Aldosterone acts to increase apical membrane permeability by activation of epithelial Na\(^+\) channels (ENaC). We have previously shown that aldosterone activates ENaC early in the course of its action by stimulating the methylation of the \(\beta\) subunit of this heteromeric channel in A6 cells. Aldosterone also stimulates the expression and methylation of k-ras in A6 cells. To determine whether aldosterone-stimulated methylations are seen in mammalian cells, we examined the effect of aldosterone on methylation and ras activation in a continuous line of cultured epithelial cells derived from mouse cortical collecting duct (CCD) and determined that \(\beta\) mENaC is a substrate for methylation by an enzyme contained in CCD cells. Aldosterone stimulated protein base labile methylation in CCD cells. Aldosterone stimulated Na\(^+\) transport in CCD cells within 1 h of addition and without an increase in cellular amount of any ENaC subunits over the first 4 h. Inhibition of methylation, using the inhibitor 3-deaza-adenosine, blocked the stimulation of Na\(^+\) transport in CCD cells within 1 h of addition and without an increase in cellular amount of any ENaC subunits over the first 4 h. Inhibition of methylation, using the inhibitor 3-deaza-adenosine, blocked the stimulation of Na\(^+\) transport induced by aldosterone at early time points (1–4 h) without affecting cellular amounts of any ENaC subunits. In contrast to 3-deaza-adenosine (3-DZA), which inhibits all methylation reactions, specific inhibitors of small G-protein methylation or prenylation had no effect on the early aldosterone-induced current. Overexpression of isoprenylcysteine carboxymethyltransferase (PCMTase), the enzyme that methylates ras, had little effect on basal transport but enhanced aldosterone-stimulated transport in A6 cells. Overexpression of PCMTase in CCD cells had no effect on either basal or aldosterone-stimulated transport. Moreover, PCMTase had no effect on ENaC activity when co-expressed in Xenopus oocytes. Aldosterone had no effect on either message or protein levels of k-ras in CCD cells. Searching a mouse kidney library, we identified a methyltransferase that stimulates ENaC activity in Xenopus oocytes without affecting surface expression of ENaC. Our results demonstrate that aldosterone stimulates protein methylation in CCD cells, and this is required for expression of the early transport response. In CCD cells this effect is not mediated via methylation of ras, which is not induced by aldosterone in these cells, and the enzyme that methylates ras has no direct effect on ENaC activity. \(\beta\) ENaC is a substrate for methylation in CCD cells. A novel methyltransferase that stimulates ENaC directly has been identified in CCD cells.

Aldosterone stimulates sodium reabsorption in responsive epithelia in part by activation of epithelial Na\(^+\) channels (ENaC). This is often viewed as occurring in several phases: an early phase in which existing channels are activated by alteration in either their trafficking and apical membrane expression (1, 2), their open probability (3, 4), or both and a later phase in which both ENaC and pump expression increases (1). These actions occur through steroid receptor binding and activation of new protein synthesis. Rapid non-genomic activation of ENaC has also been described in some tissues (5–8). Aldosterone has been shown to stimulate protein methylation early in its course of action, and this has been linked to genomic activation of ENaC (4, 9, 10). Protein methylation has also been implicated in the rapid, non-genomic ENaC activation (4, 5). Broad inhibitors of methylation reactions block the early aldosterone response in A6 and toad bladder cells (4, 9), and aldosterone has been shown to stimulate methylation of both k-ras and the \(\beta\) subunit of ENaC in A6 cells (4, 11, 12). Several lines of evidence suggest that the methylation reaction associated with stimulation of ENaC activity is likely to be at the channel: methyl donors stimulate Na\(^+\) uptake in apical membrane vesicles derived from A6 cells (10) and directly stimulate ENaC activity in cell-excised patches (13). Membranes derived from aldosterone-stimulated A6 cells contain enzymatic activity that methylates \(\beta\) ENaC in vitro and stimulates open probability of ENaC reconstituted in lipid bilayers (12). These observations suggest that methylation of \(\beta\) ENaC or a closely channel-associated protein mediates stimulation of the channel.

