Ki-ras* gene that possibly bestows aldosterone-responsiveness at the level of transcription to the Ras → MAPK signaling cascade. Sequence analysis of this region of mouse *c-Ki-ras2* reveals the presence of several potential sites responsive to corticosteroids with one hexanucleotide (TGTTCT; −50 to −45) half-site identical to those modulating corticosteroid-responsiveness in other genes (Strawhecker *et al.*, 1989). This hexanucleotide half-site is the most highly conserved portion of the palindromic GRE (5′-GGTACAnnnTGTTCT-3′) and has been shown to bind GR and *trans*-activate in response to activated receptor (Strawhecker *et al.*, 1989). It is provocative that this half-site contained in the noncoding region is absolutely conserved in sequence identity and relative position in the mouse, rat, and human *Ki-ras* genes (see accession numbers S39586, X74502, and AH005283, respectively). Sequence data for the corresponding region in the *X. laevis* *Ki-ras* gene has not been published (see accession number Y12715); therefore, it currently is unclear whether *Ki-ras* in this species also contains a similar element.

The *Ha-ras* gene also contains conserved regions similar to those in the −165–153 region of mouse *Ki-ras*. In addition, *Ha-ras* is induced by corticosteroids in some tissues, but pointedly not induced in renal and colonic epithelia (Spindler *et al.*, 1997; Stockand, 2002; Fuller, personal communication). It currently is unclear how common aldosterone effects on *Ha-Ras* expression are and what are the underlying molecular bases, if any, allowing for discretionary induction of Ki-RasA vs. *Ha-Ras* in response to aldosterone

Figure 8. Schematic diagram showing distinct but complementary mechanisms of activation of the MAPK cascade in response to classic mitogens and corticosteroids. Classic mitogens using a post-translational mechanism affect the GTP-binding state of Ras compared with steroids, which through genomic actions lead to stoichiometric increases in total and active Ki-RasA levels without changes in GTP-binding kinetics. Once stimulated by either mechanism, Ki-RasA then activates effector kinases through a common pathway with the distinction being that the genomic response compared with the posttranslational mechanism has a delayed onset and prolonged signal.

and other corticosteroids in a tissue- and species-specific manner. The effects of Ha-Ras on aldosterone effectors, moreover, have not been investigated. Although we observe a small but significant increase in total Ras levels in response to aldosterone (Figure 1; Al-Baldawi *et al.*, 2000), it is not clear what fraction of this increase results from induction of Ki-RasA vs. Ha-Ras. However, the effects of aldosterone on MAPK signaling in A6 cells reported in the current study (Figure 6) are abolished by inhibiting Ki-RasA expression with an antisense oligonucleotide, suggesting that this species of Ras and not Ha-Ras is the primary mediator of aldosterone actions in these cells. These findings are consistent with previous findings showing that Ki-RasA is necessary and sufficient to reconstitute, in part, aldosterone actions on ENaC (Mastroberardino *et al.*, 1998; Stockand *et al.*, 1999c; Al-Baldawi *et al.*, 2000).

Ki-RasA to MAPK

The current study is the first to directly link the effects of aldosterone on Ki-RasA expression with activation of the MAPK cascade. Figure 2 shows, using an *in vitro* assay, that aldosterone increases MAPK activity in A6 cells. Activity peaked between 1 and 2 h and began to wane by 4 h. Similar results were observed when activation of MAPK signaling was assessed using an *in vivo* assay of MAPK phosphorylation (Figures 3 and 7). In contrast to the time course of aldosterone effects on MAPK signaling, the classic mitogen, serum, stimulated this cascade much quicker, reaching peak activation within the first 30 min and waning thereafter (Figure 3).

