Isoprenylcysteine-O-carboxyl Methyltransferase Regulates Aldosterone-sensitive Na⁺ Reabsorption*

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The Xenopus laevis distal tubule epithelial cell line A6 was used as a model epithelia to study the role of isoprenylcysteine-O-carboxyl methyltransferase (pcMTase) in aldosterone-mediated stimulation of Na⁺ transport. Polyclonal antibodies raised against X. laevis pcMTase were immunoreactive with a 33-kDa protein in whole cell lysate. These antibodies were also reactive with a 33-kDa product from in vitro translation of the pcMTase cDNA. Aldosterone application increased pcMTase activity resulting in elevation of total protein methyl esterification in vivo, but pcMTase protein levels were not affected by steroid, suggesting that aldosterone increased activity independent of enzyme number. Inhibition of pcMTase resulted in a reduction of aldosterone-induced Na⁺ transport demonstrating the necessity of pcMTase-mediated transmethylation for steroid induced Na⁺ reabsorption. Transfection with an eukaryotic expression construct containing pcMTase cDNA increased pcMTase protein level and activity. This resulted in potentiation of the natriuretic actions of aldosterone. However, overexpression did not change Na⁺ reabsorption in the absence of steroid, suggesting that pcMTase activity is not limiting Na⁺ transport in the absence of steroid, but that subsequent to aldosterone addition, pcMTase activity becomes limiting. These results suggest that a critical transmethylation is necessary for aldosterone-induction of Na⁺ transport. It is likely that the protein catalyzing this methylation is isoprenylcysteine-O-carboxyl methyltransferase and that aldosterone activates pcMTase without affecting transferase expression.

Mean arterial pressure is maintained in homeostasis by tight control of volume reabsorption in the distal tubule of the nephron where water follows NaCl reabsorption by osmosis with activity of luminal Na⁺ channels being rate-limiting. The primary hormone regulating discretionary Na⁺ reabsorption in the distal tubule is the mineralocorticoid aldosterone. This steroid hormone increases electronegic Na⁺ reabsorption across collecting duct principal cells in a biphasic manner with an early phase (<4 h) resulting in induction of luminal Na⁺ channel activity and a secondary phase (>6 h) resulting in trophic increases in both apical Na⁺ channel and serosal Na⁺/K⁺-ATPase protein numbers (1, 2). Both phases require gene expression; however, the induced proteins remain relatively uncharacterized (3, 4). Whereas the systemic and tissue specific actions of aldosterone are clear, the cellular signal transduction pathways initiated by this steroid remain unclear.

Sariban-Sohraby et al. (5) and Wiesmann et al. (6) were the first to demonstrate an aldosterone increase in transmethylation of protein and lipid with a time course of methylation correlating with the early phase of aldosterone action. Blockage of methylation attenuated Na⁺ transport in response to aldosterone, suggesting a dependence of transport on transmethylation (5–7). Single channel analysis showed that the epithelial Na⁺ channel (ENaC), the end effector of aldosterone signaling, is activated in response to application of the methyl donor, S-adenosyl-l-methionine (AdoMet), to the intracellular face of the channel (2, 8, 9). Simultaneous addition of GTP with AdoMet potentiated ENaC activation. Moreover, inhibitors of methylation decreased ENaC activation. Because GTP often potentiates protein methylation (10, 11), these results are consistent with the actions of aldosterone requiring an essential protein methylation.

Methylation is analogous to phosphorylation with both being molecular switches that control protein activity/locale in a reversible manner. Protein methylation is mediated by a class of enzymes, the protein methyltransferases that use AdoMet as a methyl donor to transfer a methyl moiety onto a nucleophilic oxygen, nitrogen, or sulfur in a polypeptide. Protein methyltransferases are classified in two major groups, those that modify carboxyl groups to form methyl esters and those that modify sulfur and nitrogen. Methylation catalyzed by the prior enzymes is reversible and can regulate protein activity. In contrast, methylation of sulfur and nitrogen is irreversible and produces alternative forms of physiological amino acids. Four types of protein carboxyl methyltransferases are known: 1) type I modifies glutamate residues contained in bacterial chemoreceptors; 2) type II modifies aspartyl residues targeting proteins for repair or degradation; 3) type III modifies the carboxyl group of COOH-terminus isoprenylcysteines (pcMTase; EC 2.1.1.100); and 4) type IV modifies the carboxyl group of COOH-terminus leucines (for review, see Ref. 12). The most well described transmethylation involved in eukaryotic cell signal transduction is reversible methyl esterification of proteins on the carboxyl groups of COOH-terminal