On the other hand, k-ras is induced by aldosterone in A6 cells and appears to stimulate ENaC even in the absence of steroid, suggesting that levels of k-ras, at least in A6 cells, may be limiting for full channel activity. ras requires methylation for full activity and membrane localization (14), and this methylation is clearly stimulated by aldosterone in A6 cells (11). Overexpression of the enzyme that methylates ras, isoprenylcysteine carboxymethyltransferase (PCMTase), although not stimulating basal current, does enhance the effect of aldosterone in A6 cells, and inhibitors of isoprenylcysteine methylation inhibit the response to aldosterone (15). It is not known whether this enzyme methylates \(\beta\) ENaC as well, but it seems unlikely as the consensus site for methylation by this enzyme, a CAAX motif, is not present on the channel subunit. The lack of effect of overexpressing PCMTase on ENaC activity in the absence of aldosterone stimulation of k-ras is also consistent with the notion that this enzyme does not directly interact with the channel. This raises the possibility that there is a distinct methyltransferase that directly regulates ENaC in response to aldosterone stimulation. Finally, transcriptome data derived from a line of cortical collecting duct (CCD) derived from mouse kidney suggests that ras may not be an aldosterone-induced protein in mammalian kidney (16).

These observations led us to reexamine the role of methylation reactions in the response to aldosterone in cultured mammalian CCD cells. If aldosterone-stimulated methylations are important to the early transport response, we sought to determine whether k-ras was induced in Igerancysteine: GTP\(^\gamma\)S, guanosine 5’-3’-O-(thio)triphosphate; m.o.i., multiplicity of infection; MT, methyltransferase; \(I_{sc}\), short circuit current; MBS, modified Barth’s saline.
these cells and whether the response was related to ras processing. Finally we sought to determine whether β ENaC was a substrate for a membrane-bound methyltransferase in CCD cells and whether we could identify a methyltransferase that directly regulates ENaC in CCD cells.

MATERIALS AND METHODS

Cell Culture and I Cell Measurements—mpkCCD, cells (CCD cells), a line derived from cortical collecting duct of SV40-transformed mice, were the generous gift of Alain Vandewalle (17) and were maintained as described previously in Dulbecco’s modified Eagle’s medium/Ham’s F-12 with 2% fetal bovine serum in an atmosphere of humidified air, 5% CO₂ at 37 °C (18). To measure transepithelial potential difference and resistance, cells were grown on Costar Transwell 24-mm diameter inserts (Corning) until high resistance was obtained, typically 5 days. Transepithelial potential differences, resistance, and Isc were measured using the Personal FX molecular imager (Bio-Rad).

Isolation of MT773—Complementary primers (5'–GGCTGCTAAA-CTGCACCTAG and 3'–GGGTTCTAGTTCCACAC) were synthesized corresponding to the predicted sequence of MT773. These primers were used to amplify the product out of a mouse kidney cDNA library (Clontech, BD Biosciences) using Pfu DNA polymerase (Stratagene) as described by the manufacturer. The product was cloned into pCR-Blunt II-TOPO (Invitrogen) and sequenced to confirm identity.

In Vitro Expression of Full-length mENaC and MT773—Full-length β, γ mENaC and MT773 were subcloned behind the T7 promoter for use in in vitro translations. The β mENaC, γ mENaC, and MT773 proteins used in the methylation reactions were generated using the TNT coupled reticulocyte lysate system (Promega). The ENaC subunits were labeled by the addition of [3H]methionine (MP Biomedical, LLC) at a final concentration of 0.3 μCi/ml. Labeled proteins were subjected to SDS-PAGE to ensure proper size. For the studies examining the methylation of in vitro translated mENaC, the β and γ subunits were incubated with [3H]AdoMet in the presence of 100 μM GTPγS and 25 μl of an in vitro translated MT773 enzyme preparation for 1 h at room temperature. The reaction was separated on a 10% SDS-polyacrylamide gel, dried, and exposed to imaging using the Personal FX molecular imager (Bio-Rad).

Channel Expression in Xenopus Oocytes—Xenopus oocytes (stage V-VI) were pretreated with 2 mg/ml collagenase (type IV) in calcium-free saline solution. Murine ENaC cRNAs (1–3 ng/subunit in 50 nl of H₂O) were microinjected into all oocytes. Oocytes in the experimental group were additionally injected with 5 ng of cRNA of candidate methyltransferases. All oocytes were incubated at 18 °C in modified Barth’s saline (MBS) (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, and 15 mM HEPES-NaOH, pH 7.2, supplemented with 10 μg/ml sodium penicillin, 10 μg/ml streptomycin sulfate, and 100 μg/ml gentamycin sulfate). Whole cell currents were measured 24–46 h after cRNA injections.

