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### **Research Article**

Heat shock proteins (HSP) are components of the steroid receptor complex and are released into the cell cytosol after hormone binding. We tested whether HSPs released from steroid receptors mediate an increase in calcineurin phosphatase activity by steroid hormones. Aldosterone increased calcineurin activity in microdissected rat cortical collecting ducts (CCD) and connecting tubules, but not in proximal tubules, medullary thick ascending limb, or outer medullary collecting ducts. In contrast, 5 microM dexamethasone increased calcineurin activity in both CCD and proximal tubules. Aldosterone increased CCD calcineurin activity after 30 min and this response was blocked by spironolactone, but not by actinomycin D. An antibody recognizing HSP-56 did not change basal calcineurin activity, but completely blocked the stimulation of calcineurin by aldosterone. Rapamycin, an immunosuppressive drug that stabilizes the HSP-steroid receptor complex, also blocked the aldosterone response, whereas HSP-90 or HSP-70 increased calcineurin activity in permeabilized CCD. In summary, (a) aldosterone increases calcineurin activity in CCD through a transcription-independent process; (b) maneuvers inactivating HSP-56 or slowing HSP disassociation from the receptor complex blocks stimulation of calcineurin by steroid hormones; (c) HSP-90 and HSP-70 increase CCD calcineurin activity in the absence of steroid hormone. We conclude that HSPs released from transformed steroid receptors can stimulate calcineurin activity through a transcription-independent pathway.

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# Aldosterone and Dexamethasone Stimulate Calcineurin Activity through a Transcription-independent Mechanism Involving Steroid Receptor-associated Heat Shock Proteins

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## Abstract

Heat shock proteins (HSP) are components of the steroid receptor complex and are released into the cell cytosol after hormone binding. We tested whether HSPs released from steroid receptors mediate an increase in calcineurin phosphatase activity by steroid hormones. Aldosterone increased calcineurin activity in microdissected rat cortical collecting ducts (CCD) and connecting tubules, but not in proximal tubules, medullary thick ascending limb, or outer medullary collecting ducts. In contrast, 5  $\mu$ M dexamethasone increased calcineurin activity in both CCD and proximal tubules. Aldosterone increased CCD calcineurin activity after 30 min and this response was blocked by spironolactone, but not by actinomycin D. An antibody recognizing HSP-56 did not change basal calcineurin activity, but completely blocked the stimulation of calcineurin by aldosterone. Rapamycin, an immunosuppressive drug that stabilizes the HSP-steroid receptor complex, also blocked the aldosterone response, whereas HSP-90 or HSP-70 increased calcineurin activity in permeabilized CCD.

In summary, (a) aldosterone increases calcineurin activity in CCD through a transcription-independent process; (b) maneuvers inactivating HSP-56 or slowing HSP disassociation from the receptor complex blocks stimulation of calcineurin by steroid hormones; (c) HSP-90 and HSP-70 increase CCD calcineurin activity in the absence of steroid hormone. We conclude that HSPs released from transformed steroid receptors can stimulate calcineurin activity through a transcription-independent pathway. (*J. Clin. Invest.* 1997; 99:1217–1223.) Key words: calcineurin • cortical collecting duct • steroid receptor • heat shock proteins • transcription

## Introduction

The steroid hormone receptor is a heteromeric 8-9S complex of proteins which includes the steroid-binding protein and heat

shock proteins (HSP)<sup>1</sup> 90, 70, and 56. The binding of hormone to the receptor complex stimulates the release of HSPs followed by transnuclear migration and DNA binding of the activated receptor (1). The role of HSPs in receptor function is unknown, but recent studies indicate that HSP-90 facilitates anchoring of unbound glucocorticoid and mineralocorticoid receptors to the cytoskeleton and maintains the hormone binding protein in a high affinity conformation (2, 3).

Most antinatriuretic effects of aldosterone require transcription or translation of Na<sup>+</sup> transporting proteins (4–6). However, recent studies demonstrate that aldosterone can stimulate Na<sup>+</sup> uptake in the kidney and vascular smooth muscle through processes that are independent of transcription or translation (7, 8). For example, Fuji et al. (7) found that aldosterone stimulates ouabain-sensitive Rb<sup>86</sup> uptake in rat cortical collecting ducts (CCD) within 30 min. This response persisted even when tubules were incubated with actinomycin D (7). Christ et al. (8) demonstrated that aldosterone increases Na/H antiporter activity in vascular smooth muscle within 15 min; incubation with actinomycin D or cycloheximide did not block this effect. These results indicate that aldosterone has effects on Na<sup>+</sup> transport that are independent of transcription or translation and suggests that additional pathways mediate steroid hormone signal transduction.

