Methylation Increases the Open Probability of the Epithelial Sodium Channel in A6 Epithelia*

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We used single channel methods on A6 renal cells to study the regulation by methylation reactions of epithelial sodium channels. 3-Deazaadenosine (3-DZA), a methyltransferase blocker, produced a 5-fold decrease in sodium transport and a 6-fold decrease in apical sodium channel activity by decreasing channel open probability ($P_o$). 3-Deazaadenosine also blocked the increase in channel open probability associated with addition of aldosterone. Sodium channel activity in excised “inside-out” patches usually decreased within 1–2 min; in the presence of AdoMet than in untreated excised patches but less than $t_{\text{open}}$ in cell-attached patches. Sodium channel activity in excised patches exposed to both AdoMet and GTP usually remained stable for more than 10 min, and $P_o$ and the number of active channels per patch were close to values in cell-attached patches from untreated cells. These findings suggest that a methylation reaction contributes to the activity of epithelial sodium channels in A6 cells and is directed to some regulatory element closely connected with the channel, whose activity also depends on the presence of intracellular GTP.

Despite many studies, the mechanism by which aldosterone stimulates apical sodium transport is still poorly understood. It is known that the complex between aldosterone and its intracellular receptor activates gene expression and induces the synthesis of proteins (2–9); however, little is known about the cellular functions of the induced proteins except that the final result is an increase in sodium transport (2, 9–11). Originally, because of the observation that protein synthesis was required for aldosterone to increase sodium transport, it was postulated that aldosterone induced sodium channel synthesis and insertion. However, earlier studies in A6 cells showed that aldosterone increases Na$^+$ entry at the apical membrane by changing the activity of channels that are already present in the apical membrane and not by increasing the number of channels (13). Although interpretation of other electrophysiological data remains controversial (14–16), biochemical methods support the original observation that ENaC mRNA and ENaC protein in the apical membrane do not increase in the presence of aldosterone (at least in the first 2–4 h when the increase in sodium transport is most dramatic) (10, 17–19).

Since the action of aldosterone appears to involve a mechanism that increases the $P_o$ of sodium channels, an examination of post-translational modifications that alter $P_o$ may offer some insight into the mechanism of aldosterone action, but identifying signal transduction pathways that can increase sodium channel $P_o$ in A6 cells has been difficult. There have been many suggestions about potential aldosterone-induced post-translational modifications, but in the context of our previous results (20–22), one is particularly interesting. Sariban-Sohraby et al. (23) demonstrated that the amount of sodium transport that could be measured in apical membrane vesicles obtained from A6 cells, a sodium-transporting, distal-nephron cell line, was markedly enhanced by prior application of agents that methylate membrane proteins. There was no additional effect of AdoMet in the presence of aldosterone, and the effect was blocked by two methylation blockers, S-adenosylhomocysteine (AdoHcy) and 3-deazaadenosine (3-DZA). 3-DZA is a membrane-permeable drug that blocks transmethylation reactions by specifically inhibiting the S-adenosylhomocysteine hydrolase, thereby promoting the accumulation of S-adenosylhomocysteine (AdoHcy) and deaza-D-adenosylhomocysteine that produces end product inhibition of AdoMet-dependent methyltransferases (24). Since they also demonstrated that application of aldosterone leads to the methylation of membrane protein and lipid, their suggestion was that intracellular methyltransferases induced by aldosterone could be responsible for the methylation and, therefore, modulate the sodium channel protein. As a post-translational modification, methylation is analogous to phosphorylation (for reviews see Refs. 25–28). Highly specific methyltransferases promote the methylation of proteins at specific sites in the cell, so they are an attractive target for post-translational regulation of sodium channel activity. The aim of this study, therefore, was to determine whether methylation of the sodium channel protein contributed to the regulation of sodium transport by aldosterone.

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†The abbreviations used are: ENaC, epithelial sodium channel; AdoMet, S-adenosyl-L-methionine; AdoHcy, S-adenosylhomocysteine; 3-DZA, 3-deazaadenosine; FTS, S-trans,trans-farnesylthiosalicylic acid; GTPyS, guanosine 5’-3-O-(thio)triphosphate.

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The amiloride-blockable, highly selective, epithelial sodium channel (ENaC) present on the apical surface of principal cells in mammalian renal cortical collecting tubules is the primary site for the regulation of total body sodium balance and blood pressure. The cell line, A6, derived from distal tubules of Xenopus laevis nephrons, is a good experimental model for the study of these sodium channels. When grown on permeable supports in the presence of aldosterone, an examination of the aldosterone response showed that aldosterone increased sodium transport by decreasing channel open probability ($P_o$) before and after patch excision was higher in the presence of AdoMet than in untreated excised patches. Sodium channel activity by decreasing channel open probability ($P_o$) before and after patch excision was higher in the presence of AdoMet than in untreated excised patches but less than $t_{\text{open}}$ in cell-attached patches.
The present work has been previously probably membrane-associated and close to the channel. Addition of par-

methyltransferase was partially purified from the membrane fraction of A6 cells using patch clamp methods. In this way, we hoped to clarify what aspect of channel function is altered and to confirm whether the methylation effect is indeed membrane-directed.

We found that methylation inhibitors reduce ENaC activity by decreasing P.

Also, addition of the methyl donor, AdoMet, to the cytosolic surface of ENaC in excised inside-out patches significantly increased channel activity with respect to controls. The presence of AdoMet seems to maintain the single channel mean open time even after excision. Addition of partially purified methyltransferase produced little additional effect, suggesting that the endogenous methyltransferase is probably membrane-associated and close to the channel. Addition of GTP along with AdoMet increased channel activity more than AdoMet alone. The present work has been previously reported in several brief communications (13, 22).