Whole Cell Current Measurements—A two-electrode voltage clamp technique was used as described previously (18). Whole cell inward amiloride-sensitive currents were measured in control oocytes expressing αβγ ENaC alone or experimental oocytes expressing αβ ENaC + candidate methyltransferases using a DigiData 1200 interface (Axon Instruments, Foster City, CA) and a TEV 200A voltage clamp amplifier (Dagan Corp., Minneapolis, MN). Data acquisition and analysis were performed using pClamp 7.0. Amiloride-sensitive currents were defined as the difference of the current in the absence and the presence of 0.1 mM amiloride. Oocytes were bathed in a solution containing 110 mM NaCl, 2 mM CaCl₂, 2 mM KCl, and 10 mM HEPES-NaOH, pH 7.40. All measurements were made at room temperature (22–25 °C), and the bath solution was continuously perfused at 5 ml/min by gravity. Oocytes were typically incubated in the bath solution for at least 10 min before the current was recorded to allow currents to stabilize. Membrane potentials were clamped from −140 to +60 mV in 20-mV increments with duration of 900 ms. Currents were measured at a holding potential of −100 mV 600 ms after initiation of the clamp potential.

Cell Surface ENaC Labeling—The general approach was based on the method of Zerangue et al. (22) as modified by Condliffe et al. (23). Control oocytes expressing mouse α, β FLAG, and γ ENaC subunits and
experimental oocytes co-injected with αβγ ENaC and MT773 were blocked with MBS supplemented with 1 mg/ml of bovine serum albumin after 2 days of incubation. Oocytes were then exposed to MBS-bovine serum albumin with 1 mg/ml of mouse monoclonal anti-FLAG antibody (M2, Sigma) at 4 °C for 1 h. Of note, β ENaC containing the FLAG epitope (DYDKKKD) at the extracellular loop does not alter I_{Na} relative to wild type ENaC expression as first demonstrated by Firsov et al. (24). After the oocytes were labeled with primary antibody, they were washed six times in MBS-bovine serum albumin at 4 °C and incubated in MBS-bovine serum albumin supplemented with 1 mg/ml horseradish peroxidase-conjugated secondary antibody (peroxidase-conjugated AffiniPure F(ab')2 fragment goat anti-mouse IgG, Jackson Immunoresearch Laboratories, West Grove, PA) at 4 °C for 1 h. After 12 additional washes, individual oocytes were placed in 100 μl of Super Signal ELISA Femto solution (Pierce) and incubated at room temperature for 1 min. Chemiluminescence was quantified in arbitrary light units using a TD-20/20 luminometer.

Adenoviral Infection of CCD Cells—Cultured cells grown on Transwell culture inserts were infected using a recombinant adenovirus encoding either MT773 or enhanced green fluorescent protein using varying m.o.i., and the current and expression of MT773 were examined 48 h postinfection.

Semiquantitative Reverse Transcription PCR—Total RNA from cultured cells grown on Transwell culture inserts infected with varying m.o.i. of MT773 or enhanced green fluorescent protein was isolated by the guanidinium thiocyanate method (above). cDNA was generated from equal amounts of total RNA using the Protoscript (New England Biolabs). The cDNA was amplified using primers specific for either MT773 or β-actin. Multiple numbers of cycles were performed to ensure that amplification was in the linear portion of the curves to allow quantification. Quantification was performed using Bio-Rad Quantity One software.

Statistics—Median standard error is reported. t tests were performed using the Sigma Stat statistical program (Jandel Scientific). Results were considered significant if p < 0.05.
RESULTS

To examine the role of methylation in response to aldosterone in the mammalian immortalized cortical collecting duct cells we first measured the effect of aldosterone on base-labile methylation in CCD cells. CCD cells were treated with either diluent or aldosterone, and membranes were isolated by homogenization and centrifugation as described previously (12, 15) and used as a source of enzyme. The compound AFC was used as a methyl acceptor for methylation activity and isolated by heptane extraction for vapor phase analysis of base-labile methylations as described previously (15). As shown in Fig. 1, aldosterone increases AFC methylation in a GTP-dependent manner. These data indicate that CCD cells, like A6 cells and toad bladder cells in culture, respond to aldosterone by increasing protein methylation.

To estimate whether methylation reactions were involved in the aldosterone stimulation of Na⁺ transport, we initially used inhibitors of methylation that have blocked aldosterone effects in toad bladder and A6 cells (9, 12, 15). The agent 3-deaza-adenosine (3-DZA) inhibits the enzyme S-adenosylhomocysteine hydrolase and acts as a nonspecific inhibitor of methylation reactions (4). This agent has been shown to inhibit both protein and phospholipid methylations in toad bladder and A6 cells and totally inhibit the action of aldosterone in these cells. Fig. 2A shows that 3-DZA had the same inhibitory effect on aldosterone action in CCD cells. Because there was some inhibition of basal current, we examined whether 3-DZA affected the levels of ENaC subunits over the early time course of aldosterone action. Fig. 2B indicates that total CCD cell amounts of all three ENaC subunits were unaffected by either 3-DZA or aldosterone over the first 4 h of aldosterone stimulation of Na⁺ transport in these cells. These observations are consistent with results from mammalian kidney that show that total cellular amounts of ENaC subunits do not appear to increase over the early course of aldosterone action (25).