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1 The abbreviations used are: ENaC, epithelial Na⁺ channel; pcMTase, isoprenylcysteine-O-carboxyl methyltransferase; AdoMet, S-adenosyl-l-methionine; FTS, farnesylthiosalicylic acid; AFC, N-acetyl-S-farnesyl-l-cysteine; GTP·S, guanosine 5′-O-(thio)triphosphate.
isoprenylcysteines. Small, monomeric G proteins, such as p21Ras, nuclear lamin B, cyclic nucleotide phosphodiesterase, and subunits of trimeric G proteins all contain COOH-terminal cysteine isoprenoids. These proteins are post-translationally modified by transmethylation (12, 13). Methylation of these signaling molecules controls their activity and cellular localization. In particular, Ras localizes to the inner leaflet of the plasma membrane after methyl esterification. In some instances, this may be the rate-limiting step controlling Ras signaling. In particular, Ras localizes to the inner leaflet of the plasma membrane after methyl esterification. In some instances, this may be the rate-limiting step controlling Ras signaling.

The gene encoding isoprenylcysteine-O-carboxyl methyltransferase initially identified in Saccharomyces cerevisiae was recently cloned in Schizosaccharomyces pombe (15). In the past 2 years, vertebrate homologs have been identified: first in Xenopus laevis (15), and then in humans (16). Presently, cellular characterization of pcMTase protein in vertebrates is limited to a single study where antibody was used to characterize the cellular localization of human pcMTase (16).

Although there is substantial evidence that transmethylation regulates Na⁺ transport, the specific type of methyltransferase catalyzing this post-translational event in response to aldosterone remains unclear. Moreover, the direct effects of aldosterone on methyltransferase protein expression and the role this plays in aldosterone induction of Na⁺ transport remain unclear.

Regulation of the natriiferic action of aldosterone by pcMTase was tested in this study. In addition, the effects of aldosterone on pcMTase protein level and activity were determined. The cellular properties of pcMTase protein also were further characterized. Isoprenylcysteine-O-carboxyl methyltransferase activity was found to be critical for aldosterone-induced Na⁺ transport. Moreover, the activity of this enzyme can become limiting in the presence of steroid. Aldosterone increased pcMTase enzyme activity without affecting enzyme number. Overexpression of pcMTase potentiated aldosterone-induced Na⁺ transport, but failed to mimic all steroid actions suggesting that pcMTase is not itself an aldosterone-induced protein but ultimately is regulated by an aldosterone-induced protein.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

A6 cells (American Type Culture Collection) were used for all experiments. This cell line is a well characterized model of the mammalian renal, principal cell that is capable of aldosterone-sensitive vectorial Na⁺ transport. Cells were maintained in culture as described previously (7, 8). Complete media included aldosterone (1.5 μM) and fetal bovine serum (10%), whereas basic media lacked steroid and serum. This study focused on the early actions of aldosterone. Thus, all experiments were performed 4 h after addition of aldosterone or vehicle unless indicated otherwise.

**Molecular Biology**

**Amplification of Isoprenylcysteine-O-carboxyl Methyltransferase cDNA**—A full-length X. laevis pcMTase clone was amplified using a high stringency reverse transcriptase-polymerase chain reaction with specific primers (forward primer, 5'-gcttcagtcggcag-3'; reverse primer, 5'-cattggtcgctctcavec-3') developed from the published X. laevis pcMTase cDNA sequence (accession number D87750) (15). The cDNA was subsequently ligated into pGEM-T Easy (Promega) and then subcloned into pcDNA3.1Zeo(–) (Invitrogen) with NotI. Nucleotide sequence data from the expression plasmid (pXMT.zeo) was identical to that reported by Imai et al. (15). Expression of pcMTase from pXMT.zeo is controlled by the cytomegalovirus promoter. In vitro translation of pcXMT.zeo produced a protein of appropriate size (~33 kDa) indicating a complete open reading frame.

