S-Adenosyl-l-homocysteine Hydrolase Regulates Aldosterone-induced Na\(^+\) Transport*

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Aldosterone-induced Na\(^+\) reabsorption, in part, is regulated by a critical methyl esterification; however, the signal transduction pathway regulating this methylation remains unclear. The A6 cell line was used as a model epithelia to investigate regulation of aldosterone-induced Na\(^+\) transport by S-adenosyl-l-homocysteine hydrolase (SAHHase), the only enzyme in vertebrates known to catabolize S-adenosyl-l-homocysteine (SAH), an end product inhibitor of methyl esterification. Sodium reabsorption was decreased within 2 h by 3-deazaadenosine, a competitive inhibitor of SAHHase, with a half inhibitory concentration between 40 and 50 \(\mu\)M. Aldosterone increased sodium catabolism by activating SAHHase. Increased SAH catabolism was associated with a concomitant increase in S-adenosylmethionine catabolism. Moreover, SAH decreased substrate methylation. Antisense oligonucleotide complementary to SAHHase mRNA decreased SAHHase activity and Na\(^+\) current by approximately 50%. Overexpression of SAHHase increased SAHHase activity and dependent substrate methyl esterification. Whereas basal Na\(^+\) current was not affected by overexpression of SAHHase, aldosterone-induced current in SAHHase-overexpressing cells was significantly potentiated. These results demonstrate that aldosterone induction of SAHHase activity is necessary for a concomitant relief of the methylation reaction from end product inhibition by SAH and the subsequent increase in Na\(^+\) reabsorption. Thus, regulation of SAHHase activity is a control point for aldosterone signal transduction, but SAHHase is not an aldosterone-induced protein.

Endocrine regulation of NaCl and water reabsorption at the collecting tubule of the kidney is the major site in vertebrates for maintaining normal blood pressure. Aldosterone is the primary hormone modulating salt reabsorption across renal epithelial tissue. Aldosterone increases cell entry of Na\(^+\) from the urine across the apical plasma membrane by regulating the activity of the 4 \(\mu\)S, amiloride-sensitive, Na\(^+\)-selective channel (ENaC\(^+\); see Refs. 1–3). Similar to other steroids, aldosterone regulates cell activity by modifying gene expression. Whereas the end result of aldosterone action, increased Na\(^+\) reabsorption, is well known, the aldosterone-induced proteins and resulting signal transduction pathways remain poorly described and controversial.

Provocative studies by Sariban-Sohraby and colleagues (4) and Wiesmann et al. (5) established a correlation between methyl esterification and acute, aldosterone-induced Na\(^+\) transport. Several subsequent studies support further the hypothesis that a critical protein methyl esterification, in part, signals the acute actions of aldosterone (3, 6–8). The substrate of this critical methylation remains controversial and is an active area of research. Results from our laboratory suggest that aldosterone increases methyl esterification of p21ras in renal epithelial cells (9). A study by Rokaw et al. (10) provides evidence that \(\beta\)ENaC also may be an important substrate of aldosterone-induced methylation. Although methylation is a component of aldosterone regulation of Na\(^+\) transport, the signal transduction controlling this covalent modification remains to be described.

The enzymes directly regulating substrate methylation are methyltransferase and methylsterase catalyzing methyl esterification and methyl ester hydrolysis, respectively. Protein methylation is analogous to protein phosphorylation both being molecular switches controlling protein activity/locus in a reversible manner.

The predominant, intracellular methyl-donating molecule is S-adenosyl-l-methionine (AdoMet). Methyltransferase catalyzes the transfer of a methyl moiety from AdoMet onto a substrate with the end product S-adenosyl-l-homocysteine (SAH) being formed. This end product is a potent feedback inhibitor of most transmethylation reactions involving AdoMet as the methyl donor (11). The observation by Sariban-Sohraby et al. (4) that aldosterone increases methylation of both protein and lipid is consistent with the notion that a general regulator of transmethylation reactions is the control point for mineralocorticoid-induced methylation. Thus, regulation of cellular SAH concentration may be an important site for controlling methylation relevant to aldosterone signal transduction.

The only enzyme known to hydrolyze SAH in vertebrates is S-adenosyl-l-homocysteine hydrolase (SAHHase; EC 3.3.1.1; see Ref. 11). This enzyme reversibly metabolizes SAH with hydrolysis resulting in production of adenosine and l-homocysteine. The CDNA encoding SAHHase has been identified in various species including Xenopus laevis (12).