Because the methylation effect could be pleiotropic, involving more than one possible substrate, we examined the possible role of k-ras in more detail. Small G proteins are activated by sequential prenylation and terminal methylation. We therefore examined the effect of specific inhibitors of prenylation and isoprenylcysteine carboxyl methylation on the aldosterone response in CCD cells. Fig. 2C shows that the competitive inhibitors of methylation of isoprenylated substrates, AFC and AGGC, which inhibit aldosterone-induced transport in A6 cells (15), had no effect on the early aldosterone response in CCD cells at a time when 3-DZA completely inhibited aldosterone effects. Similarly inhibitors of either farnesyl- or geranylgeranyltransferase had no effect on the response to aldosterone in CCD cells even when cells were preincubated with the inhibitors for 18 h prior to hormone addition (Fig. 2D). There was some inhibition of the later aldosterone response in these CCD cells suggesting there is a role for methylated small G proteins, presumably k-ras, in the later aldosterone response (data not shown).

Although there was some inhibition of the later aldosterone response in these CCD cells by AFC and AGGC, the current data differ from our earlier results in A6 cells where k-ras is known to be an aldosterone-induced protein (14). This prompted us to re-evaluate the role of ras in the aldosterone response in CCD cells. CCD cells were examined for an effect of aldosterone on k-ras by Northern and Western analysis. In CCD cells, k-ras did not appear to be induced by aldosterone either at the protein (Fig. 3A) or message (Fig. 3B) levels. This is consistent with transcriptome analysis of CCD cells in response to steroid stimulation that did not indicate ras as an aldosterone-regulated protein (16).

Therefore, in contrast to A6 cells, in CCD cells, ras is not an aldosterone-induced protein, and ras processing does not appear to be limiting for the early aldosterone response.

These findings prompted us to re-examine the role of PCMTase on ENaC activity. In A6 cells, this enzyme, which methylates isoprenylated small G proteins such as ras, does not affect basal Na⁺ transport but does enhance aldosterone-stimulated transport when overexpressed (15). To determine whether the enzyme directly regulated ENaC, we co-expressed PCMTase with αβγ ENaC in Xenopus oocytes. As shown in Fig. 4A, PCMTase had no effect on ENaC activity in oocytes, suggesting it does not directly methylate ENaC. PCMTase was overexpressed in CCD cells and also had no effect on either basal or aldosterone-stimulated current (Fig. 4B). These results suggest that in cells where ras is not rate-limiting PCMTase activity has little effect on ENaC function.

If methylation of ras is not essential for early aldosterone action in CCD cells, we next considered whether the other target for aldosterone-stimulated methylation in A6 cells, β ENaC (12), might be a substrate for methylation in CCD cells. Mouse β and γ ENaC were translated

FIGURE 3. The effects of aldosterone on the expression of k-ras in CCD cells. A, cells were treated with or without 1 μM aldosterone (Aldo) for 4 h. Equal amounts of cell lysate were separated by SDS-PAGE and transferred to nitrocellulose. Western blot analysis shows that the protein level of Ras was not altered by aldosterone (n = 3). B, cells were treated with or without 1 μM aldosterone for 4 h. Total RNA was isolated and separated on an agarose gel. Northern blot analysis shows that aldosterone has no effect on the k-ras mRNA level (n = 2). The 28 S rRNA was used as a control for equal loading.

FIGURE 4. The effects of overexpression of PCMTase on ENaC in oocytes and CCD cells. A, PCMTase was co-expressed with αβγ ENaC in Xenopus oocytes, and amiloride-sensitive currents were compared with αβγ ENaC alone (n = 23–28). PCMTase (PCMT) has no direct effect on ENaC under these conditions. B, PCMTase was overexpressed in CCD cells as described in the text. The inset shows a Western blot demonstrating significant overexpression of PCMTase; however, there was no effect on basal or aldosterone-stimulated I₆ (n = 6). Con, control. Amps, amperes.
Methylation of ENaC in CCD Cells

FIGURE 5. β ENaC is a target for methylation in CCD cells. β and γ ENaC were in vitro translated in the presence of [35S]methionine as described. Single bands for each protein are shown on the left demonstrating the molecular mass of these translated ENaC subunits. In vitro translations in the absence of [35S]methionine were used as substrates for in vitro methylation using CCD cell membranes as the source of enzyme and [3H]AdoMet as the methyl donor as described under “Materials and Methods.” Following the reaction the subunits were immunoprecipitated, separated by SDS-PAGE, and examined using the Personal FX molecular imager. The results show that β but not γ ENaC is methylated and that this requires the presence of the enzyme preparation (prep) (n = 3).

in vitro and examined as substrates for methylation with cell membranes from aldosterone-treated CCD cells as a source of enzyme. As shown in Fig. 5, β but not γ ENaC was methylated by an enzyme found in CCD cell membranes.