**Overexpression of pcMTase**—A6 cells were transfected with pcMT.zeo using the LipofectAMINE PLUS reagents (Life Technologies, Inc.) as described previously (7). Zeocin (600 μg/ml) treatment was used to select for successfully transfected cells. Subpassages (up to 4) of the zeocin-selected (mass population) A6 cells were used for experimentation. Control transfectants contained either vector alone or pVgRXR (Invitrogen). Neither control plasmid was observed to affect Na⁺ transport or enzyme activity.

**Anti-pcMTase Antibody**—Peptides corresponding to the carboxyl-terminal 19 amino acid residues (NH₂-CYKXKVTPLPGHKVMEPCOOH; AB601), and 191–209 (NH₂-CCHIQVQKSDSHTLVTSGVCOOH; AB596) of X. laevis pcMTase were synthesized with an amino-terminal cysteine, linked to keyhole limpet hemocyanin, and used to immunize rabbits (Lofstrand Labs Ltd.) to create polyclonal anti-pcMTase antibodies. In X. laevis, the only polypeptide with identity to these antigens as described by a blastp (National Center for Biotechnology Information) search was pcMTase.

**Immunoblotting and Immunoprecipitation**—Immunoblotting and immunoprecipitation were performed on whole cell lysates harvested with detergent (1% Nonidet P-40) using standard protocols. Lysates and immunoprecipitations were separated by SDS-polyacrylamide gel electrophoresis in the presence of reducing reagent and subsequently transferred to nitrocellulose. Immunoblotting was performed in Tris-buffered saline with 5% milk and 0.1% Tween 20. Reactive proteins were detected using the enhanced chemiluminescence system. Band density was then quantified using SigmaGel software (Jandel Scientific).

**Proto-PCMTase Antigen**—Immunoprecipitation was performed on whole cell lysates harvested with detergent (1% Nonidet P-40) using standard protocols. Lysates and immunoprecipitations were separated by SDS-polyacrylamide gel electrophoresis in the presence of reducing reagent and subsequently transferred to nitrocellulose. Immunoblotting was performed in Tris-buffered saline with 5% milk and 0.1% Tween 20. Reactive proteins were detected using the enhanced chemiluminescence system. Band density was then quantified using SigmaGel software (Jandel Scientific).

**Statistical Analysis**

Statistical analysis was determined using Student’s t test for paired and unpaired data as appropriate.

**RESULTS**

Identification and Characterization of Isoprenylcysteine-O-carboxyl Methyltransferase in A6 Epithelia—Based on the published sequence (15), LKMTase should be a protein of approximately 33 kDa. Fig. 1 shows the specific reaction of anti-pcMTase antibody (AB601) with a protein in whole cell lysate of approximately 33 kDa. In a typical Western blot (n = 9), AB601 identified the 33-kDa protein, but pre-immune antiserum and antibody pre-absorbed with antigenic peptide (0.3 mg/ml) failed to react with this protein. AB601 also immunoprecipitated the...
Aldosterone failed to significantly affect pcMTase protein levels for 48 h compared with lysate from cells treated with steroid. Body on lysate prepared from A6 cells deprived of aldosterone shows a typical Western blot probed with anti-pcMTase antibody. Lane 1 was blotted with AB601, Lanes 2 and 3 were blotted with pre-immune antisera and AB601 pre-absorbed with antigen, respectively.

**Fig. 1.** Identification of a 33-kDa protein (arrow) by Western blot analysis on whole cell lysate using anti-pcMTase antibody. Lane 1 was blotted with AB601, Lanes 2 and 3 were blotted with pre-immune antisera and AB601 pre-absorbed with antigen, respectively.