In considering what other pathways might be involved, we investigated whether HSPs released from the receptor complex can activate signaling processes through interactions with other proteins. Because HSPs 90, 70, and 56 avidly bind calmodulin, we investigated whether HSPs released from the steroid receptor complex could influence the activities of calmodulin-dependent enzymes including calcineurin (9, 10). Calcineurin is a calmodulin-dependent, serine-threonine phosphatase that regulates sodium transport proteins in the distal nephron (11–13). Three isoforms ( $\alpha$ -1,  $\alpha$ -2,  $\alpha$ -3) of the calcineurin  $\alpha$  subunit have been identified that are similar in substrate affinity and kinetics properties, but differ in tissue distribution (14). We have shown that the  $\alpha$ -1 isoform predominates in the proximal tubule, whereas  $\alpha$ -2 is more common in CCD and medullary thick ascending limb (mTAL) (13).

In this article, we have investigated the effects of steroid hormones on calcineurin activity in specific segments of the rat nephron. To determine whether HSPs released from the steroid receptor complex mediate changes in calcineurin activity, maneuvers to block receptor disassociation and to bind specific HSPs after release were performed.

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1. *Abbreviations used in this paper:* CCD, cortical collecting ducts; CNT, connecting tubules; EC-1, mAb against heat shock protein; HSP, heat shock protein; mTAL, medullary thick ascending limb; OMCD, outer medullary collecting ducts; S2, proximal tubules.

## Methods

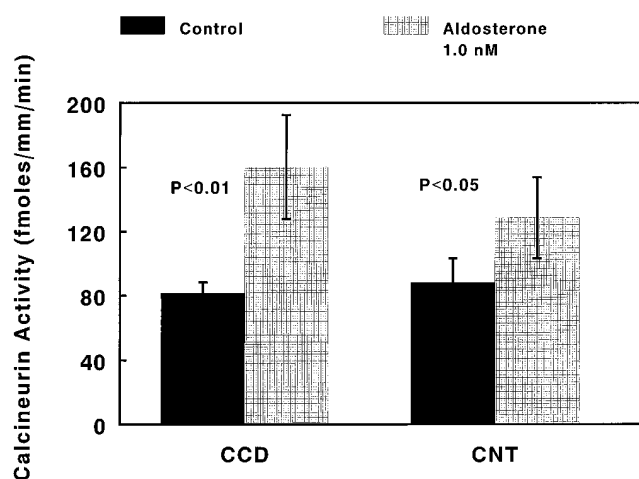
**Tissue preparation.** Pathogen-free male Sprague-Dawley rats (75–100 g) were anesthetized with Nembutal (2 mg/kg body wt i.p.; Abbott Laboratories, Chicago, IL). The left kidney was perfused with 20 ml of an ice-cold dissection solution containing 1 mg/ml of collagenase (type A; Boehringer Mannheim, Indianapolis, IN) and albumin (fatty acid free, 98–99% pure; Sigma Chemical Co., St. Louis, MO) (15). Cortical and outer medullary slices were incubated in a shaking water bath for 45 min to 1 h at 37°C in 1 ml of the collagenase solution containing (mM): NaCl 137; KCl 5; MgSO<sub>4</sub> 0.8; Na<sub>2</sub>HPO<sub>4</sub> 0.33; KH<sub>2</sub>PO<sub>4</sub> 0.44; MgCl<sub>2</sub> 1.0; Tris-HCl 10.0; and CaCl<sub>2</sub> 0.25. All samples were suffused with 100% O<sub>2</sub> during the incubation.

**Calcineurin substrate (calspec).** A calcineurin-specific substrate was synthesized from the RII regulatory subunit of protein kinase A and labeled with <sup>32</sup>PO<sub>4</sub> using the catalytic subunit of protein kinase A (Sigma Chemical Co.) and [ $\gamma$ -<sup>32</sup>P]ATP (10 Ci/mMol; New England Nuclear, Boston, MA) (13). The radiolabeled peptide was separated from unincorporated [ $\gamma$ -<sup>32</sup>P]ATP using a Sep-Pak C18 Sephadex column (Millipore Corp., Milford, MA) and 20 ml of 0.1% TFA. The peptide was eluted with 50% acetonitrile in 0.1% TFA; the protein content measured using a BCA protein assay kit (Pierce Immuno-technology, Rockford, IL). Labeled peptide was kept frozen at –80°C until needed.