A membrane-bound enzyme from both A6 cells and CCD cells can methylate β ENaC. The enzyme from A6 cells has been shown to directly stimulate the activity of ENaC expressed in planar lipid bilayers by increasing open probability of the channel (12). This is consistent with evidence from patch clamp studies that methyl donors stimulate open probability of channels in A6 cells (13). As an initial screen to identify an enzyme that could directly activate ENaC, candidate methyltransferase enzymes were co-expressed in Xenopus oocytes along with all three channel subunits, and activity was compared with ENaC alone. We began this screening process with defined methyltransferase (MT) enzymes whose functions or targets were studied previously and for which full-length DNA was available. Such proteins included isoaspartyl methyltransferase and arginine methyltransferases 1 and 5. All these failed to stimulate ENaC activity in Xenopus oocytes (data not shown). These experiments suggested the possibility that any methyltransferase stimulating ENaC might be previously uncharacterized.

As an alternative strategy to identify a unique methyltransferase, the mouse genome was searched for proteins containing binding motifs for the methyl donor S-adenosylmethionine (AdoMet). Thirty-four genes encoding AdoMet binding motifs were identified from the Ensembl mouse data base. Because previous results suggested that the enzyme was membrane-associated, proteins with putative membrane-spanning motifs were examined first. Five such genes were identified, four of which coded for unknown proteins. Initially these proteins were subjected to two screens: PCR to determine whether the protein was expressed in CCD cells followed by co-expression with ENaC in Xenopus oocytes. One protein, the product of gene ENSMUSG00000021773, was expressed in CCD cells as shown in Fig. 6. The PCR product was 851 bp in length and when sequenced at the University of Pittsburgh core facility was 100% identical at the nucleotide level with the published sequence. When in vitro translated using a reticulocyte lysate system in the presence of [35S]methionine, the protein appeared as a single band at ~30 kDa, consistent with its predicted size of 28.5 kDa (not shown). This protein, MT773, is of unknown function but contains an AdoMet binding motif, an O-methyltransferase domain, and a transmembrane domain (Fig. 7). Message for this protein is ubiquitously expressed in mouse tissues but is quite prominently expressed in kidney (Fig. 8).

Expression of the message for this protein in tissues not known to express ENaC suggests the possibility that the enzyme may have other targets in addition to ENaC.

Expression of MT773 along with wild type αβγ ENaC in oocytes resulted in marked enhancement of amiloride-sensitive current (Fig. 9A). In our earlier studies, the methyltransferase in membranes from A6 cells activated ENaC reconstituted in lipid bilayers primarily by increasing open probability and was active only when added to the intracellular side of channels (12). We therefore sought to determine whether the effect of MT773 on ENaC in oocytes was consistent with the behavior of the enzyme activating ENaC in A6 cells. As shown in Fig. 9B, MT773 had no effect on surface expression of ENaC in oocytes, suggesting that the primary effect is an increase in open probability similar to the effect of ENaC methylation in A6 cells. Because the enzyme from A6 cell membranes activated ENaC only from the intracellular side, we examined the effect of truncation of the intracellular portions of β ENaC on the response to MT773 in oocytes. Truncation of the intracellular C terminus of β ENaC resulted in very high current expression in oocytes consistent with the known effect of this mutation on surface expression of ENaC (26). MT773 had no effect on the activity ENaC when α and γ were co-expressed with the C-terminal truncation of β (Fig. 9C).

The putative methyltransferase was next evaluated in cultured mouse cortical collecting duct cells. To estimate whether MT773 might be an aldosterone-induced protein, cells on permeable supports were deprived of steroids for 48 h and then exposed to 1 μM aldosterone for 3 or 18 h. Message for MT773 was then quantitated by reverse transcript PCR. As shown in Fig. 10A, MT773 was not induced by aldosterone in CCD cells at least at the level of message. Finally to determine whether MT773 affects ENaC activity in natively expressing epithelia, CCD cells were infected with an adenovirus containing MT773. Fig. 10B demonstrates that infection with the adenoviral construct containing the methyltransferase resulted in a four-fold induction of amiloride-sensitive current even at relatively low infection levels. This seems to be a threshold effect of expression as increasing expression by increasing m.o.i. of infection was not associated with further increases in amilo-
ride-sensitive current (Fig 10C). These data demonstrate that MT773 stimulates ENaC activity in CCD cells.