**Fig. 2.** Aldosterone increases pcMTase activity without affecting protein level. A, total protein methylation in vivo increases after addition of 1.5 μM aldosterone compared with that in untreated cells. Similarly, AFC methylation in vitro (B) is increased by addition of aldosterone (gray bars). GTP-S (1 mM) enhances the ability of aldosterone to increase AFC methylation. *, versus control; **, versus GTP alone and aldosterone in the absence of GTP. A typical Western blot analysis (C) and summary densitometry graph (D) showing that aldosterone does not affect pcMTase protein level.

Aldosterone Increases Activity but Not Protein Level of pcMTase—Aldosterone addition increased protein methylation in vivo and pcMTase activity in vitro but did not affect pcMTase protein levels. Aldosterone addition (Fig. 2A and Table I) significantly increased methyl esterification of total protein from steroid-free levels of 8.0 ± 0.6 to steroid-treated levels of 20.1 ± 0.8 methyl ester cpm/μg (n = 3). Similarly, pcMTase activity as assayed by artificial substrate methyl esterification (Fig. 2B and Table I) was significantly greater in lysate prepared from aldosterone-treated cells compared with untreated cells with an activity of 5.6 ± 0.9 × 10^6 in the presence of steroid compared with 2.2 ± 0.3 × 10^6 cpm/mg in the absence (n = 12). In contrast, Fig. 2C, D and summary (Table I) shows that aldosterone failed to affect pcMTase protein level. Fig. 2C shows a typical Western blot probed with anti-pcMTase antibody on lysate prepared from A6 cells deprived of aldosterone for 48 h compared with lysate from cells treated with steroid. Aldosterone failed to significantly affect pcMTase protein levels with lysate prepared from treated cells having 3.7 ± 0.3 arbitrary units compared with 3.7 ± 0.3 for lysate from untreated cells (Fig. 2, panel D; n = 8). Fig. 2B also shows that GTP·S (1 mM) enhances the ability of aldosterone to increase pcMTase activity from 3.8 ± 1.1 × 10^6 cpm/mg with GTP·S in the absence of steroid to 9.0 ± 1.2 × 10^6 cpm/mg with GTP·S plus steroid.

Table I summarizes the changes of protein level, protein methylation, and pcMTase activity in response to aldosterone. Although pcMTase protein levels did not change in response to aldosterone, pcMTase activity and total protein methylation increased comparably by 2.6- and 2.5-fold, respectively, suggesting that the increase in protein methylesterification resulted mainly from an increase in pcMTase activity.

**Active pcMTase Is Necessary for Aldosterone Induction of Na^+ Reabsorption**—Fig. 3 shows that active pcMTase is essential for aldosterone-induced increases in Na^+ transport. Fig. 3A demonstrates that simultaneous addition of farnesylthiosalicylic acid (FTS, 100 μM), a competitive inhibitor of pcMTase (18), with aldosterone significantly decreased methylation of AFC from 43.5 ± 4.8 cpm/μg/h to 0.8 ± 0.2 cpm/μg/h (n = 3). Moreover, as shown in Fig. 3B, aldosterone-induced Na^+ reabsorption is significantly reduced by the pcMTase inhibitors, FTS and AFC.

**Overexpression of pcMTase Potentiates Aldosterone-induced Na^+ Reabsorption**—The Western blot of Fig. 4A (inset) shows that lysate prepared from two consecutive passages (lanes 2 and 3) of cells transfected with pxMT.zeo had a greater amount of pcMTase compared with control transfectants (lane 1). The data represented in the summary graph of Fig. 4A show that the relative density of pcMTase in lysate prepared from cells transfected with pxMT.zeo was 3.14-fold greater compared with control transfectant lysate (n = 8). The methyltransferase activity in lysate prepared from pcMTase overexpressing cells was significantly greater than that in lysate from cells transfected with control plasmid (Fig. 4B). Similarly, lysate prepared from HEK293 cells transiently transfected with pxMT.zeo showed increased methyl esterification of AFC (95.5 ± 20.2 cpm/μg/h) compared with that from control transfections (20.8 ± 2.4 cpm/μg/h; n = 5; data not shown).