**Calcineurin activity in microdissected tubules.** Individual nephron segments were isolated by microdissection and the length was measured before the tubules were permeabilized with imidazole (100 mM), hypotonic shock, and rapid freeze/thawing (16). Calcineurin activity was measured in the reaction buffer: Tris, pH 8.0, 20 mM; NaCl, 100 mM; DTT, 0.5 mM; CaCl<sub>2</sub>, 0.1 mM; BSA, 0.1 mg/ml; calmodulin, 100 nM; and 100 nM calyculin, a specific inhibitor of class 1 and 2A phosphatases (L.C. Laboratories, Boston, MA). Identical results were obtained with or without DMSO (the diluent for steroid hormones). Distilled H<sub>2</sub>O was added to a final reaction volume of 10  $\mu$ l. All chemicals were purchased from Sigma unless otherwise indicated. Aldosterone (1.0 nM) and dexamethasone (5.0  $\mu$ M), were dissolved in DMSO and incubated with tubules at 37°C for 30 min. Subsequently, radioactive and nonradioactively labeled calcineurin-specific peptide (calSpec) was added to each reaction tube (final concentration 50 and 200 pM, respectively) and incubated for an additional 30 min. The reaction was stopped by adding 500  $\mu$ l of an ice-cold mixture of 10% TCA and 5% activated charcoal. The reaction tubes were centrifuged at 14,000 rpm for 15 min and the supernatant filtered through a 450- $\mu$ m nitrocellulose filter (Millipore Corp.). Total radioactivity released from the <sup>32</sup>P-labeled substrate was counted by liquid scintillation and calcineurin activity calculated as femtomoles substrate hydrolyzed per mm tubule per min (13).

**Calcineurin  $\alpha$  subunit isoform expression in adrenalectomized rats.** Male Sprague-Dawley rats were adrenalectomized through a flank incision and given normal saline (154 meq/liter) to drink for 7–10 d before being killed. Individual nephron segments from the left kidney were isolated by microdissection and emulsified in 50  $\mu$ l of “high test” buffer containing proteinase inhibitors as described (17). Total protein was measured and size-separated using 7.5% SDS-PAGE (polyacrylamide gel, 7.5%). Each lane was loaded with 20  $\mu$ g protein and rat brain protein was used as a positive control for all  $\alpha$  subunit isoforms (negative controls containing no primary antibodies were included with each experiment). Separated tubule proteins were transferred to a nitrocellulose membrane by electroblotting (18) and incubated overnight with polyclonal antibodies directed against the  $\alpha$ -1 and  $\alpha$ -2 isoforms of the catalytic subunit (kindly provided by Dr. G. Weiderach, Merck Research Laboratories, Rahway, NJ). Autoradiography was performed using chemiluminescence and peroxidase-labeled goat anti-rabbit Ig antibodies according to the manufacturer’s protocol (ECL Western Blot kit; Amersham Corp., Arlington Heights, IL) (16).

Statistical significance was calculated by a Student’s *t* test with a value of *P* < 0.05 being significant. To compare results from three or

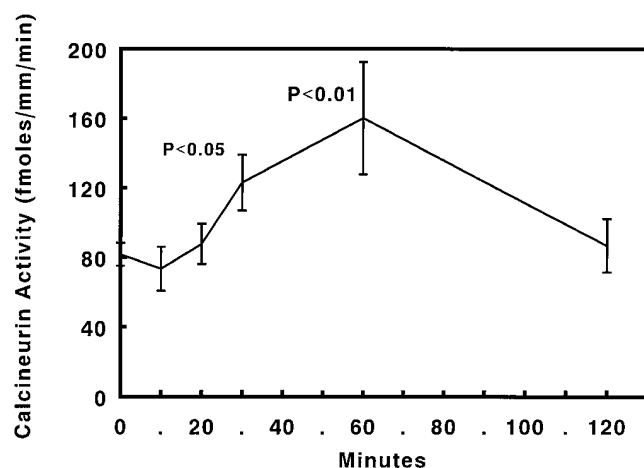


**Figure 1.** Aldosterone stimulates collecting duct calcineurin activity. Microdissected CCD (*n* = 37) and CNT (*n* = 9) were incubated for 1 h with 1 nM aldosterone. Aldosterone significantly stimulated calcineurin activity both segments.

more groups, one-way ANOVA was used followed by a multiple-comparisons, protected *t* test to determine which values were significantly different.

## Results

**Aldosterone stimulates calcineurin activity in specific nephron segments.** After 60 min, aldosterone (1.0 nM) increased calcineurin activity (*P* < 0.05) in CCD and connecting tubules (CNT) by 96 and 46%, respectively (Fig. 1). The time course for the increase in CCD shows significant (*P* < 0.05) stimulation within 30 min, with maximum activity at 60 min (*P* < 0.01), followed by a return to control values by 120 min (Fig. 2). Dexamethasone (5  $\mu$ M) increased calcineurin activity in the CCD (*P* < 0.01) (Fig. 3) and the proximal tubule (control activity: 779 ± 75, dexamethasone (5  $\mu$ M): 1,073 ± 81) (*P* < 0.01) (data not shown). In contrast, aldosterone did not change cal-



**Figure 2.** Time-dependent stimulation of calcineurin activity in the CCD. Aldosterone (1 nM) significantly (*P* < 0.05) stimulates calcineurin activity in the CCD (*n* = 8) at 30 and 60 min (*n* = 16). Enzyme activity at 20 and 120 min did not differ from control values.