DISCUSSION
The classic paradigm for aldosterone action to stimulate Na⁺ reabsorption in responsive tissues involves mineralocorticoid receptor activation with the induction of new protein synthesis (1, 17, 27). Newly synthesized proteins induce an early increase in ENaC activity, detected ~30–60 min following hormone addition, that is not dependent on the synthesis of new channel subunits and a prolonged effect that does appear to involve synthesis of ENaC subunits as well as increased expression of the basolateral Na⁺ pump (1, 28). In this classic genomic model, the early effects of aldosterone to stimulate ENaC activity may be due to trafficking of already synthesized channel subunits, under the influence of the aldosterone-induced protein SGK-1 (27, 29), or to a direct effect on channel open probability (3). Early and rapid non-genomic effects of aldosterone have also been described in a number of tissues and cell types that are not related to binding of the classic mineralocorticoid receptor, and among these effects are direct, rapid (<2 min) activation of ENaC (5–8). In the CCD cells that we have used in these studies, all the effects of aldosterone appear to be mediated via the classic, genomic pathway (17), although it seems clear that the early phase of stimulation of ENaC activity is not fully explained by activation of SGK-1, suggesting that other pathways may be involved (8, 29, 30).

Protein methylation reactions have been implicated in the early genomic action of aldosterone to stimulate the activity of ENaC (4), and they have been implicated in the rapid non-genomic activation of ENaC by aldosterone in some but not all species examined (8). In CCD cells and A6 cells, aldosterone stimulates protein methylation, and the time course of this methylation correlates well with the early (4-h) genomic actions of aldosterone (4). Inhibitors of methylation block the aldosterone response during the initial time course when activation of ENaC is thought to proceed primarily through actions on channels already present in the cell rather than synthesis of new channel subunits (1, 31, 32). This suggests that methylation reactions either activate the channel directly or are part of a signaling cascade that results in channel activation. Early observations favored the initial hypothesis. Methyl donors activate Na⁺ uptake in apical membrane vesicles derived from A6 cells (10) and subsequently were shown to increase open probability of ENaC in cell-excised patches from this cell line (13). Aldosterone stimulates the methylation of a 95-kDa protein in the apical membrane of A6 cells (25) and was subsequently shown to induce methylation of the β subunit of ENaC both in cells and in vitro (12). Finally a partially purified enzyme preparation derived from membranes of A6 cells treated with aldosterone activates ENaC reconstituted in planar lipid bilayers through an increase in open probability (12). All these findings suggest that aldosterone stimulates a methylation reaction at or near the channel that directly activates the channel through a gating effect. Direct activation of ENaC by aldosterone acting through non-genomic pathways in rabbit (8) and rat (5) collecting duct cells has been shown by several groups to be blocked by broad inhibitors of methylation, suggesting a direct effect of methylation on channel gating (5, 8). It is not clear whether similar effects on ENaC activation seen in lymphocytes of
human, canine, and rabbit (but not mouse or rat) origin are also dependent on methylation (8). It does appear, however, from this evidence as well as the varying effects on ras stimulation discussed below that different model systems may exhibit significant differences in the mechanisms by which aldosterone regulates ENaC activity.

Several groups have observed that aldosterone stimulates the synthesis of ras in A6 epithelia (11, 14, 33), and it is well known that activation of ras, as of many small G proteins, is dependent in part on sequential isoprenylation and carboxyl methylation (4). In A6 cells, aldosterone has been shown to stimulate ras methylation, and agents that inhibit or block methylation of small G proteins have been shown to partially or completely inhibit the aldosterone effect (15). When the enzyme that methylates isoprenylated cysteines of small G proteins such as ras, PCMTase, is overexpressed in A6 cells, the effect of aldosterone on transport is enhanced, although there is no effect on basal current alone. These observations suggest that the relevant methylation stimulated by aldosterone was signaling to ENaC through the ras pathway (15).