Pharmacological and molecular inhibitors of SAHHase and overexpression of SAHHase were used to test directly the notion that SAHHase activity regulates Na\(^+\) reabsorption. Since

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N-acetyl-S-farnesyl-l-cysteine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
cellular SAH levels as set by hydrolase activity may regulate Na+ transport through modulation of substrate methyl esterification, the hypothesis that aldosterone increases SAHHase activity with concomitant increases in methylation also was tested. The results of the current study demonstrate that the natriuretic actions of aldosterone are dependent on SAHHase activity with aldosterone-induced SAH hydrolysis promoting substrate methyl esterification and subsequent induction of Na+ reabsorption. Regulation of SAHHase activity by aldosterone was shown to be one control site for mineralocorticoid-induction methylated and Na+ reabsorption.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

Amphibian distal tubule principal cells (A6 cells; American Type Culture Collection, Rockville, MD) were maintained in 4% CO2 at 26 °C in a mixture of Coon’s F-12 (3 parts) and Leibovitz’s L-15 (7 parts) medium (Irving Scientific, Santa Ana, CA) supplemented with 104 mM Hepes, with 2% polyacrylamide gel electrophoresis (10%) from an appropriate size (see Ref. 12; GenBank accession number L35559) as follows: forward primer, 5′-TTCACCATGTCTGACAAACTGT-3′; reverse primer, 5′-CAGACATGACAACTGTCC-3′. Subsequent to agarose gel electrophoresis (1.2%), the fragment containing the full-length clone for xSAHHase (5′-CAGACATGACAACTGTCC-3′) was homologous to the coding sequence of the same region. In X. laevis, the only sequence with identity to these oligonucleotides as described by a blast (National Center for Biotechnology Information) search was SAHHase. Addition of exogenous single-stranded oligonucleotides to A6 cells followed a protocol similar to that previously described by this laboratory (13). Briefly, competent A6 cell monolayers washed with phosphate-buffered saline were treated for 24 h prior to experimentation with 5–10 μg oligonucleotide dissolved in basic media. Aldosterone then was repleted for 4 h in the presence of oligonucleotide.

**Northern Blot Analysis**—For Northern blot analysis, 3–5 μg of poly(A)+ RNA isolated from A6 cells was separated by electrophoresis on 1.2% agarose/formaldehyde gels, transferred to nylon membranes, and probed with radiolabeled oligonucleotides of interest. Probes were produced by nick translation and incorporation of [32P]dCTP. Northern blots were hybridized and washed under high stringency conditions and then imaged on a PhosphorImager after 1–24 h exposure. Band density was quantified using SigmaGel software (Jandel Scientific).

**Molecular Methodological Methods**

Cloning of X. laevis SAHHase—Single-stranded cDNA from A6 cells was created using the Marathon cDNA amplification kit (CLONTECH Laboratories, Inc., Palo Alto, CA) and poly(A)+ mRNA harvested with the FastTrack 2.0 Kit (Invitrogen, Carlsbad, CA). An X. laevis SAHHase full-length clone was amplified from this cDNA using a polymerase chain reaction in conjunction with specific primers developed from the reported X. laevis SAHHase sequence (see Ref. 12; GenBank accession number L35559) as follows: forward primer, 5′-TTCACCATGTCTGACAAACTGT-3′; reverse primer, 5′-CAGACATGACAACTGTCC-3′. Subsequent to agarose gel electrophoresis (1.2%), the fragment containing the full-length clone for xSAHHase was isolated and ligated into pGEM-T Easy (Promega, Madison, WI). The insert then was subcloned into pcDNA3.1(−zeo) (Invitrogen) using NotI. Nucleotide sequence data from this new expression plasmid (pxSAHHase3.1(−zeo)) was homologous to that reported by Seery et al. (12) and was consistent with the plasmid containing the full-length clone for xSAHHase. As shown in Fig. 1, in vitro translation of pxSAHHase3.1(−zeo) using the TNT T7 Coupled Reticulocyte Lysate System (Promega) produced a protein of appropriate size (~48 kDa) for a complete open reading frame.

**Transfection**—LipofectAMINE PLUS reagents (Life Technologies, Inc.) were used for transfection of A6 and HEK293 cells. Transfection followed closely the standard protocol for these reagents only differing in the fact that for A6 cells incubation time was increased to 18 h. Transient transfected HEK293 cells were maintained in tissue culture using standard techniques and used for experimentation 2–3 days post-transfection. In contrast, A6 cell transfection was prolonged by zeocin (400 μg/ml) selection. Subpassages (up to four passages after transfection) of the extended A6 cell population were used for experimentation. Control transfectants contained either pcDNA3.1(−zeo) vector or pVgRXI (Invitrogen). Neither of these plasmids affected cell activity.

**Oligonucleotide Strategy**—Antisense oligonucleotide (5′-GGACAGTTGGTGTGACATGTTG-3′) was complementary to and spanned the translation start codon (~4 to 18) of xSAHHase mRNA, whereas sense oligonucleotide (5′-CACCATGTGTGACAACTGTCC-3′) was homologous to the coding sequence of the same region. In X. laevis, the only sequence with identity to these oligonucleotides as described by a blast search was SAHHase. Addition of exogenous single-stranded oligonucleotides to A6 cells followed a protocol similar to that previously described by this laboratory (13). Briefly, competent A6 cell monolayers washed with phosphate-buffered saline were treated for 24 h prior to experimentation with 5–10 μg oligonucleotide dissolved in basic media. Aldosterone then was repleted for 4 h in the presence of oligonucleotide.

