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The *sgk*, an aldosterone-induced gene in mineralocorticoid target cells, regulates the epithelial sodium channel

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The *sgk*, an aldosterone-induced gene in mineralocorticoid target cells, regulates the epithelial sodium channel. Aldosterone increases sodium reabsorption in tight epithelia. The early phase of this stimulatory effect is thought to involve activation of apical sodium channels. To identify immediate-early genes that initiate this effect, we used a combination of polymerase chain reaction-based subtractive hybridization and differential display techniques. This review summarizes our recent findings. Aldosterone rapidly increases mRNA levels of a putative Ser/Thr kinase, *sgk* (or serum- and glucocorticoid-regulated kinase), in the native mineralocorticoid target cells, that is, in cortical collecting duct (CCD) cells. The induction of *sgk* mRNA occurs within 30 minutes of the addition of aldosterone and does not require de novo protein synthesis, indicating that *sgk* is an immediate/early aldosterone-induced gene. Induction of sgk by aldosterone is mediated through mineralocorticoid receptors (MRs), since it is prevented by ZK91857, an MR antagonist, but not by RU486, a glucocorticoid antagonist. In addition to aldosterone, RU28362, a pure glucocorticoid receptor agonist, also induced *sgk* mRNA, both in primary cultures of rabbit CCD cells and in the M-1 mouse CCD cell line. *Sgk* mRNA levels are also influenced by changes in the osmolality of the medium. In M-1 cells, incubation of cells for one hour in a mildly hypotonic medium decreased *sgk* mRNA levels, whereas incubation in hypertonic medium brought about opposite changes. To determine whether *sgk* is involved in the regulation of the epithelial sodium channel (ENaC), we coexpressed the full-length *sgk* cRNA in *Xenopus* oocytes with the three ENaC subunits. Expression of *sgk* resulted in a significant increase in the amiloride-sensitive Na current, suggesting that this protein kinase plays an important role in the early phase of aldosterone-stimulated Na transport. These results indicate that *sgk* is an aldosterone-induced immediate/early gene in native MR target cells, and is involved in the regulation of ion transport and possibly cell volume.

Aldosterone, the primary mineralocorticoid hormone, is a key regulator of sodium balance and consequently plays a central role in blood pressure regulation. Its fundamental importance is demonstrated by the severity of disturbances that result from its deficiency or excess. Aldosterone deficiency leads to life-threatening hypotension in Addisonian crisis, whereas excess aldosterone results in malignant hypertension. In addition to real and apparent mineralocorticoid excess [1], aldosterone-regulated proteins are also involved in some forms of hypertension and cardiovascular diseases. For instance, mutations in one such aldosterone-regulated protein, the epithelial sodium channel (ENaC) [2, 3], lead to constitutively elevated Na reabsorption and hypertension in Liddle disease [4] or to salt wasting in pseudohypoaldosteronism [5]. Thus, understanding the mechanism of aldosterone action has important clinical applications.

Aldosterone exerts its effects through the mineralocorticoid receptor (MR), which, like other members of the nuclear receptor superfamily, is a ligand-activated transcription factor that induces or represses specific genes. Some of these genes encode transporters involved in Na reabsorption (for example, the apical Na channel and the basolateral Na,K-ATPase) [6–11]. However, these genes are probably indirectly regulated by aldosterone through the induction or repression of early response genes.

Aldosterone effects on Na transport are usually divided into "early" and "late" phases. The early response takes place about 0.5 to 3 hours after the hormone addition. It seems likely that the early increase in apical Na permeability is mediated by activation of pre-existing channels through postsynthetic modifications (such as methylation) or association with regulatory proteins. This notion was strengthened by the observation that aldosterone increases an open probability of pre-existing Na channels in the membrane [12]. The late phase (several hours to several days) probably involves synthesis of new Na channels and Na,K-ATPase molecules.

Researchers have searched for aldosterone-induced proteins (AIPs) that initiate aldosterone's early effects for the last few decades. While early studies using two-dimensional gel electrophoresis of proteins described several AIPs, these proteins are expressed during the late phase of aldosterone effect [13, 14], and their genes have never been identified. More recently, Attali et al identified a dexamethasone-induced gene (CHIF) in the

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rat colon [15], which, however, is not induced by aldosterone in the kidney [16]. Spindler et al recently reported [17] the presence of early (1 h)-induced genes in A6 cells; however, the fact that A6 cells do not have functional MRs [18] indicates that these genes are regulated via the glucocorticoid receptor (GR).

