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Early Nongenomic Events in Aldosterone Action in Renal Collecting Duct Cells: PKCα Activation, Mineralocorticoid Receptor Phosphorylation, and Cross-Talk with the Genomic Response

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Abstract. Effects of aldosterone on its target cells have long been considered to be mediated exclusively through the genomic pathway; however, evidence has been provided for rapid effects of the hormone that may involve nongenomic mechanisms. Whether an interaction exists between these two signaling pathways is not yet established. In this study, the authors show that aldosterone triggers both early nongenomic and late genomic increase in sodium transport in the $RCCD_2$ rat cortical collecting duct cell line. In these cells, the early (up to 2.5 h) aldosterone-induced increase in short-circuit current (Isc) is not blocked by the mineralocorticoid receptor (MR) antagonist RU26752, it does not require mRNA or protein synthesis, and it involves the PKCα signaling pathway. In addition, this early response is reproduced by aldosterone-BSA, which acts at the cell surface and presumably does not enter the cells (aldo-BSA is unable to trigger the late response). The authors also show that MR is rapidly phosphorylated on serine and threonine residues by aldosterone or aldosterone-BSA. In contrast, the late (4 to 24 h) aldosterone-induced increase in ion transport occurs through activation of the MR and requires mRNA and protein synthesis. Interestingly, nongenomic and genomic aldosterone actions appear to be interdependent. Blocking the PKCα pathway results in the inhibition of the late genomic response to aldosterone, as demonstrated by the suppression of aldosterone-induced increase in MR transactivation activity, α1 Na^+/K^+/ATPase mRNA, and Isc. These data suggest cross-talk between the nongenomic and genomic responses to aldosterone in renal cells and suggest that the aldosterone-MR mediated increase in mRNA/protein synthesis and ion transport depends, at least in part, upon PKCα activation.

The steroid hormone aldosterone is involved in sodium homeostasy and control of BP. Its action consists in pleiotropic cellular effects in different tissues, including the renal cortical collecting duct (CCD). In this segment of the nephron, aldosterone regulates sodium and potassium transport (1–5) through modifications of ion transporters, such as the epithelial sodium channel (ENaC) located in the apical membrane of the cells, the Na^+/K^+/ATPase (NKA) in the basolateral membrane, and potassium channels present in both membranes (4,5). The time course of aldosterone action can be divided into three different phases as defined in the amphibian A6 cell line (3). The first phase is a latent period (approximately 1 h) during which no modification occurs either in short-circuit current (Isc) or in transepithelial resistance (R_T). During the second or early phase (1 to 3 h), Isc is increased in parallel with a decrease in R_T. Finally, the late phase (after 3 h) is characterized by an increase in Isc without further modification in R_T. The late phase of aldosterone action has been extensively studied and has been clearly shown to require synthesis of ion transporting proteins, such as the α subunit of ENaC and the α1 subunit of NKA (4–5). In contrast, much less information is available on the early phase of aldosterone response and its influence on the late phase. In recent studies, early response genes have been identified (4). In the A6 cell line, the serum and glucocorticoid-induced kinase (Sgk) and K-Ras are rapidly upregulated (4,5), and it has been shown that Sgk induces rapid translocation of intracellular ENaC into the apical membrane (6). In other studies, the authors propose that the early phase of

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aldosterone response could depend on nongenomic events (7). Aldosterone has been shown to rapidly increase intracellular calcium concentration and/or to modify intracellular pH, PKC activity, or intracellular cAMP (7,8). It has also been proposed that methylation processes could be responsible for the early increase in sodium transport, a phenomenon independent of gene transcription (9,10). In addition, rapid aldosterone signaling has been evidenced in mice with genetic disruption of the MR (11); in cells of MR knockout mice, rapid effects of the hormone on intracellular calcium and cAMP levels were observed and were even larger than in cells from wild-type mice. These observations argue for nongenomic effects of aldosterone through pathways that are not yet fully identified.

The goal of this study was to characterize the early effects of aldosterone on sodium transport in a new rat renal CCD cell line ($RCCD_2$) (12) and to bring new insight into the interactions between the early phase and the late genomic response to the hormone. Results indicate that in $RCCD_2$ cells the early phase of increase in sodium transport does not require mRNA or protein synthesis; it is not antagonized by classical antagonists of mineralocorticoid or glucocorticoid receptors (MR or GR), and it is mediated by activation of PKCα. This effect is reproduced by aldosterone-BSA (*i.e.*, aldosterone coupled to bovine serum albumine [BSA]), which prevents its entry into cells. The late phase of increase in sodium transport appears to depend on the early activation of PKCα signaling cascade. Evidence is also provided that aldosterone triggers a rapid PKCα-mediated phosphorylation of the MR on serine and threonine residues. MR phosphorylation also occurs in the presence of aldosterone-BSA or of a PKCα-specific activator. In addition, phosphorylation is specific to the MR because the glucocorticoid receptor is not phosphorylated under the same conditions. Our results also strongly suggest that the PKCα-dependent early phase of aldosterone action is required for the full development of the late response to the hormone. Indeed, inhibition of PKCα prevents the development of the late aldosterone response (*i.e.*, the transactivation activity of the MR), the transcriptional increase of the α1 subunit of NKA, and the increase in transepithelial sodium transport.

Materials and Methods

$RCCD_2$ Cell Culture

The rat CCD cell line $RCCD_2$ (12) was used between passages 5 and 20. The $RCCD_2$ cells were grown at 37°C in a humidified incubator gassed with 5% CO_2. Cells were cultured in a complete medium changed every other day and containing: DMEM/HAM F12 1:1; $NaHCO_3$ 14 mM; glutamine 2 mM; dexamethasone 50 nM; sodium selenite 5 μg/L; transferrine 5 μg/ml; insuline 5 μg/ml; EGF 10 ng/ml; T_3 50 nM; penicilline/streptomycine 100 U/ml; Hepes 20 mM pH 7.4; and fetal bovine serum 2%. Cells were seeded on either Transwell/Snapwell filters (Costar Corp.) or Petri dishes previously coated with collagen (Institut J. Boy, Reims, France). The medium bathing the apical surface of the $RCCD_2$ cells is designated as the inner well, and the medium bathing the basolateral surface of the monolayer through the porous filter is designated as the outer well. To examine hormonal effects, the complete medium was replaced by minimum medium (MM) containing: DMEM/HAM F12 1:1; $NaHCO_3$ 14 mM; glutamine 2 mM; penicillin-streptomycin 10 U/ml; Hepes 20 mM, pH 7.4.

Reagents

All reagents were from Sigma, unless otherwise specified. Aldosterone-BSA (aldo-BSA) was from Steraloids (Newport, RI); it consists of BSA coupled to aldosterone through a carboxymethyl oxyme residue on the C3 of the hormone. As specified by the manufacturer, 25 aldosterone molecules are covalently linked to each BSA molecule. To compare results obtained in the presence of aldosterone to those with aldo-BSA, the concentrations of aldo-BSA are provided as moles of aldosterone, not as moles of aldosterone-BSA (this compound has a molecular weight 200 times higher than aldosterone).

Electrophysiological Studies

The measurement of the short-circuit current (Isc; $\mu A \cdot cm^{-2}$) was performed on $RCCD_2$ cells grown on collagen-coated Snapwell filters as described (12). Briefly, Snapwell filters were incubated overnight in MM. They were then mounted into a voltage clamp system (Costar Corp., WPI). Cells were bathed on each side with 8 ml of MM thermostated at 37°C. This voltage current clamp was used to measure Isc by clamping the transepithelial potential V_T to 0 mV for 1 sec. In this condition, Isc was determined after pretreatment with various inhibitors or antagonists (see below) and treatment with aldosterone, aldosterone-BSA, or dexamethasone.

Imaging of MR in Living Cells

For living-cell imaging experiments, a doubly and stably transfected clone originating from the $RCCD_2$ cell line was used (13): this clone includes the Cre-Lox inducible system to allow conditional expression of an eGFP-tagged human MR. A first transgene allows constitutive expression of the inducible mER-Cre-mER recombinase whose activity depends on tamoxifen or 4-OH-tamoxifen stimulation. A second transgene harbors a cassette flanked by two loxP sites and contains a stop signal for transcription (poly-adenylation site). This "stop" cassette is followed by the cDNA sequence of an eGFP-hMR fusion protein. Both transgenes are under control of the cytomegalovirus (CMV) promoter. When cells from this clone are stimulated with 4-OH-Tam, the Cre-mediated recombination allows the excision of the "stop" cassette and the subsequent expression of the eGFP-hMR protein. In our experiments, cells were seeded in Lab-teck (chambered coverglass system; Nalge Nunc International) previously coated with collagen. After 24-h culture, complete medium was replaced with MM. Twenty-four hours later, cells were stimulated with 10 nM 4-OH-Tam applied for 5 h. Twelve hours later, the Lab-teck wells were mounted into a video microscopy chamber, and cells were treated with 1 nM aldosterone or 25 nM aldo-BSA. Image acquisition was performed at 37°C, under 5% CO_2, every 10 min for 1 h.

Stable Transfection of the $RCCD_2$ Cells with a Luciferase Reporter Gene and Transactivation Experiments

The $RCCD_2$ cell line was transfected with the pAGE5MMTVLu construct (kindly provided by Dr. Richard-Foy, Toulouse, France) (14) to study the transcriptional activity of endogenous mineralocorticoid (MR) and glucocorticoid (GR) receptors in $RCCD_2$ cells. The pAGEMMTVLu is derived from the mouse mammary tumor virus long terminal repeat. It can be activated by both glucocorticoids and mineralocorticoids through the interaction of the hormone-receptor complex with glucocorticoid-responsive elements (GRE) (16).

$RCCD_2$ cells plated on 3.2-cm diameter wells (about 500,000 cells) were transfected using lipofectamin (Life Technologies) with 1 μg of the plasmid pAGE5MMTVLu. For the selection of transfected cells, the medium was supplemented with 400 μg/ml G418. Stable G418-resistant clone pools were seeded at limit dilution, several single clones were isolated and tested, and one clone was chosen. Using this clone, the transcriptional activity of endogenous MR and GR was studied. Cells were seeded on 24-mm diameter Transwell filters (Costar Corp.) previously coated with collagen, grown in complete medium for 3 d, incubated overnight in MM, and then treated or not for 1 h with inhibitors (see below) before treatment with aldosterone or dexamethasone in the same medium. The luciferase activity was determined 24 h later. For this purpose, cell monolayers were rinsed twice in phosphate-buffered saline (PBS) and lysed by the addition of 0.2 ml of lysis buffer (25 mM Glycyl-glycine [pH 7.8], 1 mM EDTA, 1 mM dithiothreitol, 8 mM $MgSO_4$, 1% Triton X-100, 15% glycerol) 30 min at 4°C. The lysate was transferred to a microfuge tube and centrifuged for 5 min. Luciferase activity was assayed in 150 μl of the supernatant with 350 μl of luciferase assay buffer (25 mM Tris, 8 mM $MgCl_2$, 1 mM dithiothreitol, 1% Triton, 1% BSA, 15% glycerol, pH 7.8). Luciferase-mediated light output was determined on a Lumat LB 9501 luminometer (Berthold) by injection of 100 μl of substrate assay buffer (25 mM Tris, 8 mM $MgCl_2$, 1 mM dithiothreitol, 1% Triton, 1% BSA, 15% glycerol, pH 7.8) with 0.22 mM Luciferin and 1 mM ATP and integration of light emission peak for 10 s.