MATERIALS AND METHODS

A6 Cell Culture Preparation—For single channel experiments, we used A6 cells from American Type Culture Collection (Manassas, VA) in the 68th passage. Experiments were carried out on passages 70–80, with no discernible variation between cells from different passages. Cells were maintained in plastic tissue culture flasks (Corning, NY) at 26 °C in a humidified incubator with 4% CO2 in air. The culture medium was a mixture of Coon’s medium F-12 (3 parts) and Leibovitz’s medium (1 part) modified for amphibian cells with 104 mM NaCl, 25 mM NaHCO3, pH 7.4, with a final osmolarity of 240 mosmol/kg H2O. Besides these components, 10% (v/v) fetal bovine serum (Irvine Scientific, CA), 1% streptomycin, and 0.6% penicillin (Hazleton Biologics, KA) and, in most experiments, 1 μM aldosterone were added. Cells grown on plastic tissue culture dishes were detached when confluent by

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detoxylation of the methyltransferase substrate, N-acetyl-S-farnesyl-L-cysteine, and a blocking concentration of a methyltransferase inhibitor when appropriate (S-trans,farnesylthiosalicylic acid, 5-deazadenosine, or S- farnesyl-L-cysteine). After incubation for 1 h at 37 °C, the incubation was stopped by the addition of 50 μl of 20% trichloroacetic acid and the reaction mix vortexed for 10 s. The isoprenyl cysteine methyl esters were separated by the addition of 400 μl of heptane to the reaction mix. A fraction (200 μl) of the organic top layer containing the methyl esters was transferred to a small top-free Eppendorf tube and dried under vacuum. 200 μl of 1 x NaOH was added, and the tube was placed upright in a vial containing a small volume of scintillation fluid. The vials were sealed and incubated at 37 °C overnight. The strong base hydrolyzes the methyl esters releasing methanol vapor that partitions into the hydrophobic scintillant. The methyl esters in the vials were counted by liquid scintillation counting. The specific activity of the enzyme is defined as picomoles of methyl-1H group transferred per mg/min of enzyme protein. The preparation catalyzed the incorporation of radiolabeled isotope into N-acetyl-S-farnesyl-L-cysteine which was 70–90% inhibited by AdoHcy or FTS. The specific activity of methyl groups transformed by the enzyme was 0.12 pmol/mg protein/min.

Transepithelial Current Recording—A6 cells were grown for 14 days on permeable supports with a surface area of approximately 9 cm², at which time transepithelial potential differences (PD) and resistances (R) were measured using dual flexible electrodes, containing Ag/AgCl pellets (Millicell-ERS, Millipore, Bedford MA). The resistance of the insert was obtained by passing an alternating current of ± 20 μA at 12.5 Hz. Transepithelial current (I) was calculated from the PD and R values, and in this system, the predominant component of the current is carried by Na⁺ through amiloride-sensitive Na⁺ channels at the apical membrane (34). Because measurements were made under sterile conditions, we were able to make several measurements over time on the same cells.

Single Channel Recordings—We used either the cell-attached or the inside-out configuration of the Hamill patch clamp technique. Before sampling, the apical cell surface was carefully washed several times with our standard extracellular solution, containing (in mM): 95 NaCl, 2.5 KCl, 1 MgCl2, 1 CaCl2, 10 HEPES, sucrose, 0.8 M aldosterone, 10 HEPEs, pH was adjusted to 7.4 with NaOH. Patch pipettes contained the same solution. For inside-out experiments, the apical solution was substituted immediately before patch excision with a cytosol-mimicking solution (in mM: 85 KCl, 3 NaCl, 4 CaCl2, 1 MgCl2, 5 EGTA, potassium salt (Sigma), 1 mM adenosine trisphosphate (ATP, sodium salt, Sigma), and 10 HEPEs, pH 7.4, adjusted with KOH). Only one inside-out experiment was performed per cell. To avoid any possibility of examining cells whose properties might have been altered by extended exposure to high potassium solution. In some of the inside-out experiments, 300 μM S-adenosyl-l-methionine (AdoMet, Sigma) or GTP was added to the “intraacellular” solution. Solutions containing AdoMet, GTP, or ATP were prepared fresh every day to prevent possible degradation. All experiments were performed at room temperature (22–24 °C, very close to the physiological temperature for amphibian cells) within 45–60 min of removing the A6 cells from the incubator. Patch pipettes with a tip diameter of 1–2 μm were fabricated from WPI TW 150 glass (New Haven, CT) and fire-polished. Since the voltage dependence of apical sodium channels in A6 cells is relatively small (35), we have not corrected all our data for the small (a few mV) junction potential present after patch excision in a high potassium solution.

Data Acquisition and Analysis—Single channel currents from cell-attached patches were measured with an Axopatch 1-B current-voltage clamp amplifier (Axon Instruments, Inc. Burlingame, CA), low pass-filtered at 5 kHz, and recorded on a digital video recorder (Sony, Japan), and then the recorded signal was refiletted and digitized at twice the corner frequency (usually 1 kHz) using a Scientific Solutions A/D converter. An IBM PC-compatible computer equipped with Axtape software (Axon Instruments, CA). The data were subsequently transferred to a Micro Vax II computer (Digital Equipment) for single channel analysis. Data records from cells grown in the presence of aldosterone were low pass-filtered at 100 Hz, whereas those from aldosterone-depleted cells were filtered at 300 or 500 Hz using a software Gaussian filter. Events were detected by setting the threshold level at 50% of the estimated single
channel current amplitude. Because of the necessity for analyzing long continuous records, programs that closely follow the strategy of Colquhoun and Sigworth (36) were written for use on the VAX family of computers. These programs produce tables of event durations and amplitudes based on a 50% threshold crossing algorithm and allowed the analysis of long continuous records, necessary for the interpretation of single channel experiments on renal epithelial tissue.