**Assays of Enzyme Activity**

**SAHHase Activity**—A continuous spectrophotometric, enzyme-coupled assay similar to that reported by Palmer and Abeles (14) and Hershfield et al. (15) was used to quantify SAHHase activity. In brief, whole cell lysate was collected by Dounce homogenization washed twice with phosphate-buffered saline in 1 ml/106 cells of lysis buffer (30 mM Hepes, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 8.0). Cellular debris were precipitated by centrifugation at 10,000 × g for 30 min at 4 °C. The resulting supernatant maintained at 4 °C was used as the source of SAHHase with enzyme activity being assessed within 2 h after centrifugation. With the addition of exogenous adenosine deaminase (EC 3.5.4.4), SAHHase activity in whole cell lysate becomes rate-limiting for the metabolism of SAHHase to inosine. Enzyme activity was assayed in the hydrolytic direction in the following reaction buffer: (in mM) 25 K2PO4, 2 MgCl2, 1 EDTA, pH 7.2. The final reaction volume was 1 ml with 68 ml of reaction buffer, 100 ml of 1 mM SAH, 200 ml of α6 cell whole cell lysate, and 2 μl of adenosine deaminase (~5 units; Sigma, type VIII). The reaction was initiated after addition of lysate, and absorbance changes at 265 resulting from SAH metabolism to inosine were recorded using an Ultratec 3000 spectrophotometer (Amersham Pharmacia Biotech). The initial rate of absorbance change (1–5 min) was used to establish activity.

**Substrate Methylation**—Transmethylation was quantified by alka-lne hydrolysis of methyl esters in a vapor phase assay similar to that described by Clarke et al. (16). In brief, N-acetyl-S-farnesyl-cysteine (AFc) was used as a methyl acceptor, AdoMet as a methyl donor, and A6 whole cell lysate as the source of methyltransferase. Subsequent to washing 3 times with phosphate-buffered saline, cells were extracted at 4 °C by Dounce homogenization (1 ml/106 cells) in 50 mM Tris-HCl, 25 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, pH 8.0. Cellular debris were precipitated by centrifugation at 10,000 × g for 30 min at 4 °C. Methylation of AFC was assayed in 50 ml of lysate supplemented with 720 mM [methyl-3H]AdoMet (77 Ci/mmol; Amersham Pharmacia Biotech) and 100 μM AFC. After incubation for 1 h, the reaction was stopped with 50 μl of 60% trichloroacetic acid. Methylated substrate was isolated by organic extraction in 400 ml of heptane. The organic phase was subsequently dried and subjected to base hydrolysis (1 M NaOH). Under these conditions, only methyl esters (e.g. methylated AFC) are hydrolyzed. Liberated radioactivity was assayed with a Tri-Carb liquid scintillation analyzer (model 1900CA; Packard Instrument Co.)

**Assay of Cellular AdoMet and SAH Concentrations**—The methyl donor (AdoMet) and end product (SAH) concentrations were quantified using standard paper chromatography. Confluent monolayers of A6 cells were maintained in methionine-free, basic media for 48 h. One day prior to experimentation, cell media were supplemented with tracer amounts (50 μM) of [35S]methionine (1157 Ci/mmol; ICN Pharmaceuticals).
cals, Inc., Irvine, CA). Aldosterone (1.5 mM) was repleted for 4 h and cells collected in 50 mM Tris-HCl buffer as above. Metabolites were separated by paper chromatography on Whatman 3MM paper. To locate radiolabeled AdoMet and SAH, 5 µl of a mixture of 1 mM nonlabeled methionine, homocysteine, cysteine, AdoMet, and SAH was run in parallel with each sample. Metabolite location was identified upon development using ninhydrin reagent plus heat. Two solvents were used in series to separate metabolites as follows: 1) n-butyl alcohol/glacial acetic acid/water (65:150:20, v/v), and 2) isopropyl alcohol/ammonia/water (70:10:20, v/v). Subsequent to localization of metabolites, corresponding spots were removed from the paper, and radioactivity was quantified using the Tri-Carb liquid scintillation analyzer.

Transepithelial Electrical Measurements

For assessment of transepithelial potentials (PD) and resistances (R), A6 cells were plated on permeable, 25-mm tissue culture inserts (0.92 µm Anopore membrane; Nalge NUNC International, Naperville, IL) and grown to confluency (approximately 10 days) in the presence of complete media. Monolayer competency was determined by resistance measurement (≥1.0 kilohm/cm²). Prior to experimentation, competent monolayers were treated with basic media for 48 h. to decrease Na⁺ transport to a basal, non-steroid-induced state. This enabled the dissection of the acute from chronic actions of aldosterone. Monolayers then were repleted with aldosterone (1.5 mM) with or without experimental agents for 4 h. Transepithelial PD and R before and after treatment were measured using a Millicell Electrical Resistance System with dual Ag/AgCl pellet electrodes (Millipore Corp.). Equivalent short circuit current (Isc) was calculated using Ohm’s law: PD = V × R. Aldosterone-induced current in this system was amiloride-sensitive and carried by Na⁺.