PKC Activity Assay

PKC activity was measured by an assay (Amersham) based on the transfer of the terminal phosphate of [γ32P] ATP (Amersham) to a synthetic peptide substrate. Assays were carried out as recommendd by the manufacturer at 37°C in a final volume of 65 μl of cell lysate containing total proteins. The reaction was initiated by addition of 37.5 μM [γ32P] ATP; the assay was stopped after 15 min, and assay mixture was spotted on phosphocellulose ion exchange chromatography filter papers, which were allowed to dry for 30 sec and placed in 75 mM phosphoric acid solution (10 ml/filter). Filters were then washed twice for 10 min at 4°C in phosphoric acid. Incorporated radioactivity was determined by scintillation counting, and activity was expressed as radioactive phosphate transferred/filter/min or as percentage of control values without treatment. As inhibitors of PKC, we used chlerethrine chloride (CC, 100 nM) and GF 109 203X (GF, 100 nM). The inhibitor of PKCα was GÖ 6976 (GÖ, 10 nM), and sapintoxin D (SAPD 100 nM) was used as specific PKCα activator. In these experiments, cells were first preincubated with CC, GF, or GÖ 1 h and then treated with aldosterone or aldo-BSA or SAPD for 10 min.

Northern Blot

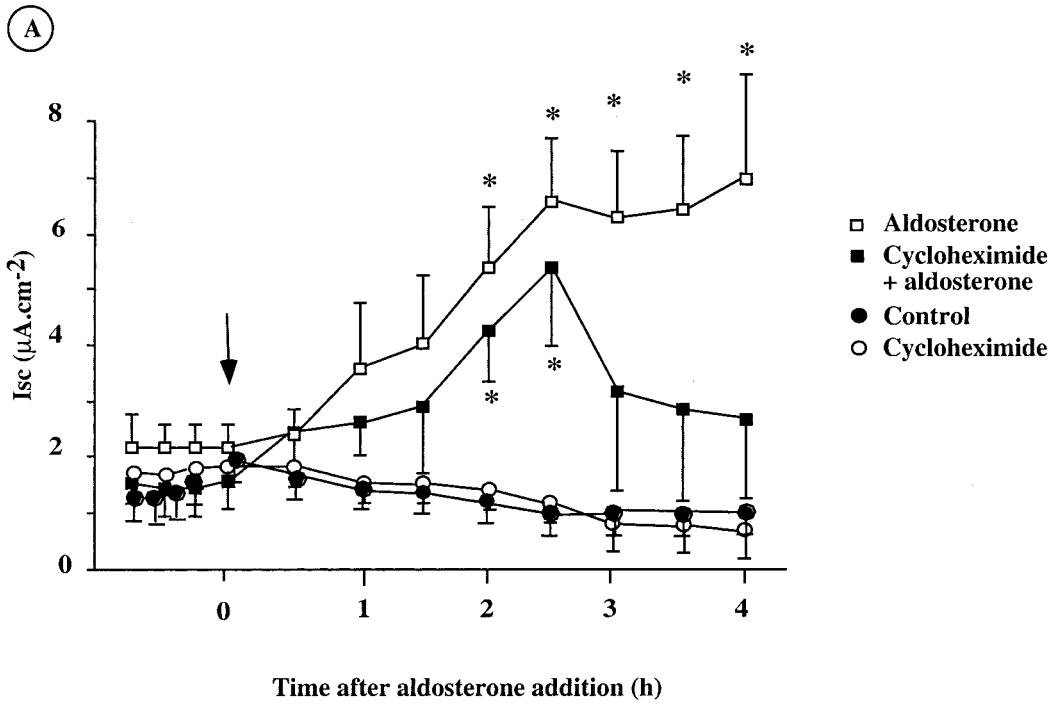
Total RNA was extracted from cells seeded on 24-mm diameter Transwell filters (Costar Corp.) previously coated with collagen as described (12). Cells were grown in complete medium for 3 d, incubated overnight in MM, and then treated or not for 1 h with inhibitors (see below) before treatment with aldosterone or dexamethasone. Total RNA (10 to 20 μg) was run on a 0.8% denaturating glyoxal agarose gel and blotted onto nylon membranes (Hybond-N, Amersham). Membranes were then hybridized with random-primed α32P-dCTP–labeled probes specific for rat α ENaC (590 bp; nt 2185 to 2775), α1 NKA (832 bp; nt 1189 to 2021), and GAPDH (851 bp; nt 20 to 871) as described (14).

Immunoprecipitation and Western Blot Experiments

Immunoprecipitation experiments were performed as described (12). Briefly, cells were treated or not for various times with aldosterone or with aldo-BSA and then washed in PBS with 1 mM sodium orthovanadate, scraped off the filters, and extracted with 200 μl of ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris HCl [pH 7.4], 2.4 mM EDTA, 1% Nonidet P40, 0.5 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C. After centrifugation (12,000 $\times$ *g*; 10 min; 4°C) to eliminate cell debris and nuclei, protein extracts were precleared with a *Staphylococcus aureus* slurry (Pansorbin, Calbiochem) before incubation overnight at 4°C under end-over-end rotation with antibodies directed against mineralocorticoid or glucocorticoid receptors (MCR-N17 for MR and GR-M20 for GR, Santa Cruz Biotechnology; 1/40; overnight at 4°C) or eGFP (Santa Cruz Biotechnology; 1/40; overnight at 4°C). With regards to endogenous MR, immunoprecipitation was performed with anti-MR together with anti–β-actin antibodies, as an internal control for protein recovery. The hMR-GFP fusion protein expressed in modified $RCCD_2$ cells was immunoprecipitated with an anti-GFP antibody. Immunoprecipitates were then incubated with protein A-Sepharose beads (CL-4B) (Pharmacia Biotech Inc.) at 4°C for 1 h. Beads were washed three times with 1 ml of high-salt buffer (500 mM NaCl, 1% NP-40, 50 mM Tris-HCl [pH 8.0]) and twice with 500 μl of low-salt buffer (20 mM Tris HCl, pH 7.5) and resuspended in 40 μl of NuPage LDS sample Buffer (Invitrogen). Samples of eluted immunoprecipitates were submitted to 6 to 12% SDS-polyacrylamide gel electrophoresis (NuPage Invitrogen), transferred onto nitrocellulose membrane (Invitrogen). Phosphorylated serine, threonine, and tyrosine residues were detected with mouse monoclonal anti-phosphoserine antibody (Sigma; 1/1000; overnight at 4°C), or with rabbit polyclonal anti-phosphothreonine antibody (Zymed; 1/500; overnight at 4°C) or with mouse monoclonal anti-phosphotyrosine antibody (Zymed; 1/500; overnight at 4°C) coupled with peroxydase before detection with ECL kit (Amersham). Membranes used for measurement of MR phosphorylation were also blotted with the anti–β-actin antibody (Santa Cruz Biotechnology; 1/200; 1 h at room temperature) to correct for protein loading. After immunoprecipitation of GR or of GFP, nitrocellulose membranes were stripped and Western blotting was done with anti-GR antibody (1/2000; 1 h at room temperature) or anti-GFP antibody (1/500; 1 h at room temperature) followed by anti-rabbit secondary antibody (Santa Cruz Biotechnology; 1/20,000; 1 h at room temperature) coupled with peroxidase and ECL. For Western blotting of PKCα, the same protocol was used with an anti-PKCα antibody (Santa Cruz Biotechnology; 1/500; 1 h at room temperature) and an anti-rabbit β-actin antibody (Santa Cruz Biotechnology; 1/200; 1 h at room temperature).

Preparation of Cytosolic and Membrane Fractions

$RCCD_2$ cells were grown on 24-mm diameter transwell filters in complete medium before preincubation overnight in MM and treatment or not for 15 min with 10 nM aldosterone. In each condition, cells from two filters were rinsed twice with cold PBS, then scraped in 1 ml ice-cold PBS + 2 mM EDTA. Cells were centrifuged at 1400 $\times$ *g* for 10 min, then resuspended in 250 μl of Tris-Mg^{2+} buffer (10 mM Tris-Hcl [pH 7], 1 mM $MgCl_2$) added with 0.5 mM PMSF and protease inhibitors cocktail. The solution was homogenized by 20 passages throught a $25^{5/8}$-gauge needle, then centrifuged at 3500 rpm for 10 min at 4°C in a microfuge. The supernatant was ultracentrifuged at 75,000 rpm for 30 min at 4°C. The supernatant corresponded to the cytosolic fraction of the cells and the pellet to the membrane fraction. The pellet was resuspended in TNE buffer (0.1 M NaCl, 0.01 M Tris-HCl [pH 7], 10 mM EDTA). Both fractions were then prepared for Western blot (see above).



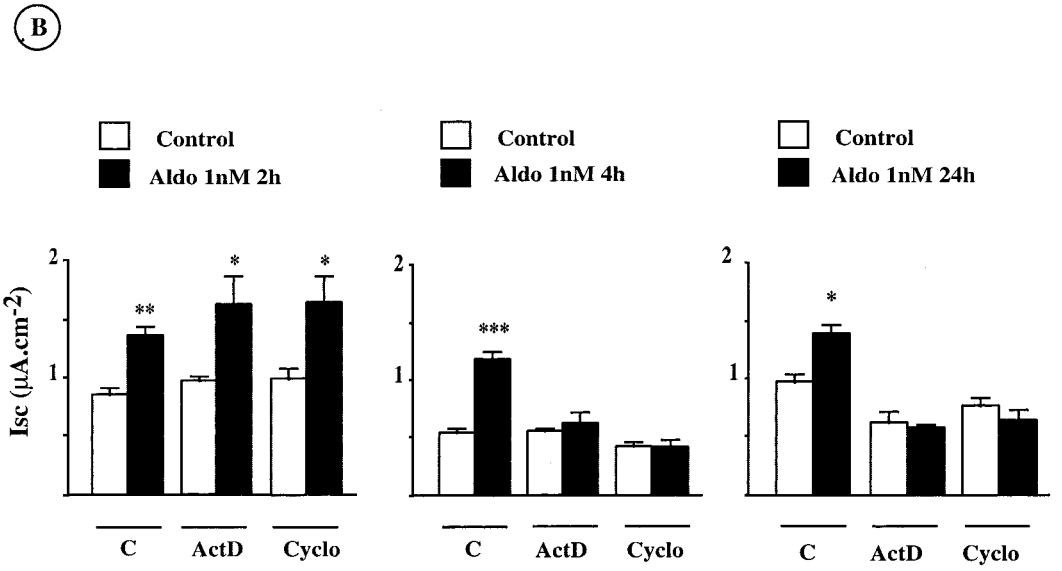


Figure 1. Effect of cycloheximide and actinomycin D (Act D) on the aldosterone-induced increase in short-circuit current (Isc) in rat cortical collecting duct cells ($RCCD_2$). (A) $RCCD_2$ cells were preincubated or not with 2 μM cycloheximide (Cyclo) and then treated or not with 1 nM aldosterone or diluent (arrow) in the presence of cycloheximide. Isc was measured every 30 min throughout the experiment. In control condition, aldosterone addition induced an increase in Isc (open squares). When $RCCD_2$ cells were pretreated with cycloheximide and then with aldosterone (closed squares), Isc was significantly increased at 2 h and 2.5 h and decreased thereafter. When cells were treated with cycloheximide only, almost no modification of Isc was observed during the experiment. In control experiments, the diluent corresponding to the inhibitor (H_2O for cycloheximide) or the hormone (ethanol 1/1000 for aldosterone) was added. Each point is the mean value of three experiments. *$P < 0.05$, experimental *versus* control period without aldosterone. (B) $RCCD_2$ cells were preincubated with 1 μM Act D or 2 μM Cyclo for 1 h and then treated or not with 1 nM aldosterone for various times (2 h, 4 h, 24 h) in the presence of the inhibitor. Cells were kept in the incubator between each Isc measurement. Both inhibitors failed to block the increase in Isc induced by aldosterone after 2 h of treatment. In contrast, they suppressed aldosterone effect after 4 h and 24 h of treatment with the hormone. In control experiments (open bars), the diluent corresponding to each inhibitor (H_2O for Cyclo and DMSO 1/10000 for Act D) or aldosterone (ethanol 1/1000) was added. Condition C corresponds to cells that were not incubated with inhibitors. No effect of DMSO 1/10000 was observed. Each point is the mean value of four to seven experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ aldosterone *versus* control without hormone.