One method for calculating $N_P$, from single channel records without making any assumptions about the total number of channels in a patch or the $P_o$ of a single channel is given by Equation 1,

$$N_P = \sum_{i=0}^{N} \frac{N_i}{T}$$

(Eq. 1)

where $T$ is the total recording time; $N_i$ is the observable number of current levels (corresponding to the apparent number of channels) within the patch determined as the highest observable current level; $i$ is the number of channels open; and $t_i$ is the time during which $i$ channels are open. If channels open independently of one another and the exact number of channels in a patch is known, then the $P_o$ of a single channel can be calculated by dividing $N_P$ by the number of channels in a patch. The total number of functional channels ($N$) in the patch was determined by observing the number of peaks detected in all points amplitude histograms constructed, when possible, from event records of long enough duration to provide 95% confidence of determining the correct $N$ according to methods we have previously described (37, 38). However, especially in some of the untreated excised patches, we could not record long enough to reach a 95% confidence level, and the values of $N$ in these patches may be an underestimate. The mean open time ($t_o$) of $N$ channels can be calculated as shown in Equation 2,

$$t_o = \frac{N_P}{n/2} = \sum_{n=-1}^{1} \frac{N_i}{n/2}$$

(Eq. 2)

where $n$ is the total number of transitions between states during the total recording period, $T$, and the other parameters are the same as in Equation 1. This value represents the average time the channel spends open (in any open state) and should not be confused with the mean residency time of the channel in a specific state (sometimes called the mean open time for the state). Nonetheless, this measure provides an easy way to distinguish whether experimental manipulations (e.g. AdoMet or 3-DZA) modify $P_o$ by affecting the open states or closed states. The open probability measured so that it can be studied in physiological solutions, i.e. without the need of any kind of ion channel blocker. Its biophysical properties have been extensively described by us and others (43).

A Methylation Inhibitor, 3-DZA, Inhibits ENaC $N_P$, and $P_o$—If methylation is important for ENaC activity, inhibitors of methylation should reduce activity. A6 cells monolayers previously treated with aldosterone were pretreated for 2.5–5 h with 300 μM methylation inhibitor, 3-DZA for comparison with untreated cells. The estimated $P_o$ of channels in the 3-DZA-pretreated patch was less than 0.004, barely discernible in the amplitude histogram (for clarity, the bottom portion of the histogram has been magnified in the inset) compared to about 0.5 in the untreated patch.

RESULTS

Sodium Channel Properties—In this work, we have studied the highly selective, amiloride-blockable, aldosterone-inducible, 4-pS sodium channels previously observed in recordings from the apical surfaces of both A6 cells and isolated rat and rabbit cortical collecting tubule cells (1, 39–42). An example of a typical recording from a patch containing three such channels is shown in Fig. 1A. In this case, the open probability measured from about 6 min of continuous recording at 0-mV pipette potential was close to 0.5 (see the amplitude histogram on the bottom). This is by far the most common type of channel found on the apical surface of A6 cells grown on permeable supports so that it can be studied in physiological solutions, i.e. without the need of any kind of ion channel blocker. Its biophysical properties have been extensively described by us and others (43).

![Fig. 1. 3-DZA reduces the open probability of ENaC channels. A6 cells monolayers were pretreated for 2.5–5 h with 300 μM methylation inhibitor, 3-DZA (right panel), for comparison with untreated cells (left panel). The patch activity was then recorded at 0-mV pipette potential ($V_p$) for at least 4–5 min. Inward currents are downward deflection; $c$ = closed level. The records were filtered at 100 Hz. A comparison of the amplitude histograms from A and B shows a dramatic difference in the open probability of sodium channels under the two conditions. The estimated $P_o$ of channels in the 3-DZA pretreated patch was less than 0.004, barely discernible in the amplitude histogram (for clarity, the portion of the histogram containing open amplitude points has been magnified in the inset) compared to about 0.5 in the untreated patch.

![A methylation inhibitor, 3-DZA, inhibits ENaC $N_P$ and $P_o$—If methylation is important for ENaC activity, inhibitors of methylation should reduce activity. A6 cells monolayers previously treated with aldosterone were pretreated for 2.5–5 h with 300 μM methylation inhibitor, 3-DZA for comparison with untreated cells. The patch activity was then recorded at 0-mV pipette potential ($V_p$) for at least 4–5 min. Usually, this time was sufficiently long to estimate confidently the number of active channels ($N$) in a control patch in A6 cells (provided that $N \leq 4–5$ channels (38, 44)). If $N$ is known with confidence, $P_o$ can also be calculated. A representative example of a single channel record from a cell pretreated with 3-DZA for 3 h is shown in Fig. 1B. A comparison of the amplitude histograms from Fig. 1, A and B, shows a dramatic difference in the open probability of sodium channels under the two conditions. The estimated $P_o$ of channels in the 3-DZA-pretreated patch was less than 0.004, barely discernible in the amplitude histogram (for clarity, the bottom portion of the histogram has been magnified in the inset). This estimate of $P_o$ is an upper limit since it depends upon accurately determining the number of channels, $N$, in a patch. If we have underestimated $N$, then will we overestimate $P_o$. Therefore, $P_o$ may actually be significantly lower than 0.004.](http://www.jbc.org/Downloaded from http://www.jbc.org/)