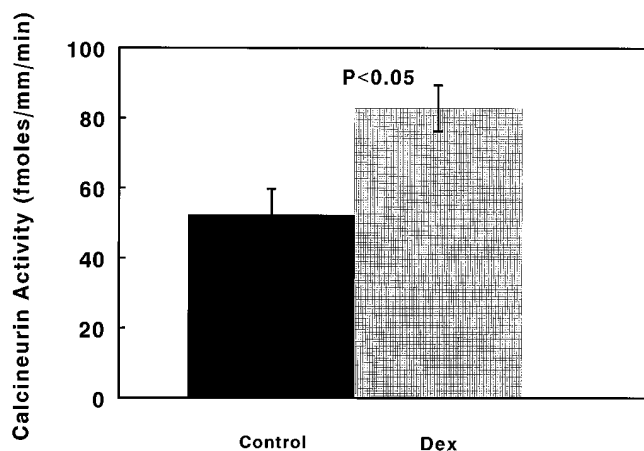


Figure 3. Dexamethasone stimulates calcineurin activity in the CCD. Dexamethasone (5  $\mu$ M) significantly ( $P < 0.05$ ) stimulates calcineurin activity in the CCD ( $n = 8$ ).

calcineurin activity in outer medullary collecting ducts (OMCD), mTAL, or proximal tubules (S2) (data not shown). To determine whether the increase in calcineurin activity induced by aldosterone requires binding to mineralocorticoid receptors, CCD were incubated with spironolactone before the addition of aldosterone. Spironolactone blocked the stimulation of calcineurin activity by aldosterone (Fig. 4).

*Adrenalectomy does not change calcineurin isoform expression along the rat nephron.* Steroid hormones could increase calcineurin activity by regulating the synthesis of calcineurin in renal epithelia. In this case, adrenalectomy should change the level of calcineurin expression and distribution along the nephron. To address this possibility, a Western blot analysis of calcineurin  $\alpha$  subunit isoform expression in CCD, S2, and mTAL isolated from control and adrenalectomized animals was performed. When plasma aldosterone levels were undetectable (data not shown), isoform expression was examined in specific nephron segments. A representative Western blot of  $\alpha$ -1 isoform expression in normal rat brain and microdissected S2, CCD, and mTAL is shown in Fig. 5 A. A doublet between 59 and 61 kD was found in CCD, S2, and mTAL. The  $\alpha$ -2 antibodies detected a single 61-kD band mTAL, but a doublet between 59 and 61 kD was found in CCD, S2, and rat brain (Fig. 5 C). To determine the relative quantities of  $\alpha$ -1 and  $\alpha$ -2 isoform expression in S2, CCD, and mTAL from control and adrenalectomized animals, the protein densities of six different experiments was measured by laser densitometry. Adrenalectomy did not change calcineurin  $\alpha$ -1 or  $\alpha$ -2 isoform expression in any segment tested (Fig. 5, B and D).

*Mechanisms for aldosterone-induced stimulation of calcineurin activity.* We incubated microdissected CCD with aldosterone (1.0 nM) and actinomycin D (5  $\mu$ M). Addition of actinomycin D (30 min) did not block the stimulation of calcineurin activity by aldosterone (Fig. 6). To assess the role of HSPs in the aldosterone-induced stimulation of calcineurin activity, we incubated CCD with an mAb against HSP-56 (EC-1). EC-1 did not change the basal level of calcineurin activity. However, the addition of EC-1 blocked the ability of aldosterone to stimulate calcineurin activity (Fig. 7). Next, we incubated CCD with aldosterone and rapamycin, a structural analogue of FK-506 that binds to the HSP-56 component of