Immunofluorescence Studies

The expression of PKCα was determined on $RCCD_2$ cells grown in complete medium on collagen-coated transwell filters. Cells were fixed with ice-cold methanol for 10 min at room temperature, incubated for 1 h with an anti-rabbit polyclonal anti-PKCα antibody (Santa Cruz Biotechnology; 1/50; room temperature), then with a cy3-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratory, Inc; 1/200; 30 min at room temperature). Each incubation step was followed by washing in PBS. In these experiments, the nucleus was stained with Sytox green (Molecular Probes, Inc.). *xz* sections of the cells were realized by confocal laser scanning microscopy LSMS 10 (Zeiss).

Inhibitors and Antagonists

Inhibitors or antagonists were added for 1 h at 37°C before addition of aldosterone, aldo-BSA, or dexamethasone and remained present throughout the experiment: inhibitor of RNA synthesis (actinomycin D 1 μM, inner and outer wells), inhibitor of protein synthesis (cycloheximide 2 μM, inner and outer wells), inhibitor of the ENaC (amiloride [ami] 10 μM and phenamil [phe] 100 nM, inner well), inhibitors of protein kinase C (chlerethrine chloride [CC] 100 nM and GF 109203 $\times$ 100 nM, inner and outer wells), inhibitor of protein kinase C alpha (GÖ 6976 10 nM [GÖ], inner and outer wells), inhibitor of protein kinase A (dihydrochloride [H89] 100 nM, inner and outer wells), sodium ionophore amphotericin B (Ampho B 10 μM, inner well), activator of protein kinase C alpha (sapintoxin [SAPD] 100 nM, inner and outer wells), mineralocorticoid receptor antagonist (RU26752 1 μM, inner and outer wells), glucocorticoid receptor antagonist (RU486 1 μM, inner and outer wells). In corresponding control conditions, diluent corresponding to that used for the inhibitor or the hormone was added to the medium. RU compounds were kind gifts of Roussel-Uclaf, France.

Statistical Analyses

Results are expressed as mean values $\pm$ SE. Comparison analysis was made using *t* test for either paired data or unpaired data after ANOVA and correction for multiple comparisons.

Results

Early Aldosterone-Induced Increase in Sodium Transport in $RCCD_2$ Cells Is Mediated by a Nongenomic Signaling Pathway, Independent of the Mineralocorticoid Receptor

The time course of the effect of aldosterone (1 nM) on Isc was evaluated in $RCCD_2$ cells grown on porous membranes and compared with the effect obtained in the presence of the inhibitor of protein synthesis cycloheximide (Figure 1A). After a control period during which Isc was stable, addition of aldosterone led to a progressive increase in Isc, which was significant at 2 h and thereafter. While absolute values of Isc vary between panels A and B of Figure 1, probably due to differences among cell batches (see also data in reference 12 and other figures from this report), the expected aldosterone-induced increase in Isc was constantly found. Of note, $RCCD_2$ cells have low values of basal and aldosterone-stimulated Isc compared with the mouse $mpkCCD_{cl4}$ cell line (in which higher doses of aldosterone were used) (15), possibly due to species differences, culture conditions, or variations in the transported ion species. When cells were preincubated with 2 μM cycloheximide for 1 h, addition of 1 nM aldosterone also led to an initial increase in Isc, as in cells without cycloheximide; then Isc decreased after 2.5 h. Treatment of the cells with cycloheximide alone or with the diluent (control) did not signif-

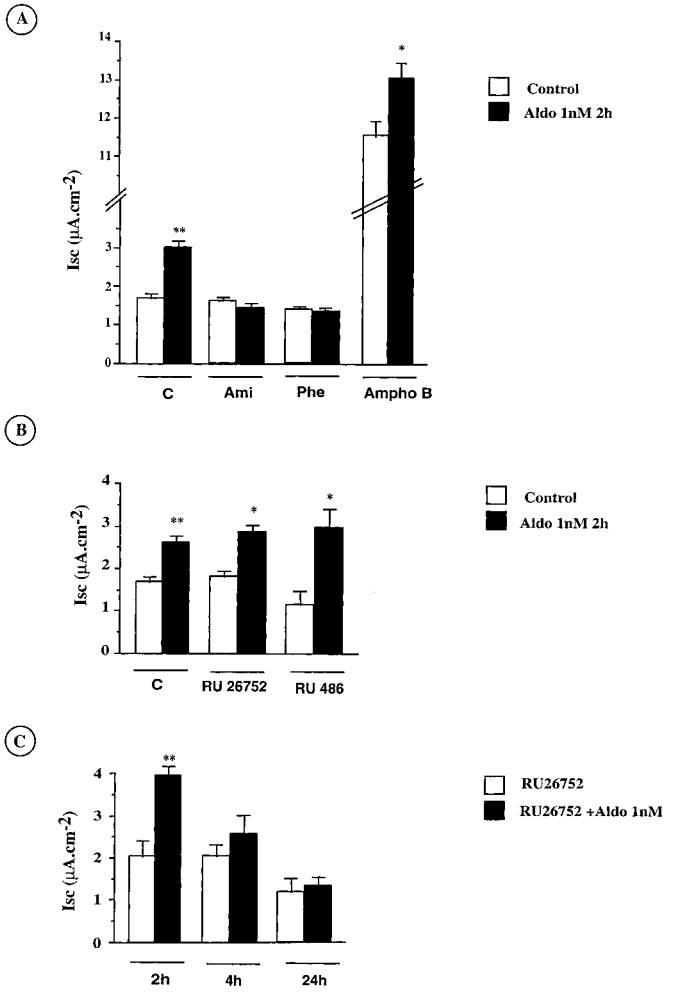


Figure 2. Effect of inhibitors of sodium transport and antagonists of mineralocorticoid or glucocorticoid receptors on the aldosterone-induced increase in Isc. (A) Amiloride (Ami, 10 μM) or phenamil (Phe, 100 nM) added apically to the cells 1 h before aldosterone (and kept throughout the experiment) blocked the early response (2 h) to the hormone. When cells were incubated with 10 μM amphotericin B (Ampho B) in the apical compartment, Isc was increased and a further significant increase was observed after 2 h of aldosterone treatment. (B) The MR or GR antagonists RU26752 (100 nM) and RU486 (100 nM), respectively, were added 1 h before aldosterone addition and were maintained in the medium throughout the experiment. These antagonists did not suppress the aldosterone-induced increase in Isc (2 h). (C) The influence of the MR antagonist RU26752 (100 nM) was examined on the aldosterone-induced increase in Isc after various times of exposure to the hormone. Whereas the effect of aldosterone was not altered by RU26752 at 2 h, it was fully blocked after 4 h and 24 h of treatment with the hormone in the presence of the antagonist. In control experiments (open bars), the diluents corresponding to amphotericin B (DMSO 1/10,000), to MR and GR antagonists, or to aldosterone (ethanol 1/1000) were added. Each bar is the mean value of at least nine filters from three to six experiments. *$P < 0.05$; **$P < 0.01$ experimental *versus* control without hormone.