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This figure summarizes the effect of aldosterone and 3-DZA on the Na\(_p\) of EnaC. The effect of aldosterone addition to aldosterone-depleted cells was to increase the Na\(_p\) of individual sodium channels from a very low level to a much higher level, and 3-DZA blocks the effect of aldosterone. Paired dishes of A6 cells were serum- and aldosterone-depleted (see “Materials and Methods”) for 48 h before one dish was treated with 300 \(\mu\)M 3-DZA for 4 h. Then 1.5 \(\mu\)M aldosterone was added to both dishes, and sodium channel activity was sampled from both dishes by repeatedly forming patches on cells selected at random from the dishes. Pretreatment with 3-DZA of aldosterone-treated cells dramatically decreased Na\(_p\) from 2.88 ± 0.731 (mean ± S.D., \(n = 37\)) to 0.297 ± 0.185 (\(n = 49\)). These values contrast with the Na\(_p\) determined in cells treated with aldosterone-free medium for 24 h of 0.117 ± 0.134 (\(n = 45\)).

### 3-DZA Blocks the Effect of Aldosterone—
Our goals in this study were 2-fold as follows: first, to examine the regulation of sodium channel activity by methylation; and second, to determine if the action of aldosterone involved a methylation event as suggested by others (23, 23, 45, 46). We had previously shown that one effect of aldosterone addition to aldosterone-depleted cells was to increase the \(P_o\) of individual sodium channels from a very low level (less than 0.01) to a much higher level (about 0.4) (37) primarily by increasing the mean open time of channels. To investigate further the relationship between methylation and the effects of aldosterone, we examined the effect of 3-DZA on single sodium channels in patches formed on aldosterone-depleted cells. First, paired dishes of A6 cells were serum- and aldosterone-depleted (see “Materials and Methods”) for 48 h before one dish was treated with 300 \(\mu\)M 3-DZA for 4 h. Then 1.5 \(\mu\)M aldosterone was added to both dishes, and sodium channel activity was sampled from both dishes by repeatedly forming patches on cells selected at random from the dishes. The results of a large number of experiments in which the patch activity was observed with or without 3-DZA incubation in the presence or absence of aldosterone are summarized in Fig. 2. A 3–4-h pretreatment with 3-DZA of aldosterone-treated cells dramatically decreased Na\(_p\) from 2.88 ± 0.731 (mean ± S.D., \(n = 37\)) to 0.297 ± 0.185 (\(n = 49\)). These values contrast with the Na\(_p\) determined in cells treated with aldosterone-free medium for 24 h of 0.117 ± 0.134 (\(n = 45\)).

### 3-DZA Probably Produces Little, If Any, Change in N—
Unfortunately, although these data clearly suggest that 3-DZA decreases sodium channel \(P_o\), a concomitant effect on the channel density could not be completely ruled out. In controls, the mean number of channels (\(N\)) in patches showing detectable channel activity (37 patches) was 7.13 ± 1.70, with \(P_o = 0.408 \pm 0.0619\). On the other hand, as mentioned above, the large decrease in mean \(P_o\) we frequently observed after incubation with 3-DZA or in aldosterone-free medium made the detection of temporally overlapping openings difficult, thus reducing the accuracy of our determination of \(N\).

In fact, only 23 out of 43 experiments performed after removal of aldosterone and 36 out of 49 after 3-DZA treatment had channel activity with a \(P_o\) large enough to determine \(N\) accurately for the recording period available (see Kemendy et al. (37) for a discussion). Thus, although there may be an apparent decrease in \(N\) after 3-DZA treatment or aldosterone removal, we cannot state with any significant statistical confidence (\(p > 0.1\) for all conditions) that there is actually any change in \(N\). Therefore, there might have been a small decrease in the mean \(N\) per patch after 3-DZA treatment or aldosterone removal (with a corresponding decrease in the mean \(P_o\)) but one that we cannot verify statistically.

### 3-DZA Does Not Alter the Current-Voltage Relationship—
Fig. 3 shows the current-voltage (I-V) relationship for sodium channels from cell-attached patches, obtained by measuring the single channel current amplitudes at various applied pipette potentials. We assumed that \(V_p\) values from −70 to +70 mV should cover the physiological range of membrane potentials. The potentials shown in the figure are the displacement of the cellular potential from the resting potential with respect to the potential of the patch pipette (or \(-V_p\) i.e. a positive potential is a cell depolarization). The shapes of the I-V curves obtained from untreated (circles) and 3-DZA-treated cells (3–5 h, squares) were statistically indistinguishable. They were also consistent with I-V curves previously obtained for highly selective sodium channels in A6 and mammalian distal nephron cells (1, 38, 41, 47).
Methylation and ENaC

Fig. 4. Methyltransferase inhibitors block aldosterone-induced increases in transepithelial current. The isoprenylcysteine methyltransferase-specific inhibitor FTS and the more general methyltransferase inhibitor 3-DZA both inhibit aldosterone-induced increases in transepithelial current but produce little if any change in basal current (A and B). In each panel, four groups of cells in three separate experiments were used as follows: one group treated with aldosterone-free medium (–aldosterone); a second treated with aldosterone-free media containing 100 μM FTS or 300 μM 3-DZA (–aldosterone + FTS or –aldosterone + DZA); a third treated with 0.1 μM aldosterone medium (+aldosterone); and a fourth treated with FTS or DZA plus 0.1 μM aldosterone medium (+aldosterone + FTS or +aldosterone + DZA). Only application of aldosterone produces a significant change in current. Current in treated monolayers are not significantly different from levels in current. Current in treated monolayers are not significantly different from levels in current. Current in treated monolayers are not significantly different from levels in current.