The goal of the present study was to identify and characterize aldosterone-induced immediate/early genes in native mineralocorticoid target cells, that is, in renal cortical collecting duct (CCD) cells. Since our previous studies demonstrated that primary cultures of rabbit CCD cells express functional mineralocorticoid receptors (MRs) and respond to aldosterone with enhanced Na transport and increased ENaC mRNA expression [8, 19], we used this model system to identify aldosterone-induced genes.

RESULTS AND DISCUSSION

Aldosterone induces the expression of *sgk*, a Ser/Thr kinase, in rabbit cortical collecting duct cells

Primary cultures of rabbit CCD cells, grown on permeable filters [19, 20], were treated with 10 nmol/L aldosterone for 60 minutes. Our previous results showed that this time point precedes any significant change in amiloride-sensitive Na current in cultured CCD cells [19]. mRNAs, differentially expressed in the control and aldosterone-treated cells, were identified using suppression polymerase chain reaction (PCR)-based subtraction hybridization [21, 22] and differential display [23] methods, as modified in our laboratory [24].

This method resulted in a highly reproducible pattern of amplified DNA fragments from the subtracted cDNA pools. Because of the less than complete subtraction of common cDNAs, inherent to any subtractive hybridization method, many cDNAs were shared in the "control" and "aldosterone" cDNA pools. However, about 30 to 40% of cDNA fragments seemed unique. One of these cDNA fragments, which was significantly enhanced in aldosterone-treated cells, exhibited a marked homology to *sgk* (serum- and glucocorticoid-induced kinase). The identity of this PCR product as the rabbit *sgk* was verified by sequencing.

Sgk is a novel member of the Ser/Thr kinase family. In contrast to most protein kinases, *sgk* is predominantly regulated at the transcriptional level by several stimuli, including glucocorticoids [25, 26], serum [25], follicle-stimulating hormone [27], and alterations in cell volume [28]. Recent in situ hybridization data indicate that *sgk* mRNA levels are also increased in the kidney of rats treated with aldosterone [26].

We determined the full-length sequences of the rabbit and mouse *sgk* and compared these with the human [28] and rat sequences [29]. This comparison revealed that *sgk* is extremely well conserved across species: The predicted amino acid sequences are 96 to 98% identical to each other [24]. This observation suggests that *sgk* plays an important role in signal transduction of several hormones and other stimuli.

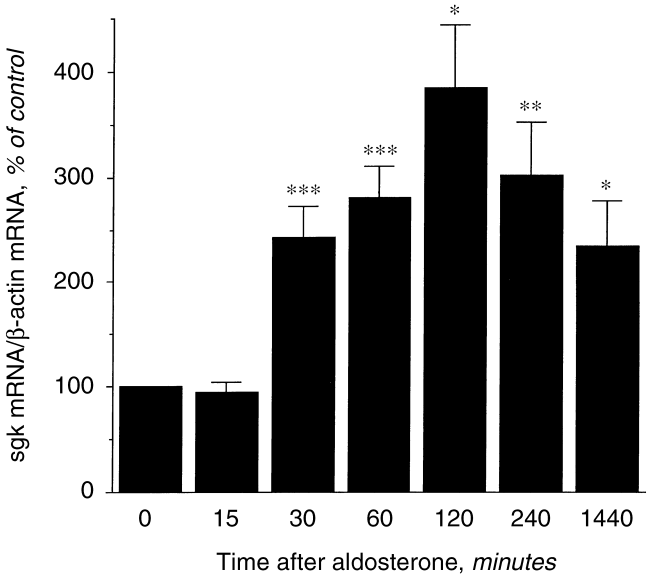

Fig. 1. Aldosterone induces *sgk* mRNA expression in cortical collecting duct (CCD) cells. CCD cells were incubated with vehicle or 10 nmol/L aldosterone at 37°C for various times (15 min to 24 h). The levels of *sgk* mRNA were determined by quantitative RT-PCR using serial dilutions of cDNA (3 to 0.12 ng) as a template. Mean values of *sgk* mRNA levels are expressed as a percentage of control. Values shown are relative amounts of *sgk* mRNA normalized for β-actin mRNA determined from the same cDNA samples. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$, using Student's paired *t*-test (two tailed) when compared with values of control samples at the same time points. *N*, number of individual cultures for each control and aldosterone treatment; $N = 2$ for 15 minutes; $N = 7$ for 30 and 120 minutes; $N = 11$ for 60 minutes; $N = 10$ for 240 minutes; $N =$ for 1440 minutes.