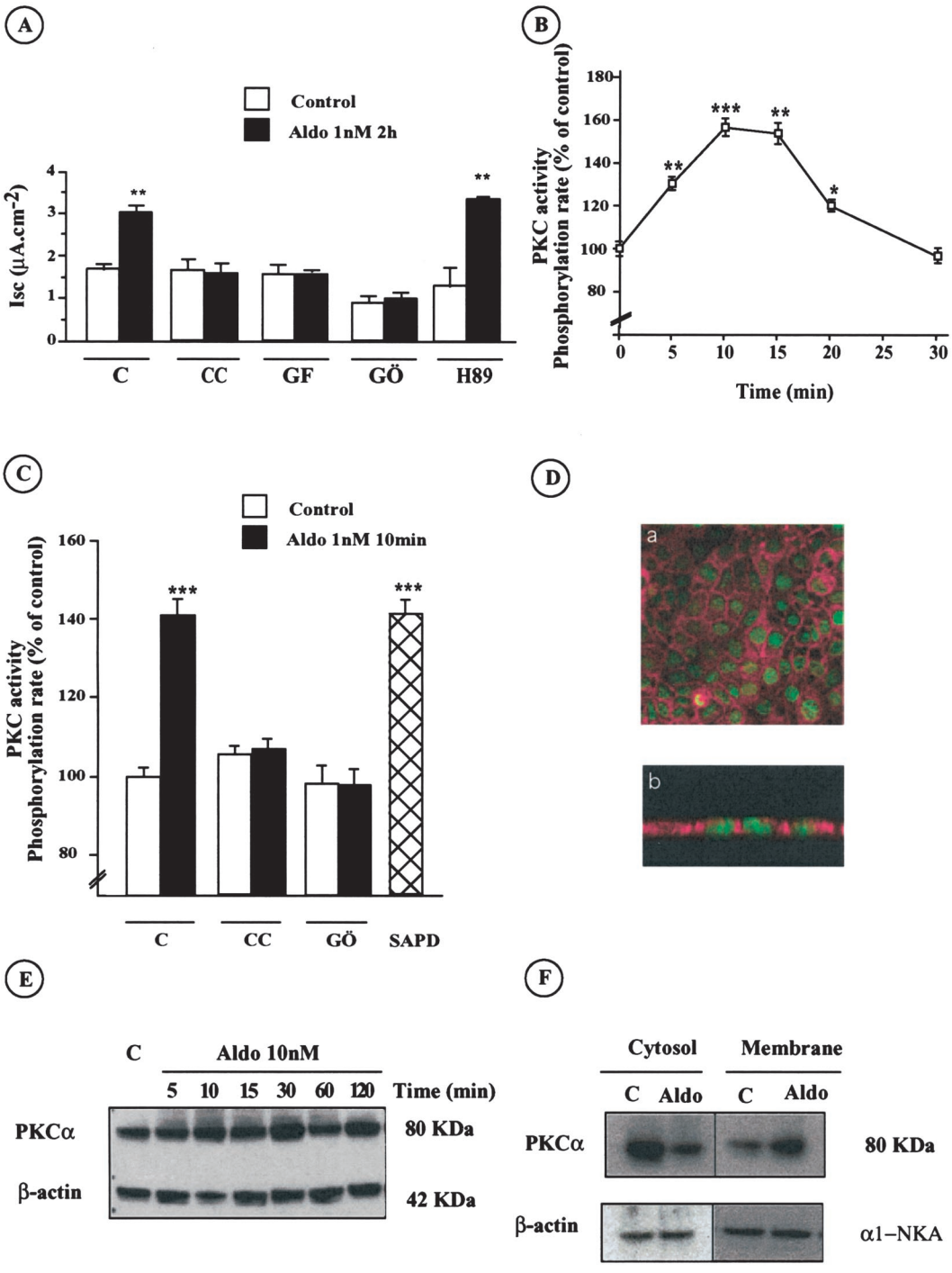


Figure 3. Influence of the PKCα signaling pathway on the early response to aldosterone. (A) Cells were incubated in the presence of the PKC inhibitors chleretrine chloride (CC, 100 nM) or GF 109203X (GF, 100 nM) or with a specific PKCα inhibitor GÖ 6976 (GÖ, 10 nM) added 1 h before aldosterone. The increase in Isc induced by 2 h of treatment with 1 nM aldosterone was blocked by these inhibitors. In contrast, it was not blocked by the PKA inhibitor H89 (100 nM). (B) Time course of activation of PKC by aldosterone in $RCCD_2$ cells. The effect of 1 nM aldosterone on PKC activity was studied using a protein kinase C enzyme assay. PKC activity was significantly increased by aldosterone after 5 min of treatment and then remained elevated up to 20 min. (C) The increase in PKC activity induced by 10-min treatment with 1 nM aldosterone was blocked when cells were preincubated (1 h) in the presence of the PKC inhibitor chlerethrine chloride (CC, 100 nM) or with the PKCα inhibitor GÖ6976 (GÖ, 10 nM). The specific PKCα activator sapintoxin D (SAPD, 100 nM) reproduced the aldosterone-induced increase in PKC activity. (D) In $RCCD_2$ cells grown on porous filters in complete medium, PKCα was evidenced by immunofluorescence using a specific anti-PKCα antibody. The nuclei are in green and PKCα in red (panels a and b). The *xz* reconstitution of the cells (panel b) shows that PKCα is present in all $RCCD_2$ cells, both in the cytoplasmic and in the membrane compartments. (E) The time course of aldosterone effect on PKCα protein expression in $RCCD_2$ cells grown on transwell was evaluated by Western blot experiments. PKCα appeared as an 80-kD band. β-actin was used as an internal standard. The amount of PKCα was not modified by aldosterone treatment. (F) PKCα protein expression in

icantly modify Isc during the 4 h of the experiment. Thereafter, we documented the effects of 2, 4, and 24 h of treatment with aldosterone in the presence of the protein synthesis inhibitor cycloheximide (2 μM) or the mRNA synthesis inhibitor actinomycin D (1 μM) (Figure 1B). The aldosterone-induced increase in Isc was not blocked by actinomycin D or cycloheximide after 2 h of treatment with the hormone. However, the late response to aldosterone (at 4 h and 24 h) was prevented by both inhibitors. The concentration of actinomycin D used here has been shown to abolish the aldosterone-induced increase in transcripts encoding for NDRG2 (an early response gene) in the same cellular model (16). We have also documented this finding at the protein level by Western blot; cycloheximide (2 μM) blunted the accumulation of NDRG2 elicited by 1 h treatment with aldosterone (not shown). Moreover, similar concentrations of each drug have been shown to suppress aldosterone-induced sodium transport in mpkCCD$_{cl4}$ cells (15). Taken together, these results suggest that mRNA and protein synthesis are not required to mediate the early (2 h) increase in Isc induced by aldosterone in contrast to the late response.

To characterize the early phase of aldosterone action in RCCD$_2$, the effect of several inhibitors or antagonists on the aldosterone-induced increase in Isc have been examined. Figure 2A shows that the aldosterone-induced increase in Isc (at 2 h) is blocked by two inhibitors of the ENaC, amiloride (10 μM), and phenamil (100 nM), suggesting an involvement of the amiloride-sensitive sodium transport mediated by ENaC. These data also indicate that in the absence of hormones (MM) no ENaC activity is present in RCCD$_2$ cells. This is compatible with patch-clamp observations of Pacha *et al.* (17), where the amiloride-sensitive currents in principal cells of cortical collecting tubules from normal rats appear negligible in the absence of aldosterone challenge. Interestingly, in the presence of the sodium ionophore amphotericin B (10 μM) added apically, Isc was also significantly increased by aldosterone (1 nM) at 2 h compared with control cells with amphotericin B alone, suggesting that the hormone also affects a basolateral sodium transporter, probably NKA. Then, we examined the influence of antagonists of the mineralocorticoid (MR) and glucocorticoid (GR) receptors on Isc (Figure 2B). Neither the MR antagonist RU26752 nor the GR antagonist RU486 were able to block the early (2 h) effect of aldosterone. In contrast, the late phase (4 h and 24 h) of aldosterone action on Isc was blocked by RU26752 (Figure 2C).

We have also evaluated the effect of aldosterone on the expression of the α subunit of ENaC and α1 subunit of NKA. Northern blot experiments showed that α ENaC and α1 NKA mRNA expression were significantly increased after 24 h of treatment with 1 nM aldosterone as expected (α ENaC: 214 $\pm$ 50%, $n = 8$, $P < 0.05$, aldo *versus* control; α1 NKA: 273 $\pm$ 47%, $n = 4$, $P < 0.05$, aldo *versus* control). In contrast, no significant effect was observed after 2 h of aldosterone treatment (α ENaC: 86 $\pm$ 9%, $n = 5$, NS; α1 NKA: 107 $\pm$ 11%, $n = 4$, NS). These experiments show that in RCCD$_2$ cells the Isc observed after 2 h of aldosterone treatment occurs without modification in α ENaC and α1 NKA mRNA, in contrast to the late effect at 24 h.

Transient Activation of PKCα Is a Key Event in the Early Effect of Aldosterone in RCCD$_2$ Cells

To test whether the PKA and/or PKC signaling pathways were involved in the early aldosterone-induced increase in Isc, cells were pretreated (1 h) either with two specific PKC inhibitors, chlerethrine chloride (CC, 100 nM) and GF 109203X (GF, 100 nM), or with the PKA inhibitor H89 (100 nM) before aldosterone addition. Whereas CC and GF blocked the early effect of aldosterone on Isc, H89 was without effect (Figure 3A). We also used the PKCα specific inhibitor GÖ 6976 (GÖ, 10 nM). Figure 3A shows that the effect of aldosterone (1 nM) on Isc was totally blocked by addition of GÖ. These experiments suggest that PKC has a role in the early effect of aldosterone in RCCD$_2$ cells and that it may involve the α isoform of PKC. To extend these results, we examined the effect of aldosterone on cellular PKC activity. Such PKC assay has been used by others (18). Figure 3B shows the time course of the increase in PKC activity elicited by aldosterone (1 nM). PKC is rapidly activated in response to aldosterone because a significant increase in the phosphorylation rate was observed as early as 5 min after hormone addition. The effect was maximal (10 to 15 min), and then PKC activity returned to control values at 30 min. As a control for the specificity of this assay, Figure 3C shows that the increase in PKC activity induced by 1 nM aldosterone is blocked by the PKC inhibitor CC and the specific PKCα inhibitor GÖ. We also show that the specific PKCα activator sapintoxin (100 nM, SAPD) can stimulate PKC activity to an extent similar to that of 1 nM aldosterone. Figure 3D shows that PKCα can be immunodetected in RCCD$_2$ cells; a clear staining was present in all cells, both in the membrane and in the intracellular compartment, when cells were grown in complete medium. Figure 3E shows that treatment of the cells for up to 2 h with 1 nM aldosterone did not modify the amount of PKCα present in the cells, as detected by Western blot. In contrast, 15-min treatment by aldosterone (1 nM) of RCCD$_2$ cells grown in MM resulted in a redistribution of PKCα from the intracellular compartment to the membrane fraction (Figure 3F). Indeed, in this condition

the cytosol and membrane fractions of control cells and cells treated for 15 min with 10 nM aldosterone. Aldosterone treatment led to an increase in the amount of PKCα present in the membrane fraction and a corresponding decrease in the cytosolic fraction. As control of gel loading, β-actin (cytosol fraction), and α1-NKA (membrane fraction) were used to allow comparison of the PKCα signals in control *versus* aldosterone-treated cells. In control experiments (open bars), the diluent corresponding to each inhibitor (H_2O for H89, CC, and GF; DMSO 1/10,000 for GÖ) was added. The diluent corresponding to aldosterone (ethanol 1/1000) was also added in control conditions. Each value is the mean of six to eight filters from at least three experiments. *$P < 0.05$; **$P < 0.01$, experimental *versus* control. Figures presented in panel D, E, and F are representative of at least three experiments.

(cells grown without hormones), aldosterone elicited a decrease in the amount of PKCα recovered in the cytosol and a corresponding increase in the membrane fraction.

Early Effects of Aldosterone Are Reproduced by Aldosterone-BSA

Since the effects produced by aldosterone in the early phase may involve a nongenomic signaling pathway, we tested whether they could be reproduced by aldosterone coupled to BSA (aldo-BSA). Indeed, in this form aldosterone may interact with a putative membrane receptor but does not likely enter the cells (19). To ensure that this is in fact the case, experiments were performed on $RCCD_2$ cells stably transfected with human MR (hMR) coupled to eGFP (13). In these cells, the addition of aldosterone (1 nM) resulted in nuclear translocation of the hMR-eGFP from the cytoplasm to the nucleus within 30 min; such translocation was not observed with 25 nM aldo-BSA (Figure 4A). This observation suggests that it is unlikely that aldosterone dissociates from BSA, and thus should not trigger the classical MR-mediated response. Alternatively, aldo-BSA may be subject to endocytosis but appears ineffective in promoting the classical ligand-dependent nuclear translocation. Figure 4B shows the effect of aldo-BSA compared with the effect of aldosterone 1 nM. It should be noted that aldo-BSA concentrations are expressed by taking into account the amount of aldosterone, not of aldosterone-BSA as indicated in Materials and Methods. A significant increase in Isc was present at 1.25 nM aldo-BSA, and no saturation was observed at 125 nM. In contrast, 1.25 nM aldo-BSA appeared ineffective in promoting the late (24 h) aldosterone increase in Isc, as shown on Figure 4C. The increase in Isc induced by 2 h of treatment with 1.25 nM aldo-BSA was blocked by the addition of 100 nM phenamil (Figure 5A), as was observed with aldosterone (see Figure 2). The specific PKCα inhibitor GÖ 6976 prevented the increase in Isc induced by aldo-BSA in the 1.25 to 125 nM concentration range, as shown in Figure 5B. We have also questioned whether PKC activity was influenced by aldo-BSA or by the glucocorticod dexamethasone compared with aldosterone. As illustrated in Figure 5C, the increase in PKC activity elicited by aldosterone was reproduced by aldo-BSA but not by dexamethasone 10 nM. In addition, the increase in PKC activity induced by aldo-BSA was inhibited by the PKCα specific inhibitor GÖ (Figure 5D).

Taken together, these results indicate that the nonpermeant molecule aldo-BSA can mimic the early but not the late phase of aldosterone action, suggesting a membrane effect.