Materials

All reagents were purchased from Sigma or Calbiochem unless indicated otherwise. Sense and antisense phosphorothioate oligonucleotides were synthesized by the Emory University Microchemical Facility. This facility also synthesized the specific X. laevis SAHHase primers used for polymerase chain reaction. For each lysate, protein concentration was established using the DC Protein Assay Kit (Bio-Rad).

Statistical Analysis

Data are expressed as the mean ± S.E. Statistical significance was determined using Student’s t test for paired and unpaired data as appropriate. For multiple comparison, a one-way analysis of variance in conjunction with the Student-Newman-Keuls test was used. A p ≤ 0.05 was considered significant.

RESULTS

Regulation of Na⁺ Transport and SAHHase Activity by 3-Deazaadenosine—Shown in Fig. 2 are the time course and dose-response profiles of the actions of DZA (4-amino-1-[β-D-ribofuranosyl]-1H-imidazo[4,5]-pyridine) on steady state Na⁺ transport in A6 cells maintained in complete media. Maximum current inhibition occurred within 2 h after application. At 2 h, relative currents were 0.64 ± 0.07, 0.44 ± 0.17, 0.31 ± 0.01, 0.18 ± 0.22, and 0.04 ± 0.02 and were significantly different than currents at time 0 h for all doses of DZA (30, 50, 100, and 300 µM, respectively; n = 6) and amiloride. In contrast, relative current at 2 h across monolayers treated with vehicle (0.95 ± 0.09) was not different compared with 0 h. Moreover, all relative currents at 2 h were different compared with vehicle at 2 h. The half inhibitory concentration for DZA was between 40 and 50 µM. Interestingly, current inhibition by DZA at concentrations <100 µM lasted only 4 h, after which (≥6 h) current tended toward levels prior to addition. Relative current at 8 h for the 30 µM group was significantly greater than cells treated with vehicle.

The effects of SAHHase inhibition on aldosterone-induced current and cellular concentrations of AdoMet and SAH are shown in Fig. 3. The changes in current induced by aldosterone in the absence and presence of DZA are shown in Fig. 3A and summarized in Table I. Addition of aldosterone (1.5 mM) for 4 h to monolayers serum and steroid deprived for 48 h significantly increased eqIsc by 1.84 ± 0.11 µA/cm² (n = 16). In the presence of 50, 100, and 300 µM DZA, aldosterone significantly increased current (ΔeqIsc = 1.05 ± 0.06, 0.82 ± 0.13 and 0.67 ± 0.13, respectively) compared with base line; however, induced current in the presence of DZA (at all doses) was significantly lower compared with that in the absence of DZA.

Fig. 3B shows the percent change in AdoMet and SAH levels in A6 cells in response to aldosterone in the absence and presence of the SAHHase inhibitor, DZA. AdoMet levels significantly decreased by 15.6 ± 1.7% (n = 6) in response to aldosterone. Similarly, aldosterone significantly decreased SAH levels by 5.8 ± 1.5% (n = 6). In the presence of DZA (300 µM), aldosterone-induced AdoMet catabolism was significantly attenuated with levels being only 2.5 ± 0.5% (n = 6) lower than the concentration prior to steroid addition. Upon simultaneous addition of DZA with aldosterone, SAH level significantly increased 14.6 ± 0.1% (n = 6), which is significantly greater compared with that in the absence of inhibitor. These observations demonstrate that inhibition of SAHHase activity decreases SAH metabolism resulting in a concomitant decrease in Metabolism.

Regulation of methyltransferase activity in A6 cells by end product inhibition was confirmed. Transmethylation of pseudosubstrate (APC) in response to enzymes contained in whole cell lysate was significantly inhibited by SAH (100 µM) from a control of 43.46 ± 4.80 cpm/µg to 20.04 ± 0.34 cpm/µg (n = 3; data not shown in a figure).
SAHHase Regulates Na⁺ Transport

Fig. 3. Regulation of aldosterone-induced current and AdoMet (SAM) and SAH catabolism by DZA. A, repleted is addition of 1.5 μM aldosterone for 4 h (n = 16); and +50 (n = 18), +100 (n = 18), and +300 (n = 17) μM are repletion in the presence of DZA (at respective concentration). Aldosterone repletion in the absence and presence of DZA increased current above baseline. *, p < 0.05 versus repleted control; **, versus +50 μM. B, changes in AdoMet and SAH levels are relative to aldosterone-depleted cells. AdoMet and SAH levels decreased approximately 15 and 6%, respectively, in response to aldosterone. However, in response to aldosterone in addition to 300 μM DZA (+DZA), AdoMet levels decreased only by about 3% and SAH levels increased nearly 15%.