Aldosterone-induction of *sgk* in cortical collecting duct cells does not require protein synthesis and is mediated via the mineralocorticoid receptor

As shown in Figure 1, in primary cultures of rabbit CCD cells, aldosterone rapidly increased the levels of *sgk* mRNA, the difference versus control was already significant at 30 minutes. *sgk* mRNA levels increased further up to four hours and then declined, although mRNA levels after 24 hours of aldosterone treatment were still significantly higher than control values. The conclusion reached, based on quantitative reverse transcription-polymerase chain reaction (RT-PCR) determinations of *sgk* mRNA levels (Fig. 1) [24], was confirmed by Northern analysis that showed the approximately 2.4 kb mRNA transcript hybridizing with the rabbit *sgk* RNA probe to be significantly increased following 30 minutes of 10 nmol/L aldosterone administration [24]. The rapid time course of induction suggests that *sgk* is a primary aldosterone-induced gene. This conclusion was confirmed by our results that the induction of *sgk* by aldosterone

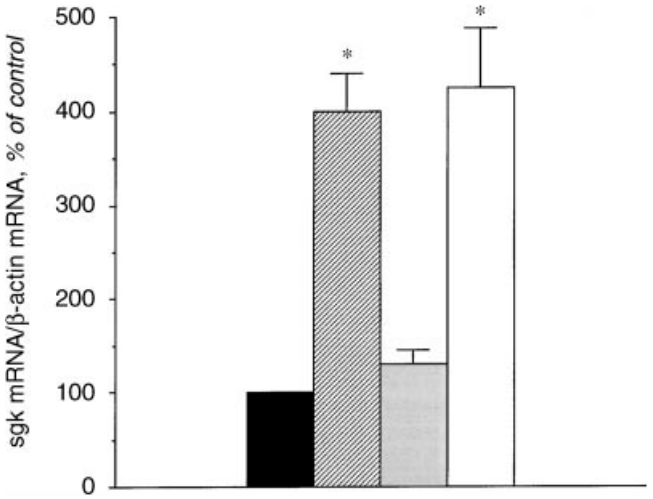

Fig. 2. Induction of aldosterone is mediated through mineralocorticoid receptors (MR). Cortical collecting duct (CCD) cells were incubated with vehicle (■), 10 nmol/L aldosterone (▨), 10 nmol/L aldosterone plus 1 μmol/L ZK91587, an MR antagonist (▩) or 10 nM aldosterone plus RU 486, a glucocorticoid receptor (GR) antagonist (□) for 60 minutes at 37°C. $*P < 0.01$ using Student's paired *t*-test when compared with values of control samples.