Aldosterone Promotes Phosphorylation of Endogenous MR

To get insight into the nongenomic mechanism of action of aldosterone, we examined whether aldosterone and aldo-BSA could influence the MR and GR phosphorylation state. Because anti-MR antibodies cannot detect MR in Western blot, we immunoprecipitated both MR and β-actin from $RCCD_2$ lysates. This allows detecting of MR phosphorylation and evaluation of the amount of immunoprecipitated proteins using the anti–β-actin antibody in the same sample. Figure 6A shows that 15-min treatment of $RCCD_2$ cells with 10 nM aldosterone resulted in an increase in endogenous MR phosphorylation on serine residues, while β-actin signals were comparable. The signal was specific to MR because it was totally displaced by

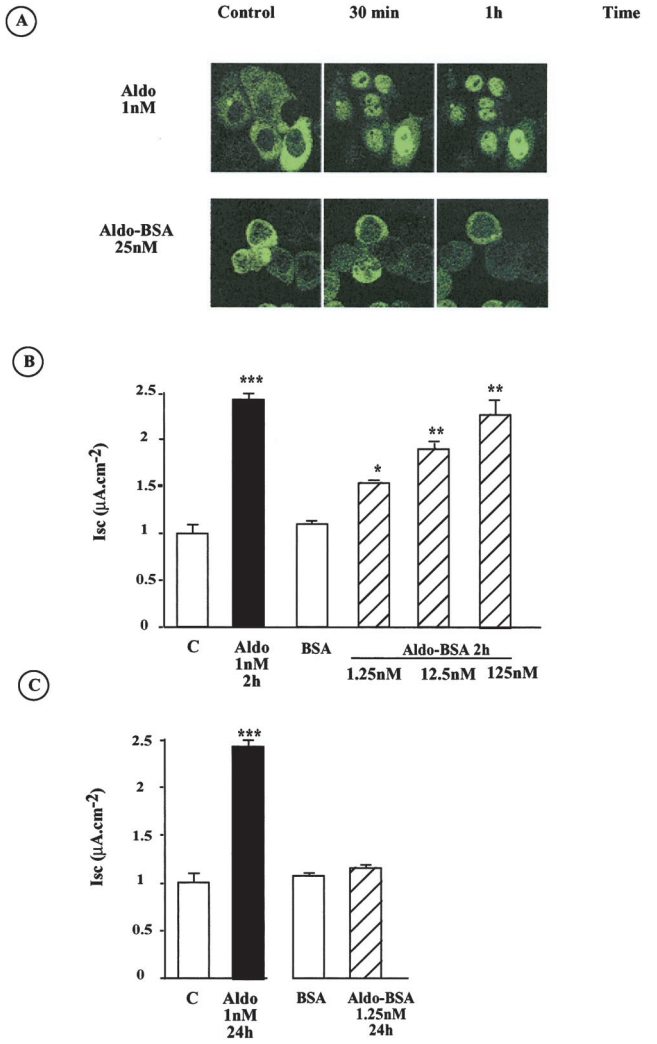


Figure 4. Effect of aldosterone–bovine serum albumin (aldo-BSA) on nuclear translocation of the eGFP-hMR and on Isc in $RCCD_2$ cells. (A) The effect of 1 nM aldosterone or 25 nM aldosterone-BSA on the localization of MR was tested on $RCCD_2$ cells stably transfected with human MR coupled to eGFP; expression of MR-GFP was induced with 4-OH-Tamoxifen (5 h, 10 nM) before incubation of the cells in MM for 2 h (see Materials and Methods). Whereas aldosterone addition resulted in a complete translocation of the eGFP-hMR from the cytoplasm to the nucleus in less than 30 min, aldo-BSA treatment was ineffective to induce MR-GFP translocation, as visualized by confocal microscopy on living cells. (B) The dose-dependency of the effect of aldo-BSA on Isc was evaluated after 2 h treatment. Isc was significantly increased in the presence of aldo-BSA within the 1.25 to 125 nM concentration range. (C) The long-term effects (24 h) of aldosterone and aldo-BSA were tested on Isc. Twenty-four–hour treatment with 1 nM aldosterone induced an increase in Isc in the cells. No increase was observed after 24 h of treatment with 1.25 nM aldo-BSA. In control experiments, the diluent corresponding to the hormone (ethanol 1/1000 for aldosterone or H_2O for BSA and aldo-BSA) was added. Each bar is the mean value of at least six filters from two to four experiments. *$P < 0.05$; **$P < 0.01$, experimental *versus* control without hormone. Panel A is representative of three experiments.

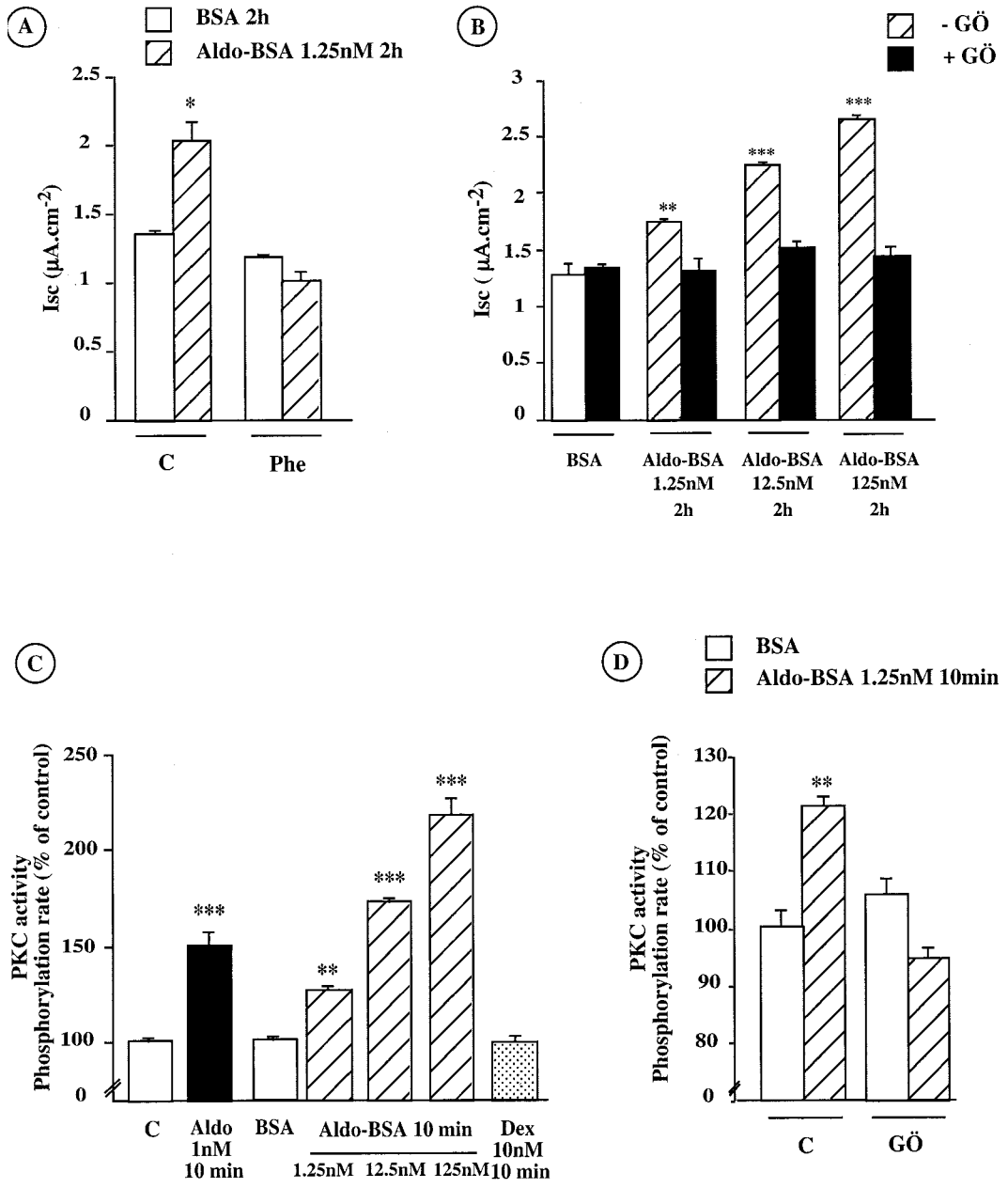


Figure 5. Characteristics of the effect of aldosterone-BSA on Isc and PKC activity. (A) Phenamil (Phe) 100 nM, an inhibitor of epithelial sodium channel (ENaC), was added 1 h before aldo-BSA (1.25 nM). The increase in Isc induced by 2 h of treatment with aldo-BSA was blocked by phenamil. (B) Cells were pretreated (or not) for 1 h with GÖ 6976 (GÖ, 10 nM) and then incubated in the presence of aldo-BSA (1.25 nM-125 nM) for 2 h. The dose-dependent increase in Isc elicited by aldo-BSA was suppressed in the presence of the PKCα inhibitor GÖ. (C) Effects of 10-min exposure to aldosterone, aldo-BSA, or dexamethasone on PKC activity in $RCCD_2$ cells. Aldosterone or aldo-BSA promoted an increase in PKC activity. The effect of aldo-BSA on PKC activity was dose-dependent. Treatment of $RCCD_2$ cells with dexamethasone was ineffective to induce an increase in PKC activity. (D) The increase in PKC activity induced by 10 min treatment with 1.25 nM aldo-BSA was blocked when cells were preincubated for 1 h in the presence of the PKCα inhibitor GÖ6976 (GÖ, 10 nM). In control experiments (open bars), the diluent corresponding to each inhibitor (H_2O for phenamil; DMSO 1/10000 for GÖ or SAPD) was added. The diluent corresponding to aldosterone or dexamethasone (ethanol 1/1000) or aldo-BSA (BSA in H_2O 1/1000) were also added in control conditions. Each bar is the mean value of six to eight filters from at least three experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, experimental *versus* control.

incubation of the anti-MR antibody with the corresponding immunizing peptide during the immunoprecipitation (Figure 6A). The same experiment (with or without aldosterone) was performed with $RCCD_2$ cells transfected with the eGFP-hMR (see Materials and Methods), except that hMR was immunoprecipitated with an anti-GFP antibody. In these experiments, membranes were also blotted with the anti-GFP antibody to verify that the same amount of proteins were immunoprecipi-