Methyltransferase Inhibitors Reduce Transepithelial Sodium Current and Block Aldosterone-induced Increases in Transepithelial Sodium Current—If 3-DZA reduces the $P_o$ of individual ENaC channels, then total transepithelial sodium current should also be reduced. Fig. 4C shows that 3-DZA at all concentrations tested reduces transepithelial current. Since 3-DZA does not reduce current at any concentration as much as 10 μM amiloride.

is consistent with a small (about 15 mV) hyperpolarization of the apical membrane, expected if the apical sodium permeability is decreased by 3-DZA. The single channel conductance with no applied potential (i.e. the resting potential) is ~4 pS under both conditions. 3-DZA does not significantly alter the rectification properties, ion selectivity, or unit conductance of apical sodium channels.

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The Methyl Donor, S-Adenosyl-L-methionine, Activates ENaC in Excised Patches—Results obtained after 3-DZA incubation demonstrate that inhibition of the cellular methylations reactions reduces ENaC activity in A6 cells. However, based only on these “cell-attached” experiments performed after long incubation times, it is impossible to decide whether the crucial methylation step involves the channel protein itself or other cellular components unrelated to the apical membrane such as histone methylation that might affect protein transcription. This point can be clarified by examining ENaC activity in excised patches whose internal surface has been exposed to the methyl donor, AdoMet, provided that the putative methyltransferase responsible for the channel methylation is membrane-associated. In both controls and AdoMet-treated patches, 1 mM ATP was always present, on the assumption that some of the processes necessary to maintain sodium channel activity might require an energy source. After obtaining a high resistance seal on an A6 cell, the recording chamber was rapidly filled with a high potassium and low calcium solution (see “Materials and Methods”). The patch was then excised, moved well away from the cell surface, and its behavior observed for at least 10 min, at $V_p = 0$ mV. The entire excision process never took more than 1 min.

A rapid spontaneous decrease in ENaC activity is very common in excised patches from a variety of epithelial cells including A6 cells (47), strongly suggesting that some diffusible intracellular factor must be present to maintain channel activity. Indeed, in 73% of our controls we could not observe any channel opening more than 2 min after excision. Fig. 5A shows an example of channel activity typical of patches shortly after excision before activity decreases. In this case, it was possible to observe channel activity after excision for only about 5 min. This is in very good agreement with previous work, in which less than 25% of the control excised patches showed long lasting activity (47).

In contrast, when excised in the presence of AdoMet, patch activity lasted at least 5 min and in 8 out of 10 experiments activity lasted at least 8 min. An example of typical channel activity after excision in AdoMet is shown in Fig. 5B with the amplitude histogram for channel activity after excision. In this particular case the channel activity remained stable for about 10 min, with no modification of current amplitude; the calculated $P_o$ was 0.12.

The small decrease in the single channel current amplitude after excision is consistent with the change in transmembrane potential across the patch (about ~40 mV before excision, the intracellular apical potential, versus 0 mV after excision, closer to the sodium equilibrium potential). As in the case of 3-DZA experiments, the single channel conductance is also about 4 pS, consistent with the properties of typical apical sodium channels in A6 cells.

Activity of untreated and AdoMet-treated excised patches with time after excision are summarized in Fig. 6. Ten consecutive 1-min windows in which $P_o$ was calculated for every excised AdoMet-treated patch and the values for each window were averaged (10 experiments) and plotted for comparison with the control values of $P_o$ measured and averaged in the...
Methylation and ENaC

AdoMet increases the activity of ENaC channels. A shows an example of channel activity typical of patches shortly after excision before activity decreases. In contrast, B shows an example of typical channel activity after excision in 100 μM AdoMet with the amplitude histogram for channel activity after excision. In this particular case the calculated channel $P_o$ was 0.12. Inward currents are downward deflections. Records are filtered at 100 Hz and held at a constant $V_m = 0$ mV. The high potassium bath solution contained 1 mM ATP in addition to the AdoMet.

FIG. 5. AdoMet increases the activity of ENaC channels. A shows an example of channel activity typical of patches shortly after excision before activity decreases. In contrast, B shows an example of channel activity after excision in 100 μM AdoMet with the amplitude histogram for channel activity after excision. In this particular case the calculated channel $P_o$ was 0.12. Inward currents are downward deflections. Records are filtered at 100 Hz and held at a constant $V_m = 0$ mV. The high potassium bath solution contained 1 mM ATP in addition to the AdoMet.

AdoMet significantly prolonged the sodium channel activity, as shown in Fig. 5. AdoMet, the mean $P_o$ measured 2–4 min after excision was 0.167 ± 0.056, significantly different from the corresponding control value (0.049 ± 0.0097). The low level of activity remaining, in both AdoMet and control patches, even after 6–7 min of recording is due to a small number of patches (about 20–25% of the total) whose activity did not decrease. Exogenous methyltransferase does not increase the effects of AdoMet on excised patches. We performed excised patch experiments in the presence of both 300 μM AdoMet and 2 μg of partially purified A6 methyltransferase (30, 48) in a volume of about 400 μl. The addition of the enzyme did not affect the decrease in channel activity in a statistically significant way. In contrast, guanosine triphosphate substantially increases ENaC activity in the presence of AdoMet. We examined the activity of single sodium channels in excised patches in the presence of 1 mM ATP, 200 μM GTP, and 300 μM AdoMet. In the presence of GTP, channel activity was significantly increased with respect to controls even 8–10 min after excision when the mean $P_o$ was 0.546 ± 0.0199 in the presence of GTP and significantly different ($p < 0.01$) from the corresponding values for control (0.0705 ± 0.00478) and AdoMet-treated (0.0980 ± 0.0169) patches.