An alternative to aldosterone affecting the MAPK cascade through genomic actions dependent on induction of Ki-RasA expression is that this steroid activates MAPK signaling independent of its nuclear effects. Indeed, Gekle *et al.* (2001) report that aldosterone modulates Na^+/H^+ exchange in Madin-Darby canine kidney cells through MAPK signaling, and that due to the rapidity of this action it is likely independent of steroid effects on gene expression. Similarly, Manegold *et al.* (1999) report that aldosterone, independent of modulating gene expression, increases phospho-MAPK levels within 3 to 5 min with levels waning soon thereafter. We believe that the current results are more consistent with aldosterone activating the MAPK cascade in A6 cells via a genomic mechanism for several reasons. This is particularly true when the maximal effects of steroid are considered. First, as mentioned above, absolute and active Ki-RasA levels increased by the same amount in response to aldosterone (Figure 1), and aldosterone's effects on MAPK signaling at peak activation are entirely dependent on Ki-RasA expression and activation of its downstream effectors, such as Raf (Figure 6) and MEK (Figure 4). Second, activation (phosphorylation) of MAPK in response to aldosterone was not apparent until 30 min after treatment (Figure 3). Third, at all time points assayed the stimulatory effects of aldosterone on phosphorylation of MAPK were attenuated by treatment with the nuclear corticosteroid receptor antagonist RU486 (Figure 3). Similarly, the maximum stimulatory effects of aldosterone (at 2 h) on phosphorylation of MAPK and MAPK activity (Figures 3 and 4) were sensitive to blockade of nuclear corticosteroid receptor, transcription, and translation. These results strongly support the contention that for aldosterone to affect MAPK signaling in A6 cells, its genomic

actions are absolutely required. However, it was not our intention to investigate nongenomic regulation of MAPK signaling by aldosterone and thus, our experimental design cannot definitively exclude this possibility for the earlier time points (<30 min; see below). It is possible that in the current study there was a nongenomic response superimposed on a slower developing genomic response.

Further support for a genomic mechanism driving the activation of MAPK in response to aldosterone observed in the current study is provided by comparison of the aldosterone-dependent time course of MAPK activity and phosphorylation vs. that of serum (Figures 2 and 3). Presumably, as shown previously for aldosterone (Manegold *et al.*, 1999; Gekle *et al.*, 2001), the nongenomic effects of this steroid would have a rapid time course more similar to traditional mitogens. This is not the case in the current study. We find that aldosterone activates the MAPK cascade via a similar genomic mechanism dependent on induction of Ki-RasA in rat cardiac fibroblasts (Stockand and Meszaros, 2002). It is not clear why some cells respond to aldosterone via nongenomic activation of the MAPK cascade and others with a genomic mechanism. It is likely that these complimentary mechanisms are manifested in a cell-specific manner. Importantly, the systemic and cellular effects of aldosterone on classic target tissues, such as the distal nephron and colon, are mediated primarily through the genomic actions of this steroid (reviewed in Booth *et al.*, 2002).

Further comparison of the maximal effect of aldosterone on MAPK activity (Figure 2) and activation of MAPK (Figures 3 and 7 and Table 1) shows that MAPK activity and phosphorylation increase ~12 and ~2-fold, respectively. This difference may reflect signal amplification where a unit increase in MAPK phosphorylation yields a higher increase in activity. Comparison also of the effects of aldosterone on MAPK activity and phosphorylation at the 5-min time point shows that although MAPK activity already has increased by 5 min, there is no apparent increase in phosphorylation of MAPK. At first glance, the rapid effects of aldosterone on MAPK activity seem to be consistent with an early nongenomic response superimposed on a genomic response. We do not believe this to be the case, as argued above. Moreover, it is not commonly accepted that A6 cells have a nongenomic response to aldosterone. It is likely that, although performed in the presence of several different kinase inhibitors, the *in vitro* assay used to quantify aldosterone actions on MAPK activity was somewhat biased by the presence of uninhibited kinases or other cellular factors that either directly impinge upon phosphorylation of MBP or MAPK activity when taken out of cellular context. However, it also is possible that the assay of MAPK activity was more sensitive than that assessing activation of MAPK. The results showing that MAPK activity is increased 12-fold, whereas MAPK phosphorylation increased only 2-fold by 2 h would also be consistent with this possibility. If this is the case then nongenomic actions of aldosterone on MAPK signaling in A6 cells during the first 30 min or so cannot be wholly excluded by the current results. However, if a nongenomic response was superimposed on a slower developing genomic response we would have expected aldosterone-stimulated MAPK activity to peak at two distinct time points, which was clearly not the case. Nevertheless, the current results definitively demonstrate that peak activation

of MAPK signaling at the 2-h time point in response to aldosterone is absolutely dependent on a genomic event and induction of Ki-RasA.