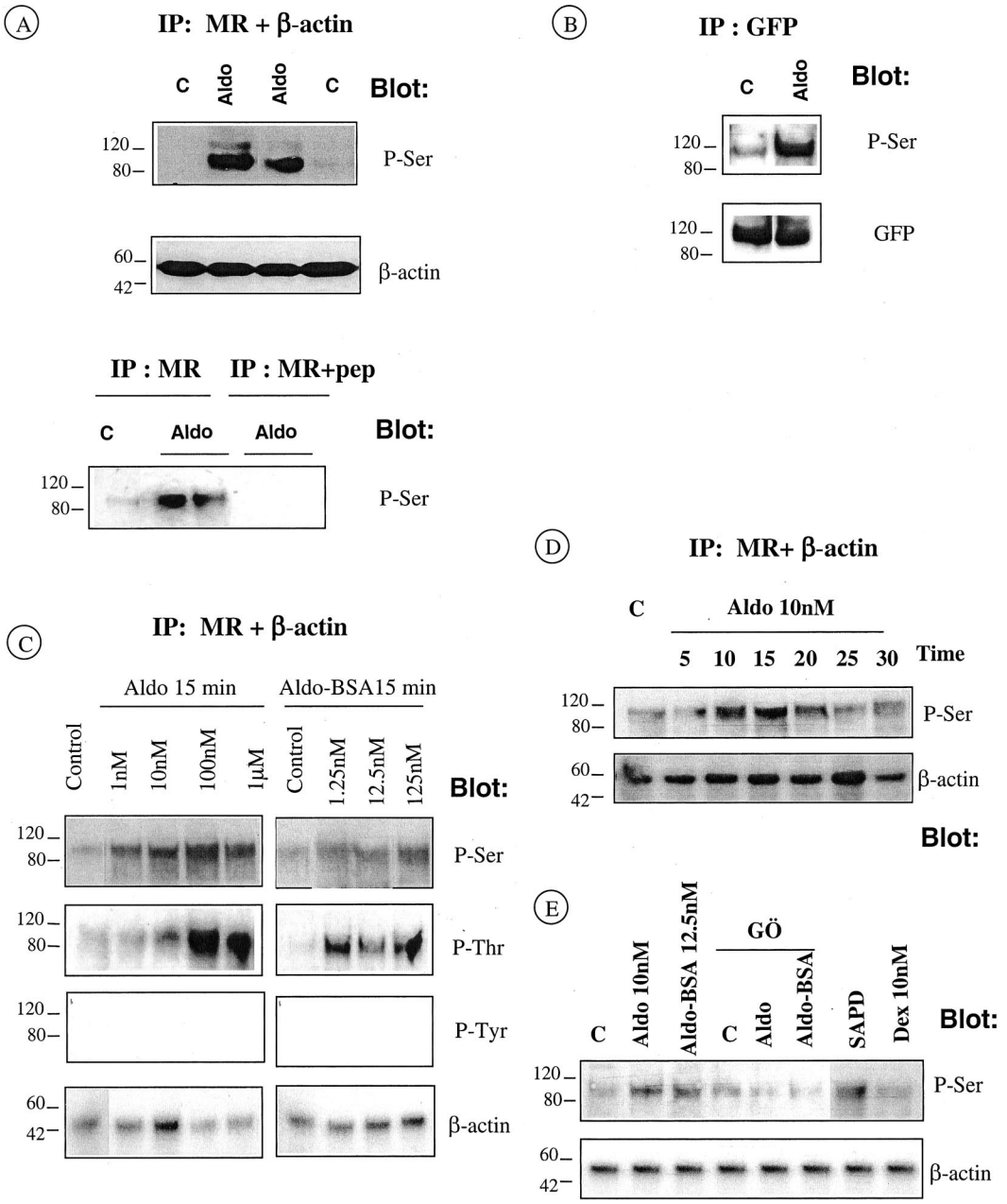


Figure 6. Phosphorylation of the MR in response to treatment with aldosterone and aldosterone-BSA in $RCCD_2$ cells. The endogenous MR and β-actin were immunoprecipitated with the anti-MR and the anti β-actin antibodies. Samples were submitted to electrophoresis. The blots were then incubated with anti-phosphoserine, anti-phosphothreonine, or anti-phosphotyrosine antibodies, as well as with an anti–β-actin antibody to allow estimation of protein loading. (A) The effect of aldosterone (10 nM) for 15 min was tested on the serine phosphorylation state of the endogenous MR in $RCCD_2$ cells. Phosphorylated MR was detected at approximately 100 kD. Aldosterone treatment increased MR phosphorylation on serine residues, while β-actin signals were comparable. The band was totally displaced when MR immunoprecipitation was performed in the presence of immunizing peptide. (B) The effect of aldosterone (10 nM, 15 min) was tested on the phosphorylation state of the eGFP-hMR in eGFP-hMR–transfected $RCCD_2$ cells (see Materials and Methods). In these experiments, the eGFP-hMR was immunoprecipitated with an anti-GFP antibody. Blots were incubated with anti-phosphoserine antibody, then with anti-GFP antibody, after stripping of the same membrane. Whereas the same amount of eGFP-hMR was immunoprecipitated, a clear increase in hMR phosphorylation was observed after treatment with aldosterone. (C) Dose dependency of aldosterone and aldo-BSA effects on endogenous MR phosphorylation. Treatment of the cells with various concentrations of aldosterone for 15 min resulted in a large increase in MR phosphorylation on serine and threonine residues without modification of tyrosines residues. The same pattern of MR phosphorylation was observed when the cells were treated with aldo-BSA for 15 min. Control for protein loading is provided by the β-actin signal. (D) Time course of aldosterone-induced MR phosphorylation on serine residues after treatment with 10 nM aldosterone. Serine phosphorylation of endogenous MR was evidenced 10 to 20 min after hormone addition. (E) Serine-phosphorylation of MR in cells exposed 15 min to aldo-BSA, sapintoxin D (SAPD), or dexamethasone. Low concentrations of aldo-BSA

tated in the two conditions. In this condition, serine phosphorylation of hMR was also detected in response to aldosterone 10 nM (Figure 6B). Figure 6C shows that treatment of $RCCD_2$ cells with increasing concentrations of aldosterone resulted in phosphorylation of the endogenous MR not only on serine but also on threonine residues, without phosphorylation on tyrosine residues. Interestingly, low doses of aldosterone (1 to 10 nM) promoted essentially serine phosphorylation of MR, while higher concentrations affected both serine and threonine residues. Aldo-BSA (1.25 nM) was also effective in inducing serine and threonine phosphorylation of MR (Figure 6C). Results presented in Figure 6D show that aldosterone-induced MR phosphorylation is rapid because it was observed 10 min after aldosterone addition. Finally, it is suggested that the effects of aldosterone and aldo-BSA may be mediated through PKCα because (*1*) MR phosphorylation (in the presence of aldosterone or aldo-BSA) was reduced in the presence of the specific PKCα inhibitor GÖ (Figure 6E) and (*2*) it was reproduced by the specific PKCα activator SAPD 100 nM (Figure 6E). It is noteworthy that dexamethasone (10 nM) was ineffective in promoting MR phosphorylation.

The phosphorylation state of the endogenous GR is also affected by the presence of aldosterone, as shown in Figure 7A; high doses of the hormone promoted serine phosphorylation of GR (without any detectable signal using anti-phosphothreonine or anti-phosphotyrosine antibodies). Dexamethasone (10 nM) also induced serine-phosphorylation of the GR (Figure 7B). In contrast, aldosterone (10 nM) or aldo-BSA (1.25 nM) were ineffective (Figure 7B).

These results indicate that aldosterone (1 to 10 nM) and aldo-BSA (1.25 nM) can phosphorylate MR but not GR. Their effect is rapid and appears to be mediated, at least in part, through the PKCα signaling pathway.

Cross-Talk between the PKCα Signaling Cascade and the Genomic Pathway Is Required to Trigger the Late Response to Aldosterone in $RCCD_2$ Cells

To establish whether early nongenomic and late genomic responses to aldosterone were linked, we evaluated whether the late aldosterone-induced increase in Isc was influenced by the PKC inhibitors CC (100 nM) and GF (100 nM) or by the PKCα inhibitor GÖ (10 nM). Pretreatment of the cells for 1 h before aldosterone addition with GF or GÖ prevented the late (24 h) aldosterone-induced increase in Isc. In contrast, pretreatment of the cells with H89, a PKA inhibitor that did not modify the early response, did not influence the late aldosterone effect (Figure 8A). In the presence of the PKC inhibitor CC, pretreatment of cells 1 h before aldosterone addition also prevented the late (24 h) aldosterone-induced increase in Isc (Figure 8B). However, when CC was added only 2.5 h after aldosterone addition (*i.e.*, after the initial phase of aldosterone action), the late aldosterone response was fully observed. In addition, treatment for 24 h with dexamethasone induced an increase in Isc, which was not prevented by 1-h pretreatment with CC (Figure 8C). This result is consistent with the absence of PKC activity stimulation by dexamethasone (Figure 5C). These results suggest that the early stimulation of PKC activity by aldosterone is necessary to obtain the late response to the hormone.

We then examined whether the inhibition of the PKC pathway could alter the transcriptional effect of endogenous corticosteroid hormone receptors on a reporter gene (Figure 9) or on an aldosterone-regulated gene, the α1 subunit of NKA (Figure 10). The aldosterone-induced or dexamethasone-induced increases in endogenous MR or GR transactivation activities were examined using a reporter gene driving luciferase (Figure 9). This activity was blocked when cells were preincubated for 1 h with 100 nM CC or 10 nM GÖ (Figure 9A). In contrast, the response to dexamethasone (10 nM) was not affected by CC (Figure 9B). We also noted that the effects of aldosterone and dexamethasone on transactivation activity were blocked by RU26752 and RU486, respectively. In these experiments, the magnitude of the transcriptional response of endogenous MR to aldosterone was much smaller than that yielded in classical transactivation assays including MR transfection. This is probably related to the difference in MR expression levels (low *versus* high) in native $RCCD_2$ *versus* transiently transfected cells; it may also be due to other transcriptional modulators that vary among cell lines (20). In $RCCD_2$ cells treated for 24 h with aldosterone (Figure 10A) or dexamethasone (Figure 10B), we observed that the aldosterone-induced increase in the amount of α1 NKA mRNA was prevented by pretreatment with 100 nM CC (Figure 10A). In contrast, H89 did not block the effect. Figure 10B shows that dexamethasone also increased the amount of mRNA encoding for α1 NKA. However, neither CC nor H89 affected the dexamethasone-induced increase in α1-NKA mRNA.

Discussion

It is generally considered that the mechanism of action of aldosterone is dependent on MR-mediated transcriptional gene activation (3–5). Some years ago, it was proposed that it could also involve nongenomic effects in mammalian cells and that part of the response to the hormone could depend on modifications of preexisting proteins (7,21–25). Thereafter, it was shown that aldosterone could increase rapidly the intracellular concentration of Ca^{2+} and/or modify pH_i, PKC activity, or intracellular cAMP concentration (8,21–25). Rapid responses to aldosterone have been shown to occur at very low hormone

(1.25 nM) elicited MR phosphorylation on serine residues at level close to that induced by 1 nM aldosterone. Pretreatment (1 h) with the PKCα inhibitor GÖ 6976 (GÖ, 10 nM) prevented MR phosphorylation. The specific PKCα activator SAPD 100 nM elicited MR phosphorylation. In contrast, dexamethasone had no effect. In control experiments (C), the diluent corresponding to each inhibitor (DMSO 1/10,000 for GÖ or SAPD) was added. The diluents corresponding to aldosterone or dexamethasone (ethanol 1/1000) or aldo-BSA (BSA in H_2O) were also added in control conditions. Each panel is representative of at least three individual experiments.