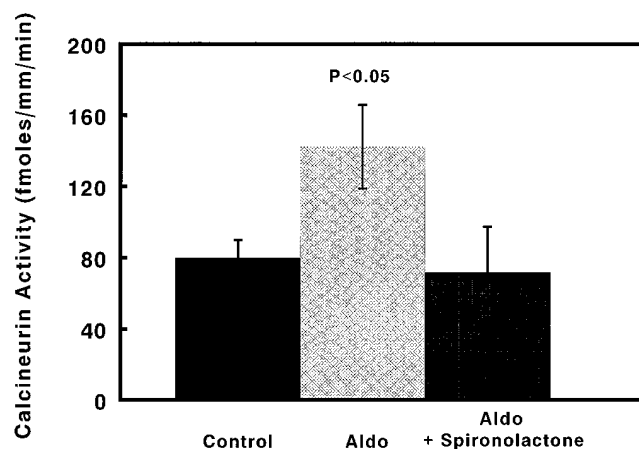


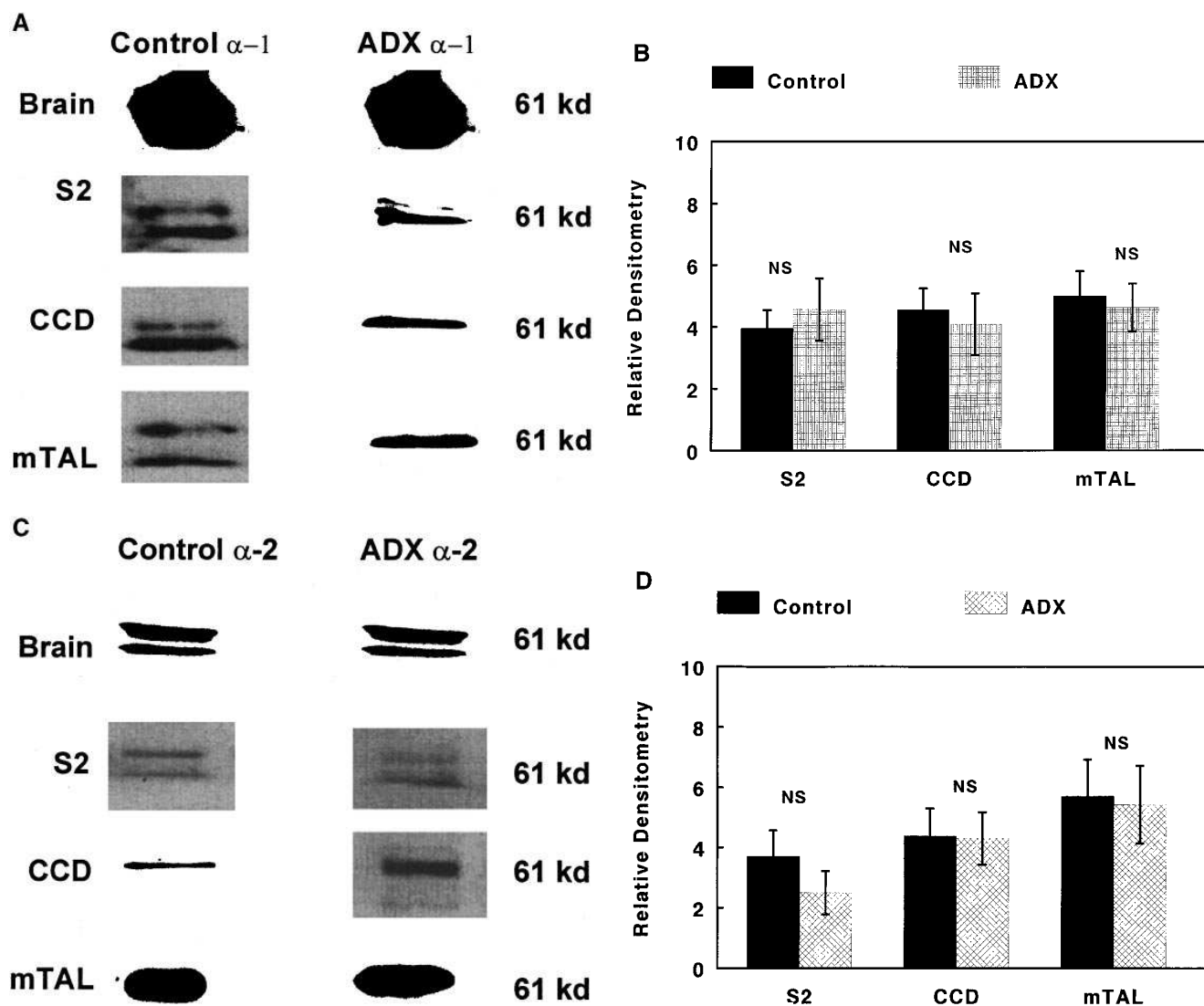
Figure 4. Spironolactone blocks the activation of calcineurin by aldosterone. Spironolactone (5  $\mu$ M) reversed the stimulation of CCD calcineurin activity by aldosterone (1.0 nM) ( $n = 6$ ).

untransformed steroid receptors (19). Recent studies have shown that rapamycin stabilizes the HSP-steroid receptor complex, slowing the release of HSPs after steroid hormone binding (20). When rapamycin was coincubated with aldosterone, it completely blocked the stimulation of calcineurin by aldosterone (Fig. 8). Lastly, we incubated CCD with aldosterone and purified HSP-90 or HSP-70 (1 nM) for 15, 30, 45, and 60 min. Adding HSP-90 significantly increased calcineurin activity at 15 ( $P < 0.05$ ) and 30 min ( $P < 0.01$ ), but this response returned to a basal levels after 60 min (Fig. 9). HSP-70 significantly ( $P < 0.05$ ) increased CCD calcineurin activity at 45 min and, like HSP-90, there was a return to basal levels within 1 h (Fig. 10).