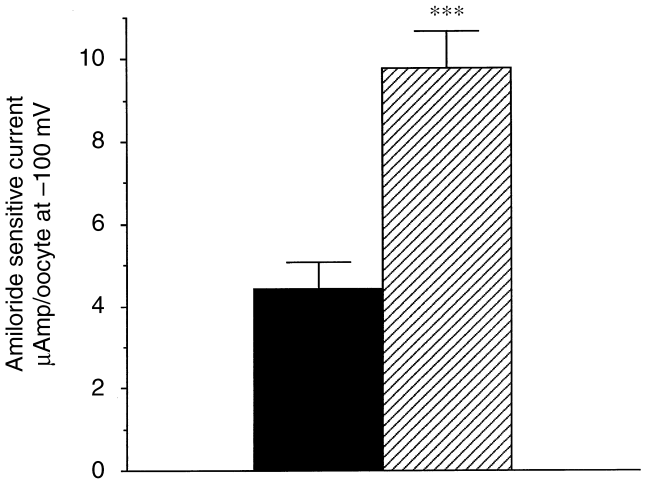

Fig. 3. Effect of *sgk* on the magnitude of the amiloride-sensitive current expressed in oocytes. *Xenopus* oocytes were injected with cRNA from epithelial sodium channel (ENaC) alone (■) or ENaC with *sgk* (▨). Oocyte currents were measured with the two-electrode voltage clamp in the presence of 100 mmol/L Na gluconate in the bathing solution. The amiloride-sensitive component is the difference of the whole-cell currents in the absence and presence of 10 μmol/L amiloride in the perfusate. Values represent mean current of oocytes at membrane potential of −100 mV. $N = 34$ oocytes for ENaC alone, $N = 29$ oocytes in the ENaC + *sgk* group. Error bars represent SEM. $***P < 0.00001$. Data are redrawn from Náray-Fejes-Tóth et al [24]; used with permission from the *Journal of Biological Chemistry.*

was unaffected by cycloheximide [24]. This observation is similar to those reported for glucocorticoid induction of *sgk* in fibroblasts [25] and A6 cells [26].

Since to some extent aldosterone binds to the GR, we tested whether its effects in rabbit CCD cells are mediated via MR or GR by using receptor-specific antagonists. As shown in Figure 2, the effect of 10 nmol/L of aldosterone could be blocked by preincubating the cells with 1 μmol/L of ZK91587, a specific MR antagonist, but not by 1 μmol/L of RU 486, which is a GR antagonist. Thus, we conclude that in CCD cells aldosterone induces *sgk* via the MR, although just like in other systems [25, 26], activation of the GR by pure glucocorticoid agonists has a similar effect [24].

sgk activates amiloride-sensitive Na current in *Xenopus* oocytes

To test if *sgk* increases the function of ENaC, we coexpressed the mouse *sgk* with all three ENaC subunits in oocytes. After 36 hours of incubation, the amiloride-sensitive component of whole-cell currents was measured with the two-electrode voltage clamp technique.

These experiments revealed that oocytes coinjected with ENaC and *sgk* exhibited significantly larger amiloride-sensitive currents than oocytes injected with ENaC alone. The difference was highly significant in every experiment ($P < 0.0001$). The results obtained with all oocytes are summarized in Figure 3 [24]. The mean amiloride-sensitive current measured at −100 mV in oocytes injected with ENaC alone was 4.42 ± 0.66 μA. Oocytes coinjected with ENaC and *sgk* expressed significantly larger currents, with a mean of 9.78 ± 0.90 μA ($P < 0.0001$). The difference between the two groups was observed at all membrane voltages [24].

These results are similar to those reported by Chen et al with the *Xenopus sgk* and indicate that *sgk* activates ENaC when coexpressed in oocytes [26]. Regulation of Na channel activity by protein kinases has been described by several laboratories [30–33]. Importantly, aldosterone was found to increase phosphorylation of specific Ser/Thr residues on the β and γ ENaC subunits in Madin-Darby canine kidney cells [34], but the kinase that mediates this effect in vivo is yet to be identified. *Sgk* seems to be a good candidate for mediating this effect. It is also interesting to note that *sgk* is also induced by serum [25, 29], which is a rich source of insulin, and insulin increases phosphorylation of the same Ser and Thr residues on β and γ ENaC as aldosterone [34]. It is conceivable that the insulin-mediated increase in ENaC activity [35] is also mediated through phosphorylation via *sgk.*

Induction of *sgk* in the M-1 mouse cortical collecting duct cell line

To expand these observations to another species, we tested the effect of corticosteroids on *sgk* mRNA levels in the M-1 CCD cell line [36]. This cell line, just like the A6 line [18], does not express functional MR. Since in CCD cells, activation of the MR or the GR has very