The present studies were undertaken to re-examine the issue of methylation and ENaC activity in an endogenously expressing mammalian cell line. Our findings indicated that, as in A6 cells, aldosterone stimulates methylation of proteins that is necessary for the early stimulation of transport, and ENaC is a substrate for methylation in CCD cells. Unlike A6 cells, k-ras was not an aldosterone-induced protein in CCD cells, and inhibitors of ras processing did not inhibit the early response to aldosterone in these cells. There was some inhibition of the latter aldosterone response by inhibitors of ras methylation (data not shown), so it is likely that ras plays a role in ENaC activation, but it does not appear to be involved in early channel activation as in A6 cells where low levels of k-ras may be limiting. For this reason, perhaps, overexpression of PCMTase had no additional effect on aldosterone-stimulated transport in CCD cells. Taken together, the current results suggest that in CCD cells, early activation of ENaC is more likely associated with β ENaC methylation as noted in A6 cells and planar lipid bilayers (12). We

FIGURE 9. The effect of MT773 on ENaC activity and surface expression in oocytes. A, MT773 was co-expressed with αβγ ENaC in Xenopus oocytes, and amiloride-sensitive currents were compared with αβγ ENaC alone. Co-expression of MT773 with ENaC significantly increases ENaC activity (p < 0.001; n = 24–38). B, to measure surface expression of ENaC αβγ were co-expressed with MT773, and surface expression was measured by luminometry as described. MT773 had no effect on surface expression of ENaC (p = 0.48; n = 19–21). C, C-terminal truncation of β ENaC was expressed with wild type α and γ in the presence and absence of MT773. MT773 had no effect on ENaC activity in the presence of this mutation (p = 0.45; n = 18–20). Amps, amperes.

FIGURE 10. Expression of MT773 in CCD cells. A, MT773 is not an aldosterone-induced protein. Total RNA was isolated, and CCD cells were treated with aldosterone for 0, 3, or 18 h and converted to cDNA. Amplification using primers for MT773 and β-actin was performed. The amplified products were run out on an agarose gel (p = 0.59; n = 3). As shown, RNA expression of MT773 does not increase with exposure to aldosterone. B, overexpression of MT773 by adenoviral infection increases ENaC activity in CCD cells. Adenoviral infection of m.o.i. ranging from 50 to 500 significantly increases amiloride-sensitive ENaC activity by 4-fold (p < 0.001; n = 4). The inset shows reverse transcription PCR at varying m.o.i. demonstrating that increasing m.o.i. increases the message for MT773. Multiple cycles were performed to ensure amplification was within the linear range. Amps, amperes.
therefore sought to identify a methyltransferase capable of activating ENaC, and our initial screen used the oocyte expression system.

We identified a protein containing a methyltransferase and AdoMet binding domain that is present in CCD cells. This protein activated ENaC when co-expressed in Xenopus oocytes and appeared to do so through a primary effect on channel open probability. These effects are consistent with our previous findings on activation of ENaC in planar lipid bilayers by membrane-bound methyltransferases derived from A6 cells as is the observation that the enzyme appears to act at an intracellular site most likely located on the C terminus of β ENaC (12). The methyltransferase does not appear to be an aldosterone-induced protein in CCD cells. PCMTase, which regulates ras methylation in A6 cells, is also not an aldosterone-induced protein, and it has been proposed that stimulation of protein and lipid methylations by aldosterone is mediated by an effect on the regulating enzyme S-adenosylhomocysteine hydrolase (34). The specific cellular localization of the enzyme and the specific sites on the C terminus of β ENaC that are the target of this methyltransferase are currently under study. MT773 appears to be a reasonable candidate to be the enzyme that has been suggested to regulate ENaC activity acutely via both genomic (1) and non-genomic (5, 8) pathways, but this remains to be proven definitively.