Table I

| Group observations | Depleted b | Repleted, 4 h | ΔW, mA/cm² |
|--------------------|------------|--------------|-------------|
| Wash, n = 18       | 0.59 ± 0.21| 0.71 ± 0.18  | 0.12 ± 0.24 e |
| Control, n = 45    | 0.60 ± 0.07| 2.3 ± 0.10 c | 1.7 ± 0.06  |
| +DZA, n = 18       | 0.58 ± 0.08| 1.41 ± 0.10 c | 0.82 ± 0.13 |
| +Sense, n = 63     | 0.47 ± 0.05| 2.1 ± 0.08 c | 1.6 ± 0.10  |
| +Antisense, n = 107| 0.62 ± 0.06| 1.4 ± 0.07 c | 0.82 ± 0.10 f |
| pControl, n = 22   | 0.48 ± 0.07| 2.1 ± 0.20 c | 1.6 ± 0.13  |
| pxSAHHase, n = 18  | 0.72 ± 0.08| 3.3 ± 0.16 c | 2.6 ± 0.16 f |

All values in this column are not different.

% change

Fig. 4 shows that aldosterone increases SAHHase activity in cell lysate but does not increase significantly xSAHHase mRNA levels. Active SAHHase was inhibited by DZA. As shown in Fig. 4A (and summarized in Table II), application of aldosterone for 4 h to serum- and steroid-starved cells significantly increased SAHHase activity from 1.19 ± 0.19 (n = 5) to 2.38 ± 0.26 (n = 8) nM/min/mg. However, aldosterone-induced SAHHase activity was significantly attenuated to 0.39 ± 0.10 (n = 8) upon addition of DZA (10 μM) to cell lysate during enzyme assays. Northern analysis in Fig. 5, A and B, shows that mRNA identified by a radiolabeled xSAHHase probe does not increase in response to aldosterone. In such experiments (representative blot shown in Fig. 4B and 5 experiments summarized in Fig. 4C), the ratio of xSAHHase to GAPDH mRNA did not significantly change in response to aldosterone application for 4 h with the steroid-depleted and repleted ratios being 1.04 ± 0.30 (n = 3) and 1.58 ± 0.34 (n = 5), respectively.

Antisense Inhibition of Aldosterone-induced SAHHase Activity and Current—Application of oligonucleotides complementary to the region of the xSAHHase sequence around the transcription start site reduced SAHHase activity and aldosterone-induced Na⁺ transport (Fig. 5). Fig. 5A (and summarized in Table ID) shows that the SAHHase activity in cells treated with aldosterone for 4 h and sense oligonucleotide overnight (2.76 ± 0.42 nM/min/mg; n = 7) was not significantly different from the SAHHase activity in cells treated with steroid alone (2.38 ± 0.26 nM/min/mg; n = 8; Fig. 4A). However, the SAHHase activity in cells that were treated with aldosterone for 4 h and antisense oligonucleotides overnight (1.26 ± 0.21 nM/min/mg; n = 7) was significantly lower than cells treated with aldosterone and sense oligonucleotides or cells treated with steroid alone.

Aldosterone-induced current also was inhibited by antisense but not sense oligonucleotides (Fig. 5B and Table I). In sense-treated cells, aldosterone significantly increased eqIsc by 1.6 ± 0.10 μA/cm² (n = 63), an increase similar to the response of cells treated with steroid alone. In contrast to sense-treated cells, pretreatment with antisense oligonucleotides significantly decreased aldosterone-induced current by approximately 50% to 0.82 ± 0.10 μA/cm² (n = 107). Note that simply washing steroid and serum-starved cells with basic media failed to significantly change current (n = 18).

Overexpression of SAHHase Increased Aldosterone-induced SAHHase Activity, Methylation, and Na⁺ Reabsorption—Overexpression of SAHHase in HEK293 cells increased hydrolase activity and AFC methylation (Fig. 6). Fig. 6A shows that the hydrolase activity in pxSAHHase3.1(−)zeo-transfected HEK293 cells of 1.66 ± 0.27 nM/min/mg (n = 6) is significantly greater than the 0.90 ± 0.16 nM/min/mg (n = 6) observed in control-transfected cells. Moreover, the methylation of AFC, depicted in Fig. 6B, of 71.4 ± 11.77 cm³/μg × 10⁻² (n = 7) in lysate from pxSAHHase3.1(−)zeo-transfected HEK293 cells is significantly greater compared with the 39.6 ± 8.3 (n = 9) cm³/μg/min × 10⁻² in control transfectants.