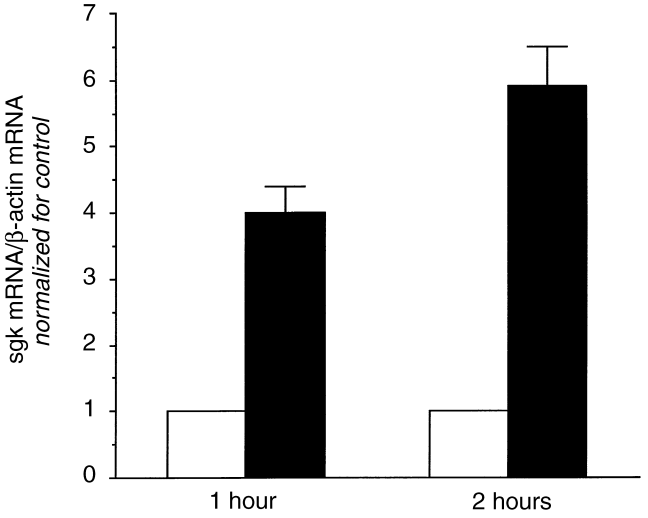

Fig. 4. *sgk* mRNA is induced by dexamethasone in the M-1 CCD cell line. M-1 cells were incubated with vehicle (□) or 100 nmol/L dexamethasone (■) for one or two hours. The levels of *sgk* mRNA were determined by quantitative RT-PCR. For each time point, the mean values of four individual cultures are shown.

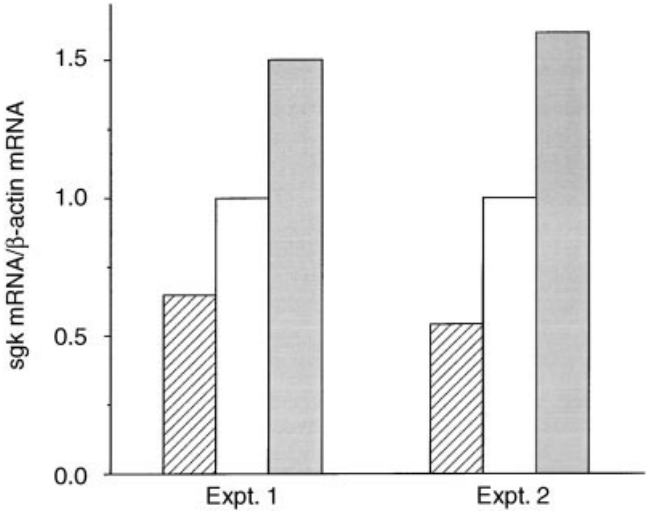

Fig. 5. Effects of media osmolality on *sgk* mRNA levels in M-1 cells. M-1 cells were incubated in 240 mOsm/L (hypotonic, removal of 25 mmol/L NaCl, ▨), 290 mOsm/L (isotonic, □) or 340 mOsm/L (hypertonic, addition of 50 mmol/L raffinose, ▩) media for 60 minutes (experiment 1) or for 120 minutes (experiment 2). The levels of *sgk* mRNA were determined by quantitative RT-PCR. For each time point the mean values of two individual cultures are shown. Values represent the fold increase as compared with values obtained in cultures incubated in isotonic medium.

similar if not indistinguishable effects [19], we tested the effect of a synthetic glucocorticoid, dexamethasone, which binds predominantly to the GR. Data in Figure 4 demonstrate that *sgk* mRNA levels were rapidly induced by dexamethasone in M-1 cells.

The results of Waldegger et al indicate that in human hepatoma cells, *sgk* is induced by changes in cell volume: shrinkage increases, whereas swelling reduces *sgk* mRNA levels [28]. To determine whether similar changes take place in CCD cells, we incubated M-1 cells in mildly hypotonic (240 mOsm/L), isotonic (290 mOsm/L), and mildly hypertonic (340 mOsm/L) medium for 60 to 120 minutes. As shown in Figure 5, incubation in hypotonic medium brought about a modest decrease in *sgk* mRNA levels, whereas incubation in hypertonic medium increased *sgk* expression. These data suggest that *sgk* might play an important role not only in the regulation of Na entry, but also in the control of cell volume. On one hand, aldosterone induces *sgk*, which activates Na channels, thereby increasing intracellular Na and resulting in an increase in cell volume. On the other hand, cell swelling rapidly decreases the expression of *sgk*, creating a negative feedback in cell volume regulation. Such a feedback could also explain the decline in *sgk* mRNA levels at 24 hours, despite the continuous presence of aldosterone (Fig. 1) [24].

CONCLUSIONS

The main finding of this study is that aldosterone rapidly increases the mRNA levels of *sgk*, a Ser/Thr kinase in the native mineralocorticoid target cells. This effect is direct since it does not require de novo protein synthesis and is mediated through MRs. *Sgk*, when coexpressed in *Xenopus* oocytes with ENaC, leads to a significant increase in amiloride-sensitive Na current, suggesting that this protein kinase plays an important role in the early phase of aldosterone-stimulated Na transport.

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