## Discussion

Our results demonstrate that: (a) aldosterone increases calcineurin activity in CCD and CNT but not in proximal tubule, mTAL, or OMCD; (b) dexamethasone increases calcineurin activity in the CCD and the proximal tubule; (c) aldosterone significantly stimulates calcineurin activity within 30 min by a mechanism that does not require gene transcription; (d) adding HSP-90 or HSP-70 increases CCD calcineurin activity within 15 min and parallels the response to aldosterone; (e) an antibody to HSP-56 blocks the stimulation by aldosterone as does spironolactone and rapamycin; (f) adrenalectomy does not change calcineurin protein isoform expression in the rat nephron, suggesting that stimulation of calcineurin activity by steroid hormones is not due solely to changes in isoform expression.

Few studies have investigated the relationship between steroid hormones and the activity of cellular phosphatases. Liu and Greengard (21) found that aldosterone increased the activity of an unidentified phosphatase and stimulated dephosphorylation of a 49-kD protein (protein D) in toad bladders. This effect was rapid ( $< 15$  min) and could be reversed by spironolactone, actinomycin D, or cycloheximide. Their results are consistent with our data demonstrating that aldosterone increases CCD calcineurin activity within 30 min by a mechanism that does not require transcription. Christ et al. (8) demonstrated that aldosterone (1 nM) rapidly ( $< 5$  min) increases



**Figure 5.** Adrenalectomy does not change calcineurin  $\alpha-1$  or  $\alpha-2$  isoform expression in the rat nephron. (A) A representative Western blot of  $\alpha-1$  calcineurin subunit isoform expression in S2, CCD, and mTAL from control and adrenalectomized (ADX) animals. Rat brain was used as a positive control. (B) A comparison of  $\alpha-1$  isoform expression in S2, CCD, and mTAL was made by averaging laser densitometry measurements of band intensity for six separate but identically performed experiments. No differences in calcineurin  $\alpha-1$  isoform expression between control and adrenalectomized animals was detected in any segment tested. (C) A Western blot analysis of  $\alpha-2$  expression in S2, CCD, and mTAL from control and adrenalectomized rats. Rat brain was used as a positive control. (D) A comparison of  $\alpha-2$  isoform expression in S2, CCD, and mTAL was made by averaging laser densitometry measurements of band intensity for six separate but identically performed experiments. No differences in calcineurin  $\alpha-2$  isoform expression between control and adrenalectomized animals were detected in any segment tested.

Na/H antiporter activity through a process involving generation of  $IP_3$  and release of intracellular calcium (22). This effect was not blocked by actinomycin D, cycloheximide, or spironolactone (8). The rise in intracellular calcium was rapid, reaching a maximum within 3–5 min of aldosterone administration (22). The high affinity of calcineurin for calcium allows for rapid (< 1 min) increases in enzyme activity in response to rising intracellular calcium. We found that aldosterone did not significantly increase calcineurin activity before 30 min, making it unlikely that a change in intracellular calcium is the sole mechanism for this response.

The unoccupied steroid receptor is a heteromeric 8-9S complex of proteins that includes the steroid-binding protein and HSPs 90, 70, and 56. Hormone binding to the receptor

complex releases HSPs into the cytosol allowing the occupied receptor to migrate into the nucleus and bind DNA (1). Whereas the function of HSPs after their release from the receptor complex is unknown, there are data indicating that HSPs could modify the activity of one or more phosphatases. For example, Mivechi et al. (23) showed that HSP-70 stimulated dephosphorylation of reticulocyte proteins in a dose-dependent manner within 5 min. Experiments with okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A (23), showed that dephosphorylation of reticulocyte proteins was partially reduced, suggesting that HSP-70 stimulates protein phosphatases 1 and 2A (24). To determine whether HSPs released from the steroid receptor complex mediate aldosterone-induced activation of calcineurin, we incubated CCD