Cells overexpressing pcMTase, as shown in Fig. 5, had significantly more aldosterone-induced Na^+ reabsorption compared with control transfectants at both 4 and 24 h. The relative (compared with time 0) increase in Na^+ current in response to aldosterone addition for 4 and 24 h in cells overexpressing pcMTase was 8.1 ± 1.7- and 8.6 ± 1.0-fold, respectively, but was only 3.2 ± 0.3- and 3.6 ± 0.3-fold, respectively, for control transfectants (n = 12). Whereas the aldosterone-induced currents of pcMTase overexpressing A6 cells were greater compared with control transfectants, the basal currents in the absence of steroid and serum in both transfectants were similar with 0.5 ± 0.1 and 0.3 ± 0.1 μA/cm^2 in pxMT.zeo and control transfectants, respectively. Current in all transfectants was amiloride sensitive.

**DISCUSSION**

The data presented support the hypothesis that carboxyl methyl esterification is critical to the aldosterone signal transduction, which culminates in increased Na^+ reabsorption. These results demonstrate that the enzyme catalyzing this critical methyl esterification is likely isoprenylcysteine-O-carboxyl methyltransferase. Though active pcMTase was essential to aldosterone-induced transport, and steroid increased transferase activity in vivo and in vitro, pcMTase was not established as an aldosterone-induced protein, because protein levels did not change with aldosterone treatment. This observation was strengthened further by the results showing...
Nacatalyzed by pcMTase is the transmethylation most relevant to AFC. Aldosterone-induced methylation of the artificial pcMTase substrate, produced current by the isoprenylcysteine transferase inhibitor, FTS. A, FTS (100 μM) significantly inhibited aldosterone-induced methylation of the artificial pcMTase substrate, AFC. B, FTS significantly attenuated aldosterone-induced Na\(^+\) current from 2.9 ± 0.1 to 0.6 ± 0.2 μA/cm\(^2\) (n = 6). Similarly, AFC (200 μM) attenuated aldosterone-induced current to 0.2 ± 0.1 μA/cm\(^2\) (n = 6). That overexpression of pcMTase failed to affect Na\(^+\) transport in the absence of aldosterone but potentiated transport in the presence of steroid. Potentiation of aldosterone-induced Na\(^+\) transport by pcMTase overexpression suggests that in A6 cells, pcMTase activity becomes limiting after the addition of steroid.

**Cellular Characterization of pcMTase in Transport Epithelia—** Isoprenylcysteine-O-carboxyl methyltransferase was identified in A6 epithelia as a 33-kDa protein using Western blot analysis and immunoprecipitation with two distinct antibodies. This is consistent with the predicted size of pcMTase in X. laevis (15). The current results, moreover, show that the product of in vitro translation of X. laevis pcMTase cDNA is in fact 33 kDa and that this protein is immunoreactive with anti-pcMTase antibody. In the only other study directly characterizing pcMTase protein, Dai et al. (16) also showed this protein to be 33 kDa in human myeloid cells.

The cellular localization of the pcMTase that regulates Na\(^+\) transport is unclear. Single channel experiments on ENaC activation by AdoMet suggest luminal localization for pcMTase (2, 8, 9). Moreover, biochemical experiments on A6 epithelia also suggest apical localization (for review, see Ref. 19). However, pcMTase in both human myeloid cells and yeast cells is also suggest luminal localization for pcMTase. The mechanism of channel regulation, however, remains unclear. It is possible that either a subunit of ENaC or a regulator of ENaC is modified by transmethylation. Both possibilities are active areas of research by this and other laboratories. In fact, aldosterone recently has been shown to increase the methyl esterification of the β subunit of ENaC (8) and p21ras (25). Thus, both proteins are possible effectors of aldosterone-induced methylation. Interestingly, transcription of K-ras2A mRNA in A6 cells is increased by aldosterone suggesting that this Ras is an aldosterone-induced protein (3). It will be interesting to test further whether these or other effectors of transmethylation, which also regulate ENaC activity (for review, see Refs. 2, 12, and 19) are the signaling proteins responsible for the natriergic actions of aldosterone and AdoMet.