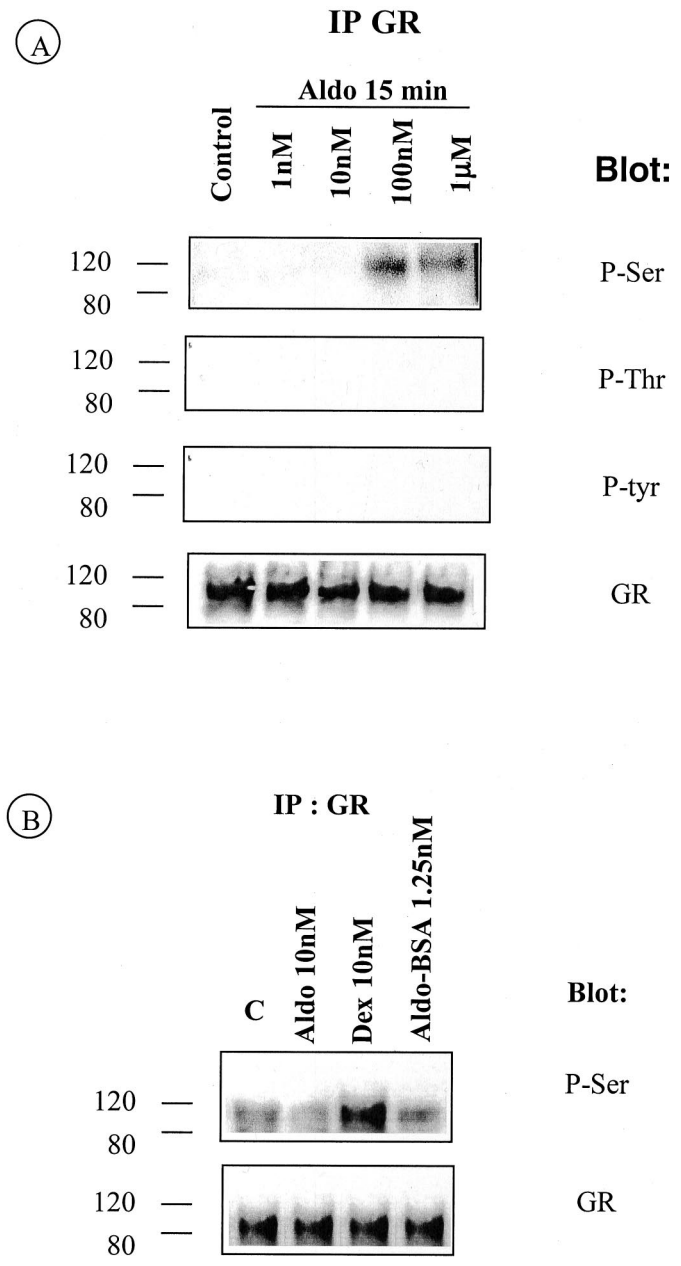


Figure 7. GR phosphorylation in response to aldosterone, aldosterone-BSA, and dexamethasone. Endogenous GR was immunoprecipitated using an anti-GR antibody. Western blot of the immunoprecipitated protein was done with antibodies against phosphoserine, phosphothreonine, and phosphotyrosine residues, as well as with the anti-GR antibody to assess for protein loading. (A) Effect of aldosterone on phosphorylation of GR in $RCCD_2$ cells. GR phosphorylation on serine residues was apparent only with the two highest aldosterone concentrations tested (100 nM and 1 μM), while no phosphorylation on threonine and tyrosine residues was apparent. GR signals were equivalent in all lanes. (B) Effect of aldosterone, aldo-BSA, and dexamethasone on GR serine phosphorylation. In conditions where the same amounts of GR were immunoprecipitated, a clear increase in GR phosphorylation could be observed in response to dexamethasone, whereas no effect of aldosterone or aldo-BSA was evidenced.

concentrations and with steroid specificities somewhat different from those involving the nuclear MR (7,21).

To get new insight into the early phase of aldosterone action and its influence on the late phase of aldosterone response, we used the $RCCD_2$ rat collecting duct cell line, which expresses the MR and is sensitive to physiologic doses of aldosterone (12). Several mammalian CCD cell lines with aldosterone responsiveness features have been generated, such as the M1 cells (with essentially nongenomic effects [24]) and the mpkCCD_{cl4} (15) originating from mouse or MDCK (from dog) (22,23). Both mouse mpkCCD_{cl4} and rat $RCCD_2$ cells express the MR and the GR and exhibit an increase in Isc after exposure to low doses (an effect via MR occupancy) as well as high doses of aldosterone (via the GR). Aldosterone (500 nM) effects in mpkCCD_{cl14} have been characterized and attributed mainly to GR occupancy (15). On the other hand, the consequences of exposure to low doses of the hormone (1 to 10 nM) have been specifically examined in $RCCD_2$ cells (12), including its ability to increase transcripts encoding for NDRG2, an aldosterone-specific early response gene (16). Interestingly, the early response (2 h) to aldosterone varies somewhat with experimental conditions. It has been shown in mpkCCD_{cl4} cells that 2-h exposure to 500 nM aldosterone (acting presumably through the GR) induces an increase in Isc that is fully dependent on transcription and translation (15). Conversely, concentrations of actinomycin and cycloheximide similar to those used in mpkCCD_{cl4} did not suppress the early response to low doses of the hormone (1 nM) in $RCCD_2$ cells. This may be indicative of distinct early events in CCD cells, depending on the dose of aldosterone. Both nongenomic and genomic phenomena appear to be sequentially detectable at low aldosterone concentrations, while high concentrations trigger essentially a (GR-mediated) genomic response.

We have shown that the early phase of response to low hormone concentrations in $RCCD_2$ cells is characterized by an early (2 h) increase in amiloride-sensitive Isc that is not prevented by actinomycin D or cycloheximide, although these inhibitors do prevent the late (4 and 24 h) aldosterone-induced increase in Isc. Such an effect is insensitive to MR and GR antagonists; it is reproduced by aldo-BSA (1.25 nM). The early increase in Isc (elicited by aldosterone or by aldo-BSA) is suppressed in the presence a PKC inhibitor (CC), or PKCα inhibitor (GÖ), but not by PKA inhibitor (H89). Both aldosterone and aldo-BSA promote a transient stimulation of PKC activity, which is not reproduced by dexamethasone, a GR ligand. The early response to aldosterone reported here appears to correspond to a sequence of events, including an initial and transient increase in PKC activity (5 to 20 min) accompanied by MR phosphorylation, leading to an increase in Isc (which reaches statistical significance 2 h after aldosterone addition). Although the integrated consequence of such events (ion transport) develops rather slowly, these successive events have characteristics of an early, nongenomic response, in view of the overall slow kinetics of aldosterone action in collecting duct cells. From a general point of view, nongenomic actions of steroid hormones are characterized by (*1*) the insensitivity of the steroid hormone effect to inhibitors of transcription and

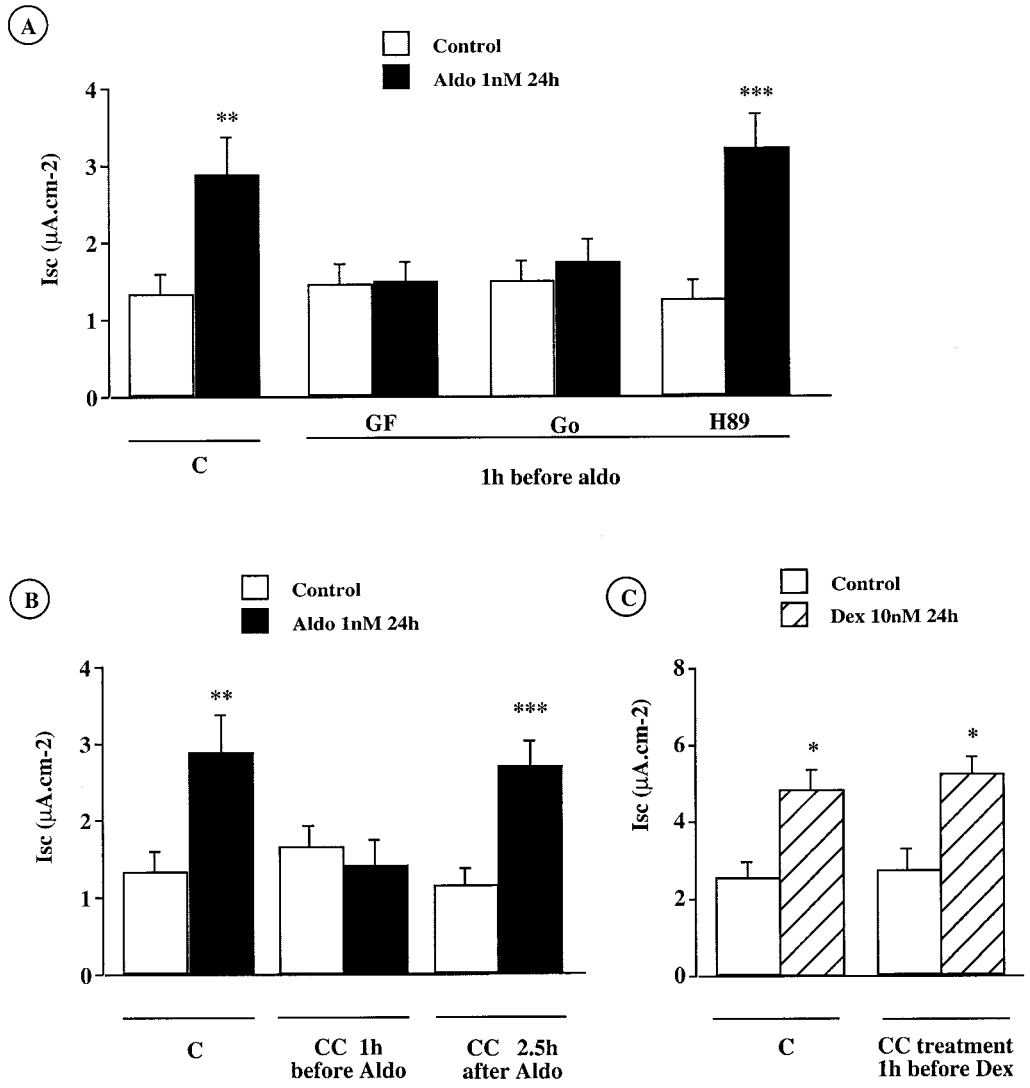


Figure 8. Effect of pretreatments with inhibitors of PKC or PKA on the long-term aldosterone-induced increase in Isc. (A) $RCCD_2$ cells were pretreated (1 h) with the PKC inhibitor GF 109203X (GF, 100 nM), the specific PKCα inhibitor GÖ 69076 (GÖ, 10 nM), or the PKA inhibitor H89 (100 nM) before aldosterone addition and thereafter. Isc was measured 24 h later (late aldosterone response). Treatment of $RCCD_2$ cells for 24 h with 1 nM aldosterone resulted in a significant increase in Isc. Pretreatment of the cells with GF or GÖ for 1 h before aldosterone addition blunted aldosterone effect. In contrast, H89 did not modify aldosterone effect. (B) Effects of the PKC inhibitor chlerethrine chloride (CC). Pretreatment of the cells with CC (100 nM) for 1 h before aldosterone addition did not alter the aldosterone-induced Isc. In contrast, when CC was added 2.5 h after addition of the hormone, this inhibition was not observed, although CC was maintained up to 24 h. (C) Exposure of $RCCD_2$ cells to dexamethasone (10 nM) for 24 h resulted in an increase in Isc; such effect was not prevented by pretreatment with the PKC inhibitor CC (100 nM). Situation C corresponds to the absence of inhibitors. In control experiments (open bars), the diluent corresponding to each inhibitor (H_2O for H89 and CC, DMSO 1/10,000 for GÖ) or to the hormone (ethanol 1/1000 for aldosterone and dexamethasone) was added. Each bar is the mean value of four to nine filters from four experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, experimental *versus* control without hormone but with inhibitor.

translation; (*2*) their reproductibily using steroids coupled to high molecular weight molecules that do not enter the cells; (*3*) a rapid time-course; and (*4*) a much higher sensitivity to the hormone than that mediated by the classical nuclear receptor (*7*). Data obtained in this study met some of these criteria, suggesting nongenomic effects of aldosterone in $RCCD_2$ cells. The existence of a membrane receptor for aldosterone has been suggested by several investigators, but it has yet to be identified. Early nongenomic effects of steroid hormones such as glucocorticoids, progesterone, estrogens, and androgens are not yet fully understood (7,26); these nongenomic processes may involve classical steroid receptors located in the membrane, G-protein–coupled receptors or membrane-associated steroid-binding proteins (26).