Transfection with pxSAHHase3.1(−)zeo of A6 cells, shown in Fig. 7, increased both xSAHHase mRNA level and SAHHase...
activity. As shown in the representative Northern blot (Fig. 7A), A6 cells transfected with pxSAHHase3.1(−)zeo had more xSAHHase mRNA compared with control transfecteds. Four such experiments are summarized in Fig. 7B. The ratio of relative band densities for xSAHHase to GAPDH is significantly increased 2.65-fold in pxSAHHase3.1(−)zeo versus control (relative density of xSAHHase/GAPDH = 1) transfectants. Fig. 7C (summarized in Table II) demonstrates that similar to message level, SAHHase activity in the absence of aldosterone and serum is increased in A6 cells transfected with pxSAHHase3.1(−)zeo (2.36 ± 0.37 nM/min/mg; n = 5). Aldosterone addition significantly increased activity in pxSAHHase3.1(−)zeo transfectants to 4.91 ± 0.66 nM/min/mg (n = 8). The activity in response to aldosterone for SAHHase-overexpressing cells was significantly greater compared with that for control transfectants (2.82 ± 0.50 nM/min/mg; n = 8). Note that the activity in aldosterone-treated control transfectants was not different than that in steroid-treated, non-transfected cells.

Overexpression of SAHHase in addition to increasing enzyme activity potentiated aldosterone-induced \( i_{\text{Na}} \) (Fig. 8 and Table I). Cells overexpressing SAHHase did not have a significantly elevated basal current compared with control transfectants and non-transfected cells but did have a significantly increased current response to aldosterone repletion (1.5 μM; 4 h). Aldosterone significantly increased current by 1.6 ± 0.13, 1.7 ± 0.06, and 2.6 ± 0.16 μA/cm² in control-transfected (n = 22; 1st black bar), nontransfected (n = 45; not shown in this figure), and pxSAHHase3.1(−)zeo-transfected (n = 18; 1st gray bar) A6 cell monolayers, respectively. The aldosterone-induced current in SAHHase-overexpressing cells was significantly greater compared with that in control transfecteds and non-transfected cells. Aldosterone-induced current in all transfecteds was amiloride (5 μM)-sensitive with inhibitor causing a significant decrease in steroid-increased Na⁺ transport. In the presence of aldosterone and amiloride current in pxSAHHase3.1(−)zeo transfectants decreased from basal levels by −0.281 ± 0.04 μA/cm² (2nd gray box). Currents in the presence of aldosterone after amiloride addition for control and pxSAHHase3.1(−)zeo transfectants were 0.53 ± 0.10 and 0.65 ± 0.06 (n = 12) μA/cm², respectively. These currents are not significantly different to currents in the absence of steroid.

A summary of the short circuit current induced by aldosterone repletion to A6 cells overexpressing SAHHase and in the presence of molecular and pharmacological SAHHase inhibitors is shown in Table I. Aldosterone (1.5 μM) addition for 4 h significantly increased current in control A6 cells, whereas merely changing the bathing medium without addition of steroid had no effect. Aldosterone also increased current in transfected cells and cells treated with antisense and DZA. Basal (depleted) currents for DZA and antisense-treated cells and transfected cells were not different from appropriate controls. In DZA and antisense-treated cells, aldosterone-induced current was decreased compared with control cells and sense-treated cells, respectively. Aldosterone increased current greater in pxSAHHase3.1(−)zeo transfectants compared with control cells and control transfecteds.

The regulation of SAHHase activity by aldosterone in the presence of pharmacological and molecular inhibitors of SAHHase and in overexpressing A6 cells is summarized in Table II. Aldosterone repletion increased activity in control and overexpressing cells; however, the activity in aldosterone-treated pxSAHHase3.1(−)zeo-transfected cells was greater compared with treated, non-transfected and transfected controls. Activity in cells treated with aldosterone and antisense was lower compared with that in cells treated with aldosterone alone or in addition to sense oligonucleotide. Similarly, DZA decreased SAHHase activity compared with control.

**DISCUSSION**

The current study directly assesses the role SAHHase plays in the signal transduction initiated by aldosterone which culminates in increased Na⁺ reabsorption. Aldosterone increased the activity of SAHHase through post-translational regulation. Moreover, as shown by both pharmacological and molecular inhibitors, induction of SAHHase activity was critical for aldosterone to increase and maintain Na⁺ reabsorption across renal epithelial cells. The current results also demonstrate that
although overexpression of SAHase potentiates aldosterone-
induced current and enzyme activity, overexpression alone is
insufficient to mimic completely all the natriferic actions of the
steroid. Thus, these results support the hypothesis that the
critical methylation required for aldosterone signal transduc-
tion is regulated by SAH catabolism with SAHHase activity
being the control point.