FIG. 6. Summary of changes in ENaC $P_o$ with time after patch excision into different media. Ten consecutive 1-min windows in which $P_o$ was calculated for each excised patch treated with different substances (11 untreated patches, 10 in AdoMet (SAM), 5 in AdoMet plus methyltransferase (Mtase), and 12 in AdoMet plus GTP) and the values for each window are averaged and plotted for comparison with the control values of $P_o$ measured and averaged in the same way. AdoMet maintained sodium channel activity longer than untreated excised patches. In AdoMet-treated cells, the mean $P_o$ measured 2–4 min after excision was 0.167 ± 0.056, significantly different from the corresponding control value (0.049 ± 0.0097). The low level of activity remaining, in both AdoMet and control patches, even after 6–7 min of recording is due to a small number of patches (about 20–25% of the total) whose activity did not decrease. Exogenous methyltransferase does not increase the effects of AdoMet on excised patches. We performed excised patch experiments in the presence of both 300 μM AdoMet and 2 μg of partially purified A6 methyltransferase (30, 48) in a volume of about 400 μl. The addition of the enzyme did not affect the decrease in channel activity in a statistically significant way.

indistinguishable from control values about 7–10 min after excision. In AdoMet-treated cells, the mean $P_o$ measured 2–4 min after excision was 0.167 ± 0.056, significantly different from the corresponding control value (0.049 ± 0.0097). The low level of activity remaining, in both AdoMet and control patches, even after 6–7 min of recording is due to a small number of patches (about 20–25% of the total) whose activity did not decrease (47).

Guanosine Trisphosphate Substantially Increases ENaC Activity in the Presence of AdoMet—If the G-protein-like 95-kDa subunit of the A6 cell sodium channel complex is really the target for transmethylation reactions (46, 49, 50) and remembering that many methylation reactions are GTP-stimulated (27), we would expect GTP analogues to influence the activity of sodium channels in the presence of AdoMet. We therefore examined the activity of single sodium channels in excised patches in the presence of 1 mM ATP, 200 μg GTP, and 300 μg AdoMet. Fig. 6 shows the time course of this activity compared with activity in controls and AdoMet-treated patches. In contrast to the results obtained with AdoMet alone, in the presence of GTP, channel activity was significantly increased with respect to controls even 8–10 min after excision when the mean $P_o$ was 0.546 ± 0.0199 in the presence of GTP and significantly different ($p < 0.01$) from the corresponding values for control (0.0705 ± 0.00478) and AdoMet-treated (0.0980 ± 0.0169) patches. Furthermore, the number of channels per patch and $P_o$ were close to those normally found in cell-attached patches from A6 cells grown in the presence of aldosterone. In particular, by considering the period with maximum activity in the presence of GTP (2nd to 5th min), $N$ was 3.0 ± 0.60 and $P_o$ was
and on the channel records under each condition, and on the left are the interval histograms. The single channel records are from patches containing one active channel held at $V_0 = 0$ mV from the inner surface of excised patches should mimic the action of aldosterone (albeit more quickly since there is no required gene expression). Fig. 8 and Table I show that this is only partially true. Excised patches from cells depleted of aldosterone for 48 h have a very short mean open time as we have previously described, and the mean open time is not much different than that of channels in untreated excised patches. However, when the same patch was treated with 0.1 mM AdoMet and 0.2 mM GTP on the cytosolic surface, the open probability and mean open time of the channel increased but to a value less than that of channels in patches on aldosterone-replete cells. The implication is that there must be some aldosterone-induced elements that are critical to maintain channel activity, and therefore, methylation is not the only required event in the activation of channels by aldosterone.

**DISCUSSION**

*Apical Sodium Channels Are Activated When Methylation Is Stimulated in A6 Cells*—Sariban-Sohraby et al. (23) showed that methylation increases sodium uptake into membrane vesicles isolated from the apical surface of cultured A6 cells. Furthermore, Wiesmann et al. (45) demonstrated that aldosterone stimulates phospholipid methylation and protein carboxymethylation and ENaC.

$S$-Adenosylmethionine increases ENaC mean open time. Event information from at least five patches (more than a thousand events for each condition) with a single channel (no evidence for a second current level) for each condition was pooled, and interval histograms with logarithmic bin sizes were generated. On the right are typical single channel records under each condition, and on the left are the interval histograms. The single channel records are from patches containing one active channel held at $V_0 = 0$ mV and filtered at 100 Hz. The interval histograms were fit with a single exponential function (although in cell-attached patches there was some evidence of a small contribution of a second class of short duration open and closed events). The mean open and closed times and calculated $P_o$ are given in Table I and summarized in Fig. 8.