Time Course and Effects of Aldosterone-stimulated MAPK Signaling

The current results show for the first time the temporal effects of aldosterone on the active levels of several different effectors and effectors of MAPK (Figure 7 and Table 1). Similar to MAPK, phosphorylation of Raf in response to aldosterone was delayed and prolonged. Phosphorylation of Raf was dominant between 2 and 6 h and thus Raf was phosphorylated (on Ser259) at a time point later than phosphorylation of MAPK. This finding is consistent with Raf being phosphorylated in response to aldosterone in a negative feedback manner. In fact, the Western blots in Figure 7 actually assessed negative regulation of Raf that had previously been active. The serine/threonine kinase Akt/PKB an effector of phosphatidylinositol 3-kinase (PI3-K) is responsible for phosphorylation of active Raf at Ser259, which then leads to inactivation (Rommel *et al.*, 1999; Zimmermann and Moelling, 1999). PI3-K is also a first effector of Ki-Ras (Yan *et al.*, 1998) and activated by aldosterone (Blazer-Yost *et al.*, 1999). Thus, this feedback regulation of Raf may reflect parallel activation by aldosterone-induced Ki-RasA of both the MAPK and PI3-K signaling cascades. Consistent with these findings are those showing that the MEK inhibitors PD-98059 and U-0126 attenuate aldosterone-sensitive phosphorylation of MAPK but not Raf; and that the PI3-K inhibitor LY 294002 decreases aldosterone-sensitive Raf phosphorylation but not phosphorylation of MAPK (our unpublished observations).

In addition to MAPK, MEK, and Raf, aldosterone increased the levels of phospho-RSK1 beginning at 30 min, reaching a peak by 4 h. RSK-1 is well known to be a target regulated at the posttranslational level in response to MAPK signaling. Interestingly, aldosterone induced expression of Fra-2 and MKP-1, which are known to be regulated at the level of transcription in response to MAPK signaling. Fra-2 is a transcription factor related to Fos. Thus, this action may enable aldosterone to secondarily impinge upon subsequent rounds of transcription. An alternative mechanism is that aldosterone directly affects transcription/translation of these proteins independent of MAPK signaling. Indeed, recent results from Spindler *et al.* (1999) support such a mechanism of aldosterone action on Fra-2. To date, a direct effect of aldosterone on MKP-1 transcription/translation has not been demonstrated. Interestingly, induction of MKP-1, which is a phosphatase that dephosphorylates MAPK in a negative feedback manner to dampen MAPK signaling, in conjunction with feedback phosphorylation of Raf on Ser259 by Akt may result in the deactivation of MAPK signaling after 4 h observed in the current study.

All of the current results are consistent with the idea that activation of the MAPK cascade in response to aldosterone is mediated by induction of Ki-RasA and activation of signaling constituents, such as Raf and MEK, that lie between this Small, monomeric G protein and MAPK. Consequently, after increases in total Ki-RasA and active Ki-RasA:GTP levels, aldosterone-dependent MAPK signaling follows a normal progression with the major exception being that signaling is prolonged. The explanation for prolonged MAPK signaling

in response to aldosterone clearly then must come from the mechanism of initiation: transcriptional control of Ki-RasA. Such a mechanism is distinct from that used by classic mitogens, and leads to a delayed but prolonged signaling event. The delay results from the latent period required for increased transcription/translation of Ki-RasA. Prolongation results from stoichiometric increases in total and active Ki-RasA with absolute but not relative levels of Ki-RasA:GTP increasing. MAPK activity in response to aldosterone would then primarily be dependent on Ki-RasA protein turnover and feedback regulation. It is predicted that prolonged steroid-dependent MAPK signaling produces unique changes in cellular activity compared with a classic response.

Although it is known that activation of Ki-RasA by aldosterone is necessary and sufficient for induced Na⁺ transport and ENaC activity in A6 cells and for ENaC activation when this G protein is overexpressed along with the channel in *X. laevis* oocytes (Mastroberardino *et al.*, 1998; Stockand *et al.*, 1999c; Al-Baldawi *et al.*, 2000), the actions of MAPK signaling on transport and ENaC seem to be inhibitory (Zentner *et al.*, 1998; Lin *et al.*, 1999). Thus, it can be speculated that the prolonged activation of the MAPK cascade described in the current study is either a component of a feedback system or impacts Na⁺-transporting epithelia independently of directly affecting Na⁺ transport. The possible physiological and pathophysiological roles for genomic activation of MAPK signaling by corticosteroids at the tissue and systemic levels remain to be elucidated.

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