REFERENCES

1. Garty, H., and Palmer, L. G. (1997) Phys. Rev. 77, 359–396
2. Alvarez de la Rosa, D., Li, H., and Canessa, C. M. (2002) J. Gen. Physiol. 119, 427–442
3. Kemendy, A. E., Kleyman, T. R., and Eaton, D. C. (1992) Am. J. Physiol. 263, C825–C837
4. Stockand, J. D., Edinger, R. S., Eaton, D. C., and Johnson, J. P. (2000) News Physiol. Sci. 15, 161–166
5. Le, M. C., Cluzeaud, F., Fay, M., and Blot-Chabaud, M. (2004) Cell. Mol. Biol. (Noisy-Le-Grand) 50, 833–840
6. Chun, T. Y., and Pratt, J. H. (2004) Trends Endocrinol. Metab. 15, 353–354
7. Arima, S. (2006) Steroids, in press
8. Zhou, Z. H., and Bubien, J. K. (2001) Am. J. Physiol. 281, C1118–C1130
9. Wiesmann, W. P., Johnson, J. P., Miura, G. A., and Changhai, P. K. (1985) Am. J. Physiol. 248, F43–F47
10. Sariban-Sohraby, S., Burg, M., Wiesmann, W., Changhai, P., and Johnson, J. P. (1984) Science 225, 745–746
11. Al Baldawi, N. F., Stockand, J. D., Al Khalili, O. K., Yue, G., and Eaton, D. C. (2000) Am. J. Physiol. 279, C429–C439
12. Rokaw, M. D., Wang, J.-M., Edinger, R. S., Weisz, O. A., Hui, D., Middleton, P., Shlyonsky, V., Berdiov, B. K., Israelov, I. I., Eaton, D. C., Benos, D. J., and Johnson, J. P. (1998) J. Biol. Chem. 273, 28746–28751
13. Bocchetti, A., Kemendy, A. E., Stockand, J. D., Sariban-Sohraby, S., and Eaton, D. C. (2000) J. Biol. Chem. 275, 16550–16559
14. Stockand, J. D., Spier, B. J., Worrell, R. T., Yue, G., Al Baldawi, N., and Eaton, D. C. (1999) J. Biol. Chem. 274, 35449–35454
15. Stockand, J. D., Edinger, R. S., Al-Baldawi, N., Sariban-Sohraby, S., Al-Khalili, O., Eaton, D. C., and Johnson, J. P. (1999) J. Biol. Chem. 274, 26912–26916
16. Robert-Nicoud, M., Flahaut, M., Elalouf, J. M., Nicod, M., Salmass, B., Mena, J., Doucet, A., Wincoller, P., Artiguenave, F., Horisberger, J. D., Vassal, A., Rossier, B. C., and Fischli, D. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2712–2716
17. Bens, M., Vallet, V., Chiracaud, F., Vascoul-Letallec, L., Kahn, A., Rafestin-Oblin, M. E., Rossier, B. C., and Vassal, A. (1999) J. Am. Soc. Nephrol. 10, 923–934
18. Lebowitz, J., Edinger, R. S., An, B., Perry, C. J., Onate, S., Kleyman, T. R., and Johnson, J. P. (2004) J. Biol. Chem. 279, 41895–41990
19. Mohan, S., Burns, J. R., Weixel, K. M., Edinger, R. S., Burns, J. B., Kleyman, T. R., Johnson, J. P., and Weisz, O. A. (2004) J. Biol. Chem. 279, 32071–32078
20. Butterworth, M. B., Edinger, R. S., Johnson, J. P., and Frizzell, R. A. (2005) J. Gen. Physiol. 125, 81–101
21. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
22. Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. (1999) Neuron 22, 537–548
23. Condliffe, S. B., Carattino, M. D., Frizzell, R. A., and Zhang, H. (2003) J. Biol. Chem. 278, 12796–12804
24. Fischio, D., Schild, L., Gauthscih, L., Merillatt, A. M., Schneeberger, E., and Rossier, B. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15370–15375
25. Sariban-Sohraby, S., Fisher, R. S., and Abramow, M. (1993) J. Biol. Chem. 268, 26613–26617
26. Shimkets, R. A., Warnock, D. G., Bositis, C. M., Nelson-Williams, C., Hinsman, J. H., Schambelan, M., Gill, J. R., Yllick, S., Milora, R. V., Findling, J. W., Casana, C. M., Rossier, B. C., and Linton, R. P. (1994) Cell 79, 407–414
27. Moffing, J., Zecnic, M., Feraillle, E., Kaisling, B., Asher, C., Rossier, B. C., Firestone, G. L., Pearce, D., and Verrey, F. (2001) Am. J. Physiol. 280, F675–F682
28. Johnson, J. P. (1992) Pharmacol. Ther. 53, 1–29
29. Flores, S. Y., Moffing-Cueni, D., Kamykyna, E., Daidie, D., Gerbec, C., Chabanel, S., Dudler, J., Moffing, J., and Staab, O. (2005) Am. J. Soc. Nephrol. 16, 2279–2287
30. McCormick, J. A., Bhalla, V., Pao, A. C., and Pearce, D. (2005) Physiology (Bethesda) 20, 134–139
31. Mallamalani, S., Kim, G.-H., Mitchell, C., Wade, J. B., and Knepper, M. A. (1999) J. Clin. Investig. 104, R19–R23
32. Fischio, G., Mallamalani, S., Knepper, M. A., and Palmer, L. G. (2001) Am. J. Physiol. 280, F112–F118
33. Verrey, F. (1999) Am. J. Physiol. 277, F319–F327
34. Stockand, J. D., Al Baldawi, N. F., Al-Khalili, O. K., Worrell, R. T., and Eaton, D. C. (1999) J. Biol. Chem. 274, 3842–3850