**Regulation of Na\(^+\) Transport by Transmethylation**—Shown
in Fig. 9 is a schematic depiction of the critical enzymes and
metabolites for the aldosterone-induced transmethylation re-
action that modulates Na\(^+\) channel activity. Sariban-Sohraby
and colleagues (4) and Wiesmann et al. (5) were the first to
document the association of aldosterone-induced Na\(^+\) trans-
port with the methylation reaction. These investigators showed
that methyl donors, such as AdoMet, could mimic the actions of
aldosterone. In addition, inhibition of transmethylation
blocked induction of transport by aldosterone demonstrating that a critical methylation was necessary for \( \text{Na}^+ \) reabsorption. Interestingly, aldosterone increased transmethylation of disparate classes of molecules, including both proteins and lipids, suggesting that the mineralocorticoid regulated methylation in general but did not regulate a specific transmethylation reaction. The current results show that one mechanism through which aldosterone regulates methylation is by altering the activity of SAHHase. Our results demonstrate that aldosterone increases \( \text{Na}^+ \) transport by first increasing the activity of SAHHase through an as yet undefined post-translational modification. An aldosterone-induced increase in hydrolysis of SAH increases the rate of most transmethylation reactions by reducing end product feedback inhibition.

Several other investigators also have confirmed that \( \text{Na}^+ \) transport and ENaC activity are regulated by transmethylation (3, 6–8); however, the substrate for this aldosterone-induced methylation remains unclear. Recent results from our laboratory suggest that methylation of p21ras is increased by aldosterone (9), and results from the laboratory of Johnson (10) support the notion that \( \beta \varepsilon \text{NaC} \) also is an aldosterone-sensitive methylation substrate.

Characterization and Regulation of A6 Cell SAHHase—Deazaadenosine decreased aldosterone-induced SAH hydrolysis (Fig. 3B) and SAHHase activity (Fig. 4A) in intact and cell-free assays, respectively. This finding is consistent with that reported by others (11, 12). Similar to DZA, antisense oligonucleotide complementary to the region around the translation start site of xSAHHase mRNA decreased enzyme activity (Fig. 5A).

The activity of SAHHase as measured by SAH hydrolysis in steroids-deprived A6 cell lysate (Fig. 4A) was similar to hydrolytic activity reported in crude extract of guinea pig cardiac muscle (18) and bovine kidney (19); however, activity was greater compared with crude extract of cat, rabbit, rat, and dog heart (18). Aldosterone repletion for 4 h to A6 cell monolayers doubled SAHHase activity to a level similar to that observed in rat liver extract (20). This induction of activity demonstrates that SAHHase is regulated by aldosterone signal transduction, an observation consistent with that reported previously by Finkelstein and Harris (21) showing that adrenal steroids regulate SAHHase activity in rat epithelial tissue. The mechanism for modulation of SAHHase by steroid, however, remains to be determined.

Three mechanisms could account for increased activity in response to aldosterone as follows: 1) increased enzyme concentration, 2) increased activity through post-translational modification of the enzyme, and 3) relief of the enzyme from metabolic regulation by increased degradation of an inhibitor, such as adenosine. Aldosterone failed to significantly increase SAHHase mRNA to the extent that SAHHase enzyme activity increased (Fig. 4) suggesting that an increase in enzyme concentration was unlikely. The observation that activity was higher in crude extract from cells treated with aldosterone compared with untreated cells suggests some form of post-translational modification, since SAHHase presumably has been relieved of end product inhibition by the inclusion of exogenous adenosine deaminase.

Translation of pxSAHHase3.1(−)zeo produced a protein of size (Fig. 1) consistent with that reported previously for SAHHase (11, 19). Overexpression of SAHHase increased enzyme activity in both HEK293 (Fig. 6A) and A6 cells (Fig. 7C). The observation that overexpressing A6 cells deprived of aldosterone had 2.65-fold increase in SAHHase mRNA levels compared with control transfectants but only 1.56-fold the activity supports the notion that post-translational events regulated by aldosterone control enzyme activity. Moreover, aldosterone application to overexpressing cells had an additive effect on SAHHase activity demonstrating that distinct from regulating protein number, steroid increased activity through some other mechanism.

These results combined show that aldosterone increases SAHHase activity through a signal transduction event culminating in post-translational regulation of the enzyme. The primary amino acid sequence of SAHHase contains multiple consensus phosphorylation sites for both serine/threonine and tyrosine kinases. In addition, a number of consensus sequences for fatty acylation exist. Although the mechanism of SAHHase regulation by aldosterone remains to be determined, it is possible that this enzyme is regulated by post-translational modifications, such as phosphorylation or acylation. It will be interesting to test whether SAHHase is regulated by the recently characterized, aldosterone-induced serine/threonine protein kinase, Sgk, which recently has been shown to be transcriptionally regulated in response to aldosterone (22).