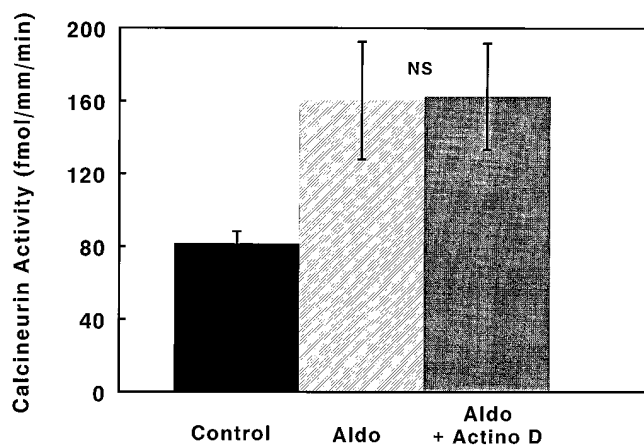


Figure 6. Aldosterone-induced activation of calcineurin activity in CCD is transcription independent. Actinomycin D (5  $\mu$ M) did not block aldosterone-induced stimulation of CCD calcineurin activity ( $n = 8$ ) after 30 min.

with aldosterone and an antibody (EC-1) that recognizes rat HSP-56. We reasoned that if HSPs released from the mineralocorticoid receptor mediate the activation of calcineurin by aldosterone, binding “free” HSPs with a specific antibody should block the response to aldosterone. Indeed, when CCD were incubated with aldosterone and EC-1 antibodies, activation of calcineurin by aldosterone was completely blocked. To confirm the role of receptor-associated HSPs in the stimulation of calcineurin activity, we performed experiments to slow the release of HSPs from mineralocorticoid receptor complex. Recent studies by Renoir et al. and others have shown that rapamycin or FK-506 stabilize the HSP-progesterone receptor complex through interactions with HSP-56 (20, 25–27). Sedimentation studies by Renoir et al. demonstrate that progesterone receptors isolated after incubation with rapamycin remain in the 9S or untransformed state, suggesting that the receptor

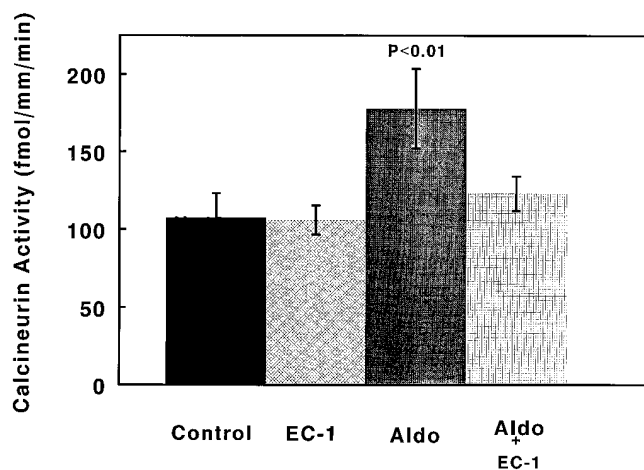


Figure 7. HSP-56 antibodies block activation of calcineurin by aldosterone. HSP-56 antibodies (dilution 1:200) did not change the basal level of calcineurin activity in microdissected CCD ( $n = 14$ ). Aldosterone (1.0 nM) increased calcineurin activity at 60 min, but when HSP-56 antibodies were incubated with aldosterone, this stimulation of calcineurin activity was blocked ( $n = 7$ ).

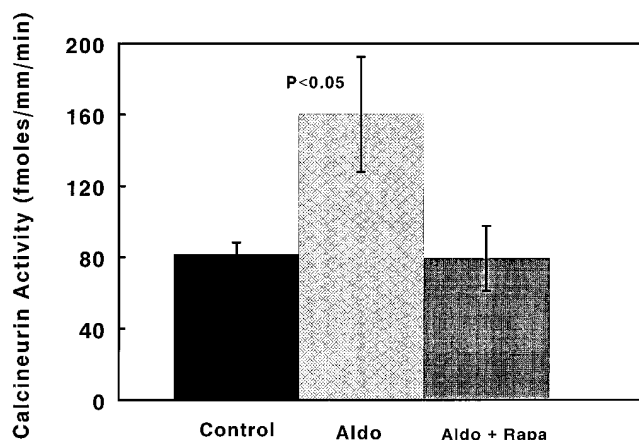


Figure 8. Rapamycin blocks the activation of calcineurin by aldosterone. Rapamycin (12.5  $\mu$ M) blocks stimulation of CCD calcineurin activity by aldosterone at 60 min ( $n = 5$ ).