Aldosterone increased activity of pcMTase 2.6-fold. Similarly, steroid increased total protein methylation 2.5-fold suggesting that the majority of methylesterification in response to aldosterone resulted from elevated pcMTase activity. Activation of pcMTase by AdoMet strongly potentiated aldosterone-induced Na\(^+\) transport in A6 cells suggesting that methyl esterification of the artificial pcMTase substrate, AFC, also a competitive inhibitor of pcMTase (21), decreases aldosterone-induced current, and the results of Blazer-Yost et al. (22) demonstrating that N-acetyl-S-geranylgeranyl-cysteine inhibits Na\(^+\) reabsorption. This later isoprenoid-cysteine analog is an inhibitor of esterification of both S-farnesyl and S-geranylgeranylated COOH-terminal isoprenylcysteines (23, 24).

Our result showing a dependency of Na\(^+\) transport on pcMTase activity is consistent with results of Eaton et al. (2), demonstrating that the majority of methylesterification in response to aldosterone resulted from elevated pcMTase activity. Activation of pcMTase by AdoMet strongly potentiated aldosterone-induced Na\(^+\) transport in A6 cells suggesting that methyl esterification of the artificial pcMTase substrate, AFC, also a competitive inhibitor of pcMTase (21), decreases aldosterone-induced current, and the results of Blazer-Yost et al. (22) demonstrating that N-acetyl-S-geranylgeranyl-cysteine inhibits Na\(^+\) reabsorption. This later isoprenoid-cysteine analog is an inhibitor of esterification of both S-farnesyl and S-geranylgeranylated COOH-terminal isoprenylcysteines (23, 24).
tion of pcMTase did not result from an increase in pcMTase protein. Because pcMTase activity in response to steroid is elevated in vitro, it is likely that some post-translational event mediates the increase. This notion is consistent with the results of Wong et al. (26) demonstrating that phenylethanolamine N-methyltransferase activity in adrenal medulla chromaffin cells is regulated by glucocorticoids independent of the steroid increasing transferase protein number.

The relative protein levels of pcMTase in cells transfected with pxMT.zeo were 3.14-fold higher than that in cells transfected with control plasmid. However, pcMTase activity was 10 times greater. This dramatic increase in activity in cells expressing only 3.14-fold more protein likely results from additional actions of aldosterone on transferase regulation. If aldosterone increases activity approximately 2.6-fold and protein levels are 3.14-fold higher, then pcMTase activity in pxMT.zeo transfectants should be 8.2-fold higher than control transfec-
tants. This number is close to the observed 10-fold increase.

The observation that overexpression of pcMTase potentiates the actions of aldosterone suggests that in the presence of steroid, transferase can become rate-limiting for Na⁺ transport. Moreover, the observations that cells overexpressing pcMTase have greater transferase activity, but no significant increase in Na⁺ transport in the absence of steroid suggest that pcMTase activity is in excess at rest or that a regulator or downstream effector of pcMTase is limiting. Because the increase in methyltransferase activity in lysate prepared from pcMTase overexpressing cells (10-fold) does not correlate well with the aldosterone-induced increase in current (2.5-fold), it appears that pcMTase activity is no longer rate-limiting for steroid-induced Na⁺ transport in cells overexpressing pcMTase as it is in control cells. Methyl esterification and Na⁺ transport are, in part, under metabolic regulation by the activity of S-adenosyl-l-homocysteine hydrolase (5–7) (an enzyme that catabolizes the end-product, negative-feedback regulator of protein methyltransferase). Thus, the current results in conjunction with this earlier observation suggest that aldosterone increases Na⁺ reabsorption by activating pcMTase through some yet to be described post-translational event, in addition to relieving the enzyme from end-product inhibition.

Thus, a critical methyl esterification is necessary for the aldosterone signal transduction that culminates in increased Na⁺ channel activity, and pcMTase likely is the enzyme mediating this critical esterification. Aldosterone does not affect pcMTase protein levels but increases its activity, which increases protein methylation in vivo. That aldosterone-induced but not basal Na⁺ current is potentiated by overexpression of methyltransferase suggests that pcMTase is not an aldosterone-induced protein but is regulated by one. The aldosterone-induced protein regulating pcMTase activity, and the downstream effectors of pcMTase relevant to aldosterone signaling remain to be determined and will be the focus of future studies.

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