**Regulation of the Transmethylation Reaction by SAHHase Activity**—It is well established that SAH is an end product inhibitor of most transmethylation reactions involving AdoMet as the methyl donor (11). Sariban-Sohraby and colleagues (4) first showed that DZA and SAH inhibited the acute actions of aldosterone as measured by \( \text{Na}^+ \) flux across apical membrane vesicles prepared from A6 cells. Moreover, application of aldosterone increased both lipid and protein transmethylation in a DZA-sensitive manner in epithelia suggesting that aldosterone affected a general control point regulating all forms of methylation. Eaton et al. (3) have shown through single channel analysis that DZA regulates \( \text{Na}^+ \) channel activity likely through regulating the transmethylation reaction. Although all of these studies support the hypothesis that SAHHase activity regulates substrate methyl esterification, this mechanism was not the focus of any of these studies or investigated directly, and only a single pharmacological inhibitor was used. Moreover, DZA was used only at a single concentration in all previous studies. Because of this, nonspecific effects of DZA could not be ruled out, and it was unclear if control of SAHHase was a physiological mechanism for regulating transmethylation reactions relevant to aldosterone-induced \( \text{Na}^+ \) transport in epithelial cells.

The current study is the first to investigate directly the role...
SAHHase plays in regulation of aldosterone-induced methylation and Na$^+$ current. The results of the current study show that aldosterone addition to A6 cells increases both SAH and AdoMet metabolism (Fig. 3B). Inhibition of SAHHase by DZA decreased SAH metabolism to such an extent that cellular concentrations rose in response to aldosterone treatment presumably because AdoMet metabolized by methyltransferase increased SAH, which could not be catabolized by an inhibited SAHHase. This notion also can account for the decrease in AdoMet metabolism observed in the presence of DZA with increased SAH decreasing methyltransferase activity and, thus, AdoMet catabolism. Methyltransferase activity in A6 cell lysate also was decreased by end product inhibition demonstrating that SAHHase activity through regulation of cellular SAH levels can control substrate methylation.

Overexpression of SAHHase resulted in an increase in both SAHHase activity and substrate methyl esterification (Fig. 7C). These results are consistent with aldosterone increasing metabolism of the end product inhibitor of methyl esterification reactions, SAH, resulting in a concomitant increase in AdoMet metabolism in A6 cells. Thus, in Na$^+$-transporting epithelial cells, one regulatory site controlling substrate methylation is modulation of SAHHase activity.

**Regulation of Na$^+$ Current by SAHHase Activity—Simultaneous addition of DZA with aldosterone for 4 h inhibited induced current in a dose-dependent manner (Fig. 3A). Also, addition of DZA to monolayers reabsorbing Na$^+$ inhibited transport within 2 h in a dose-dependent manner (Fig. 2). After 4 h, Na$^+$ current in the group treated with 30 μM DZA began to return with current levels overshooting baseline by 8 h. The time courses for maximal inhibition of existing and aldosterone-induced transport are consistent with previous findings also showing that DZA inhibits Na$^+$ transport in toad bladder and A6 epithelia (4, 5). Also consistent with our observations that DZA inhibits aldosterone-induced Na$^+$ reabsorption are the findings of Eaton et al. (3) that application of DZA for 2–4 h inhibits aldosterone-induced activation of ENaC in cell-attached patches.

The results of the current study demonstrating that DZA blockade of steady state Na$^+$ reabsorption begins to recover after 4 h can be explained by metabolism of DZA to a less potent inhibitor of SAHHase. It is likely that DZA, which also is a substrate for SAHHase in the synthetic direction (11), was metabolized to deaza-D-adenosylhomocysteine after 4 h. Since the $K_i$ for deaza-D-adenosylhomocysteine is 100 times that of DZA (23), this metabolism would enable current recovery. Since DZA is absent in antisense experiments, results from those experiments showing no current recovery are consistent with this mechanism.

Direct support for the hypothesis that aldosterone induces Na$^+$ transport, in part, by regulating SAH hydrolysis is provided by antisense and overexpression experiments. Antisense oligonucleotide but not sense decreased both aldosterone-induced enzyme activity and Na$^+$ current (Fig. 5) demonstrating that active SAHHase is necessary for salt reabsorption. Overexpression of SAHHase increased enzyme activity in an aldosterone-sensitive manner (Fig. 7). Moreover, overexpression of SAHHase potentiated aldosterone-induced current but did not affect basal currents (Fig. 8) suggesting that increased SAH-activity alone was insufficient to signal increased transport.

The current results support the hypothesis that a critical methyl esterification in response to aldosterone application signals the increase in ENaC activity at the apical membrane of epithelial cells. Since methyltransferase and SAHHase are metabolically linked by SAH concentration, the latter enzyme is a likely site for regulating methyl esterification. The results of the current study demonstrate that aldosterone first must increase SAHHase activity to initiate the subsequent increase in both methyl esterification and Na$^+$ transport. Thus, regulation of SAHHase activity by aldosterone-induced signal transduction is a control site for steroid induction of methylation and subsequent Na$^+$ reabsorption.

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