protein remains complexed to its HSPs. Moreover, progesterone receptors isolated by FK-506 column chromatography yield receptor proteins complexed to HSPs 90, 70, and 56, suggesting that FK-506 also stabilizes the HSP–steroid receptor complex (20). Because rapamycin binds to HSP-56 but does not change the basal level of calcineurin activity (25, 26), we used rapamycin to investigate whether slowing the release of the receptor-associated HSPs would block the effects of aldosterone. When CCD were incubated with aldosterone and rapamycin, activation of calcineurin by aldosterone was completely blocked. Lastly, we investigated whether raising the level of intracellular HSPs would mimic the effects of steroid hormones upon calcineurin activity. To investigate this possibility, we incubated permeabilized CCD with 1 nM HSP-90 or HSP-70 and found that there was stimulation of calcineurin activity that paralleled the response to aldosterone. Kost et al. (28) noted that after injection of progesterone into chicken oviduct cells, a “lag time” of 15–30 min was required before

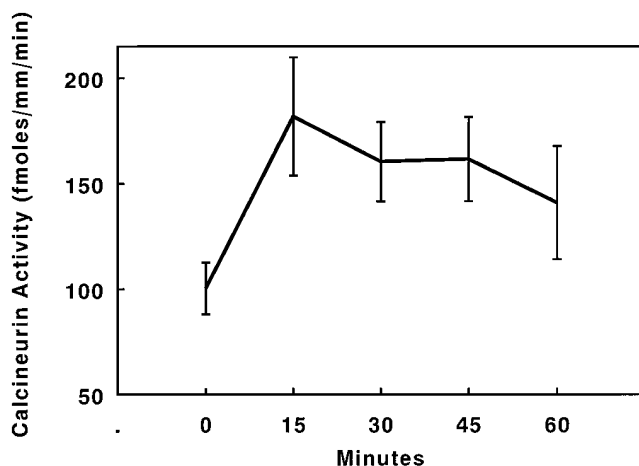


Figure 9. Addition of HSP-90 stimulates calcineurin activity in the CCD. HSP-90 (1 nM) was incubated with CCD for 15, 30, 45, or 60 min. HSP-90 significantly increased calcineurin activity at 15 ( $n = 6$ ) ( $P < 0.05$ ) and 30 min ( $n = 6$ ) ( $P < 0.01$ ). Calcineurin activity returned to baseline levels within 1 h.

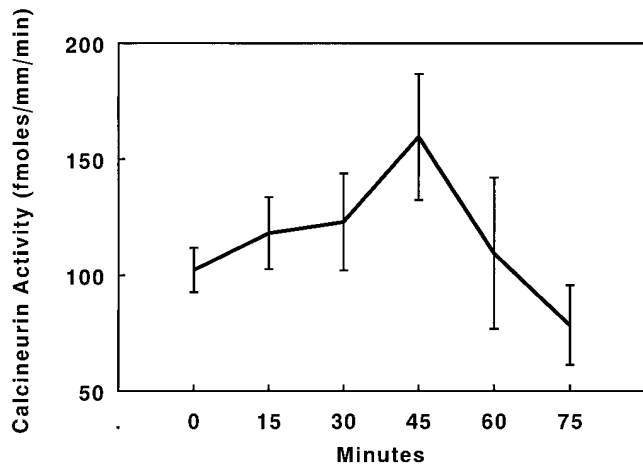


Figure 10. Addition of HSP-70 stimulates calcineurin activity in the CCD. HSP-70 (1 nM) was incubated with CCD for 15, 30, 45, or 60 min. HSP-70 significantly ( $P < 0.05$ ) increased calcineurin activity at 45 min ( $n = 5$ ). Calcineurin activity returned to baseline levels within 1 h.

changes in cytosolic HSP-90 could be detected. If HSPs released from the mineralocorticoid receptor are required to activate calcineurin, then adding HSPs to permeabilized tubules should decrease the time required for calcineurin activation. Indeed, when we added HSP-90 to permeabilized CCD, calcineurin activity increased after only 15 min. This is in contrast to the effects of aldosterone in the CCD, which requires 30 min for activation of calcineurin. Results from these experiments are consistent with the formulation that HSP-90 acts to stimulate calcineurin activity and that the longer period required for stimulation of calcineurin by aldosterone could be due to disassociation of HSP-mineralocorticoid receptor complex. We can not explain why the time-course for activating calcineurin by HSP-70 was slower since results in Figs. 9 and 10 indicate that both HSP 90 and HSP 70 increase calcineurin activity.

In summary, aldosterone increases calcineurin activity in the CCD and CNT by 96 and 46%, respectively, but does not activate the enzyme in S2, mTAL, or the OMCD. In contrast, dexamethasone stimulates calcineurin activity in S2 as well as the CCD. Aldosterone-induced stimulation of calcineurin activity in the CCD is time dependent and does not require gene transcription but is reproduced by the addition of HSP-90 or HSP-70. We conclude that a novel transcription-independent effect of mineralocorticoids and glucocorticoids is to stimulate calcineurin phosphatase activity by a pathway that involves the release of HSPs from the steroid receptor complex.

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