It appears from our study that the PKCα signaling pathway may be essential for mediating the early response to aldosterone in a cell line derived from the CCD. These results are in good accordance with previous reports in which a nongenomic

effect of aldosterone on the PKC activity has been documented in different tissues, such as the kidney or the distal colon (18,21,25). In these studies, the effect of aldosterone on PKC activity was made clear, but its relationship to the stimulation of ion transport, particularly transepithelial sodium transport, was not investigated. In this study, we show that PKC activation is necessary to observe the early aldosterone-induced increase in sodium transport. In addition, we provide evidence that both aldosterone and aldo-BSA (but not dexamethasone) are able to increase PKC activity in a time-dependent and dose-dependent manner. The effect is rapid and transient. Treatment of $RCCD_2$ cells with aldosterone leads to a rapid translocation of PKCα from the cytosolic to the membrane fraction. Such a translocation has been reported to correspond to an activation of PKC in response to various stimuli (27). Along the same line, it has been recently proposed that aldosterone could activate PKCα in the colon by direct binding to this protein; this could constitute one of the important initial events in aldosterone action (18).

In this study, we show that one of the primary effects of aldosterone consists of rapid phosphorylation of the endogenous MR, which is reproduced by aldo-BSA. Phosphorylation has been documented for the thyroid or estrogen nuclear receptors, which are rapidly phosphorylated on serine residues after hormone addition (28,29). Along this line, it has also been shown that the rat kidney native MR may undergo different phosphorylation states that influence its activity (30). Galigniana (30) showed the importance of MR phosphorylation in determining its activation and showed that it could be modulated through the activity of kinase/phosphatases affecting serine/threonine residues. Our results are compatible with these data because aldosterone led to an increase in the phosphorylation of MR on serine and threonine residues (without effect on tyrosine residues). Several putative phosphorylation sites are predicted within the rat MR protein. Depending on the program used (Phosphobase; Scansite), 10 to 12 PKC-dependent serine phosphorylation sites and 2 threonine phosphorylation sites can be identified in rat MR. Further studies should address the functional relevance of these phosphorylation sites. We also found that aldosterone effect is specific to MR because GR phosphorylation is not modified except in the presence of high concentrations of aldosterone. In these conditions, GR is phosphorylated only on serine residues. It is interesting to note that phosphorylation of GR on serine residues has been reported in response to different stimuli (31). The aldosterone-induced MR phosphorylation was blocked by the PKCα specific inhibitor GÖ 6976 and was reproduced by aldo-BSA, indicating that a membrane-initiated signaling pathway is probably involved in the phenomenon. Along this line, we observed that the specific PKCα activator sapintoxin D can also phosphorylate MR. In contrast, Aldo-BSA was ineffective at phosphorylating GR, indicating an MR-specific pathway. Whether PKCα phosphorylates MR directly or indirectly will have to be determined by further studies. Of interest, Massaad *et al.* (32) have shown that the human mineralocorticoid receptor function can be modulated by PKA phosphorylation of an unidentified protein, probably indirectly, by relieving the effect of an MR repressor. Likewise, a recent study by Wong *et al.* (33) describes a nuclear receptor-interacting protein designated as modulator of nongenomic activity of estrogen receptor (MNAR) that affects estrogen receptor (ER) transcriptional activity, and ultimately ER-mediated gene expression, through activation of the Src/Erk phosphorylation cascade. This appears to be essential in the interrelation between ER genomic and nongenomic activities.

A main issue from this study is that full aldosterone action involves a putative cross-talk between genomic and nongenomic pathways. We propose that late aldosterone effects on ion transport may be modulated by the nongenomic PKC

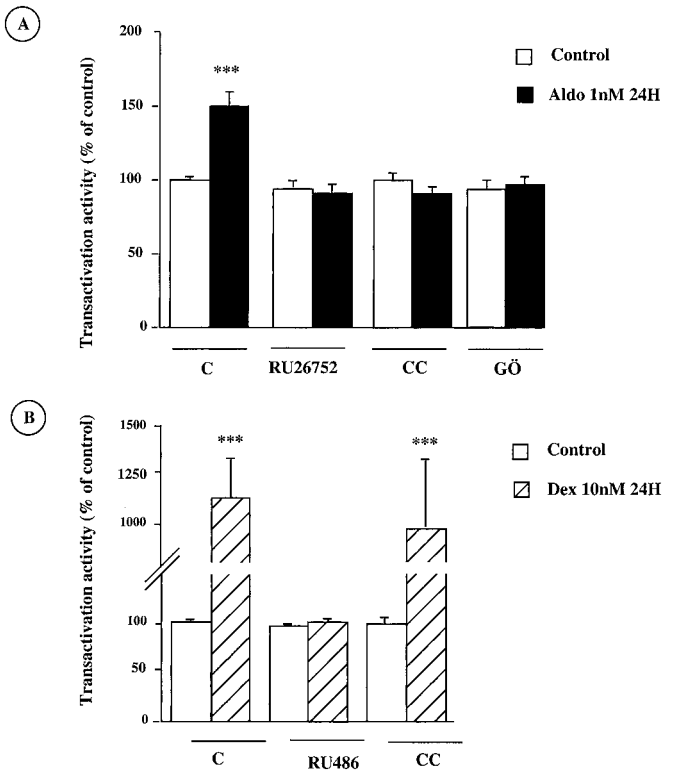


Figure 9. Effect of PKC inhibitors on the transactivation activity of the endogenous corticosteroid hormone receptors MR and GR. (A) Treatment with 1 nM aldosterone of $RCCD_2$ cells stably transfected with a reporter gene (see Materials and Methods) resulted in a significant increase in transactivation activity of the endogenous MR. Pretreatment (1 h) of the cells with CC (100 nM) and GÖ (10 nM) blocked the long-term aldosterone-induced increase in transactivation activity. This activity was also blocked by the MR antagonist RU26752. (B) Treatment of $RCCD_2$ cells with dexamethasone 10 nM resulted in a large and significant increase in transactivation activity. Pretreatment (1 h) of the cells with the specific antagonist of the glucocorticoid receptor RU486 (1 μM) blocked this increase (thus corresponding to endogenous GR), whereas pretreatment of the cells with CC had no effect. Situation C corresponds to the absence of inhibitors. In control experiments (open bars), the diluent corresponding to each inhibitor or antagonist (H_2O and CC; DMSO 1/10,000 for GÖ; ethanol 1/1000 for RU26752 and RU486) or to the hormone (ethanol 1/1000 for aldosterone and dexamethasone) was added. Bars are the mean values of six different experiments. ***$P < 0.001$, aldosterone or dexamethasone *versus* control without hormone.

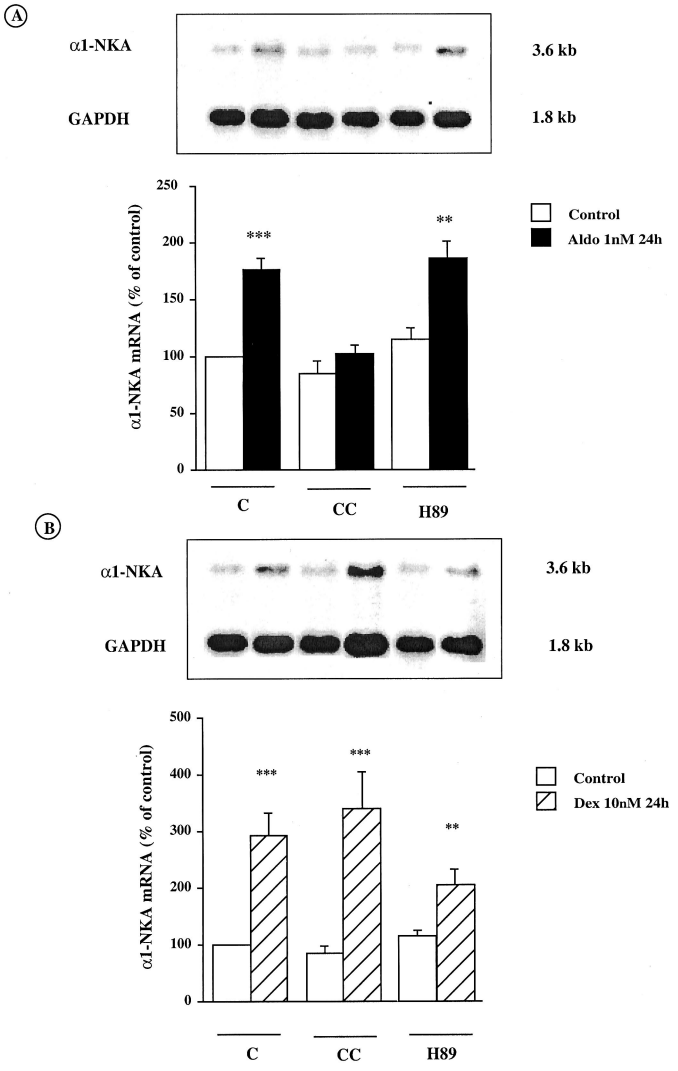


Figure 10. Effect of a PKC inhibitor on the long-term aldosterone-induced increase in α1 NKA mRNA expression. $RCCD_2$ cells were pretreated (1 h) with either the PKC inhibitor chlerethrine chloride (CC, 100 nM) or the PKA inhibitor (H89, 100 nM), followed by aldosterone or dexamethasone treatment. The α1 NKA mRNA expression was examined by Northern blot. (A) Treatment of $RCCD_2$ cells with aldosterone (1 nM) resulted in a significant increase in the amount of mRNA encoding for α1 NKA. A representative gel is shown in inset. Pretreatment of the cells with CC blocked this effect, whereas H89 had no inhibitory effect. GAPDH was used as an internal control, and results are normalized for the GAPDH mRNA abundance. (B) Treatment of $RCCD_2$ cells with dexamethasone (10 nM) resulted in a significant increase in the amount of mRNA encoding for α1 NKA. The inset is a representative experiment. In contrast to results obtained with aldosterone, pretreatment of the cells with CC did not block dexamethasone effect; H89 did not reduce it as well. Situation C corresponds to the absence of inhibitors. In control experiments (open bars), the diluent corresponding to each inhibitor (H_2O for H89 and CC) or to the hormone (ethanol 1/1000 for aldosterone and dexamethasone) was added. Bars are the mean values of six different experiments. $^{**}P < 0.01$; $^{***}P < 0.001$, aldosterone or dexamethasone *versus* control without hormone.

signaling cascade. This hypothesis is based on the effects of the PKC inhibitor chlerethrine chloride, which prevents the late aldosterone-induced increase in Isc, and the transactivation activity of the MR on a transfected reporter gene as well as on an endogenous promoter (α1 subunit of NKA). Interestingly, the addition of the PKC inhibitor after the early response (Figure 8) allows development of the late aldosterone-induced increase in Isc. Other elements participating in this cascade of events are still unkown, for aldosterone as well as for other steroid hormones. Indeed, links between genomic and nongenomic signals promoted by steroid hormones are complex and far from being understood. As recently evoked by Hammes (26), they "will likely be critical for understanding the diverse biologic responses to steroids."

In conclusion, our experiments show that in the $RCCD_2$ rat CCD cell line the early increase in transepithelial sodium transport elicited by low doses of aldosterone does not depend on transcriptional events and is mediated through the PKCα signaling pathway. It is accompanied by serine and threonine phosphorylation of the endogenous MR. Interestingly, activation of this PKCα signaling cascade appears as a key event in the development of the genomic response; blockade of this initial pathway prevents the late response to aldosterone. Future studies should help to clarify the sequence of cellular events leading to activation of the aldosterone-induced signaling cascades and their molecular counterparts.

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