Equation 3,

$$P_o(t) = Ae^{-\tau t}$$

(Eq. 3)

where $A$ is a constant corresponding to the $P_o$ value at some defined beginning time; $t$ is the time after the beginning time, and $\tau$ is the time constant of the exponential decrease. We fit Eq. 3 to the data in Fig. 6 to obtain time constants for the decrease in sodium transport. The time constants for untreated and AdoMet-treated patches are similar ($\tau = 2.28 \pm 0.336$ min in controls and $3.16 \pm 0.283$ min in AdoMet); however, the time constant in AdoMet plus GTP is, as expected, much larger (18.5 \pm 4.80 min). The implication appears to be that AdoMet and, especially, AdoMet plus GTP reduce the rate at which ENaC loses activity in excised patches.

$S$-Adenosylmethionine increases ENaC mean open time. It seemed clear from Fig. 5 that one effect of AdoMet was to increase the mean open time above that observed in excised patches. To examine this question more carefully, we combined event information from at least five patches (more than a thousand events for each condition) with a single channel (no evidence for a second current level) for each condition, plotted interval histograms with logarithmic bin sizes, and determined mean open and closed times from the interval histograms. Such histograms are shown in Fig. 7. The interval histograms were fit with a single exponential function (although in cell-attached patches there was some evidence of a small contribution of a second class of short duration open and closed events). The mean open and closed times and calculated $P_o$ are given in Table I and are summarized in Fig. 8. Patch excision appears to strongly destabilize the open state and nominally stabilize the closed state of the channel, and AdoMet and AdoMet plus GTP appear to return the channel to a state similar to cell-attached patches.

AdoMet Plus GTP Increases the Open Probability of Sodium Channels from Aldosterone-depleted Cells but Not as Much as Aldosterone-replete Cells—If aldosterone stimulation of channel activity only involves an essential transmethylation of the sodium channel, itself, or a closely associated membrane regulatory component, then addition of AdoMet plus GTP to the inner surface of excised patches should mimic the action of aldosterone (albeit more quickly since there is no required gene expression). Fig. 8 and Table I show that this is only partially true. Excised patches from cells depleted of aldosterone for 48 h have a very short mean open time as we have previously described, and the mean open time is not much different than that of channels in untreated excised patches. However, when the same patch was treated with 0.1 mM AdoMet and 0.2 mM GTP on the cytosolic surface, the open probability and mean open time of the channel increased but to a value less than that of channels in patches on aldosterone-replete cells. The implication is that there must be some aldosterone-induced elements that are critical to maintain channel activity, and therefore, methylation is not the only required event in the activation of channels by aldosterone.

**Fig. 7.** $S$-Adenosylmethionine increases ENaC mean open time. Event information from at least five patches (more than a thousand events for each condition) with a single channel (no evidence for a second current level) for each condition was pooled, and interval histograms with logarithmic bin sizes were generated. The single channel records are from patches containing one active channel held at $V_0 = 0$ mV and filtered at 100 Hz. The interval histograms were fit with a single exponential function (although in cell-attached patches there was some evidence of a small contribution of a second class of short duration open and closed events).
Methylation and ENaC

**TABLE I**

| Condition | Mean open time | Mean closed time | Calculated $P_o$ |
|-----------|----------------|------------------|-----------------|
| Cell attached patches before excision ($n = 5$) | 2.06 ± 0.064 | 2.88 ± 0.089 | 0.417 ± 0.183 |
| Patches excised into intracellular saline ($n = 7$) | 0.0452 ± 0.0286 | 10.3 ± 0.652 | 0.004 ± 0.0028 |
| Patches excised into intracellular saline + 0.1 mM Ado Met ($n = 6$) | 0.437 ± 0.0145 | 2.76 ± 0.098 | 0.137 ± 0.066 |
| Patches excised into intracellular saline + 0.1 mM Ado Met + 0.2 mM GTP ($n = 5$) | 2.36 ± 0.094 | 1.14 ± 0.042 | 0.674 ± 0.0366 |
| Patches excised into intracellular saline from aldosterone-depleted cells ($n = 11$) | 0.0349 ± 0.0080 | 4.16 ± 0.735 | 0.008 ± 0.0024 |
| Patches excised into intracellular saline + 0.1 mM Ado Met + 0.2 mM GTP from aldosterone-depleted cells ($n = 7$) | 0.158 ± 0.031 | 3.34 ± 0.565 | 0.045 ± 0.0117 |

**FIG. 8.** AdoMet plus GTP increases the open probability of sodium channels from aldosterone-depleted cells but not as much as aldosterone-replete cells. Patches were formed and channels recorded from cells depleted of aldosterone for 48 h and from cells treated with 1.5 μM aldosterone. Channels from aldosterone-free cells have a very short mean open time that is not significantly different than that of channels in untreated excised patches. When patches from the same cells were excised into 0.1 mM AdoMet (SAM) and 0.2 mM GTP on the cytosolic surface, the open probability and mean open time of the channel of the channel increased but to a value significantly less than that of channels in patches on aldosterone-treated cells. AdoMet + GTP appeared to restore the mean closed time regardless of whether the cells had been treated with aldosterone. Asterisks and brackets indicate a significant difference between treatments ($p < 0.05$).

Methylation in cultured epithelial cell line TB-6c. In this case, 3-DZA inhibition of methyltransferases was correlated with inhibition of short circuit current response to aldosterone. We have shown (51) and Blazer-Yost et al. (52) have also shown that Na⁺ transport is attenuated by inhibitors of the protein methyltransferase that modifies C-terminal isoprenylcysteine. These observations pointed to a membrane effect of methylating agents linked to renal sodium transport. We thus tried to verify whether the putative methylation effect on sodium transport was accompanied by a modification of the properties of individual apical sodium channels. We observed that 3-DZA did reduce $P_o$ and mean open time in a manner reminiscent of aldosterone removal (but faster). We also showed that AdoMet applied to the cytosolic surface of excised patches prolongs the lifetime of apical sodium channels. This information and data obtained from apical vesicles (23) imply that the methylation reaction stimulating sodium transport involves some membrane or membrane-associated target protein, possibly sodium channel proteins themselves, rather than some intracellular protein. Furthermore, data from excised patches indicate that the methylation reaction affects the channel open probability, even though a small effect on channel density cannot be completely ruled out (since the $P_o$ after DZA treatment is so low that the number of sodium channels per patch cannot be accurately determined). Since, in the absence of AdoMet, channel activity in excised patches is generally unstable, the methylation reaction in A6 cells is probably easily reversible, in keeping with the reversibility of methylation of other membrane proteins (48, 53, 54). In addition, for ENaC expressed in lipid bilayers, methylation is capable of activating sodium channels (30). This makes methylation a credible candidate as a regulatory mechanism controlling sodium absorption in renal cells.

However, the sodium channels of all epithelial cells do not appear to respond to AdoMet and GTP in the same way. Frindt and Palmer (55) examined sodium channels in principal cells of rat cortical collecting duct and could not stimulate sodium channel activity in whole cell recordings in which AdoMet and GTP were included in the pipette solution. We presume this means that there is little methyltransferase associated with the apical membrane or that something about the whole cell recording conditions alters methyltransferase activity, but it could also mean that methylation is not a critical step in the activation of channels by aldosterone. It would be interesting to examine the effects of 3-DZA or FTS on the activity of single sodium channels in this preparation.

**Action of Exogenous Methyltransferase**—It is clear from Fig. 6 that the presence of supplementary methyltransferase does not slow the decrease in activity compared with patches treated with AdoMet alone, so that this decrease in activity does not seem to depend on inactivation of methyltransferase in “inside-out” excised patches.

The small effect of methyltransferase addition in excised patches was not too surprising to us, since some methyltransferases are membrane-associated (56). It seems likely that, under our experimental conditions (i.e. in the presence of aldosterone), there already would be membrane-associated meth-
Methylation and ENaC

...ulfonates in the vicinity of the channels in an excised patch. The AdoMet results imply that the endogenous enzyme is usually sufficient to methylate either channels, themselves, or other closely associated regulatory proteins in the presence of the substrate. This observation is consistent with the observation that no addition of enzyme is necessary to obtain in vitro methylation of apical membrane vesicles (23). On the other hand, our observations do contrast with those of Frindt and Palmer (55) who, as mentioned above, could not see channel activation by methyl donors in excised patches. It would be interesting to test if addition of exogenous methyltransferase would activate sodium channels in rat principal cells.

**GTP Stimulation of Sodium Channel Activity in the Presence of S-Adenosylmethionine**—Sariban-Sohraby et al. (46) showed that aldosterone-induced membrane methylation targets a 90–95-kDa protein that has previously been suggested as a subunit of the amiloride-sensitive Na+ channel complex. In those experiments, methylation of the 95-kDa protein was stimulated by GTP analogues. Consistent with that observation, we found that addition of GTP increases the ability of AdoMet to stimulate channel activity in excised patches. There are two interpretations of that data that are not mutually exclusive. The first possibility is that the methyltransferase associated with the apical membrane of A6 cells is directly stimulated by GTP as other methyltransferases are (28, 56–63). The second possibility is that some ENaC regulatory protein requires both methylation and GTP to be active (for example a small G protein).

In previous work on membranes prepared from aldosterone-depleted A6 cells, GTPS was very effective in stimulating methylation whereas, in membranes prepared from aldosterone-replete cells, GTPS stimulated methylation poorly, if at all (46). The comparison with our results has interesting implications. In aldosterone-depleted cells, AdoMet does increase channel activity as expected from the results observed in vesicles; on the other hand AdoMet does not increase the activity to the level seen in aldosterone-treated cells. Moreover, in aldosterone-treated cells, neither AdoMet alone nor AdoMet plus GTP applied to the cytosolic surface of excised patches significantly increased the open probability of sodium channels beyond that observed in cell-attached patches. However, both treatments maintained the activity of channels in excised patches. These results seem to imply that the aldosterone-induced methyl ester that increases sodium channel activity is stable in a normal cellular (or vesicular) environment but that, in excised patches, the ester rapidly breaks down leaving inactive channels in the patch. In the presence of substrates (GTP and AdoMet) that promote formation of new methyl esters, channel lifetime is significantly prolonged.

**The Protein Target for Methylation**—To understand the role of methylation in altering sodium channel activity, it is useful to identify the protein targets for methylation. As mentioned above, Sariban-Sohraby et al. (46) demonstrated that a 95-kDa protein was methylated in the presence of aldosterone. Since this size is consistent with the size of a glycosylated sodium channel subunit, some investigators (49) have suggested that the target for methylation is one of the sodium channel subunits. Indeed, in recent work, Rokaw et al. (64) demonstrated methylation of the β subunit of sodium channels in A6 cells. In addition, βENaC appears to be activated in lipid bilayers by methylation donors (like AdoMet) to increase the open probability of sodium channels (at least ones treated with dithiothreitol) (30). The difficulty with these experiments is that the channels are reconstituted from oocyte proteins (which may contain other proteins that are targets for methylation but which must necessarily associate with βENaC), and methylation enzymes are found in a total cell cytosolic lysate. Therefore, the oocyte lysate may contain many methyltransferases other than isoprenylcysteine methyltransferase that could methylate βENaC or associated regulatory proteins.

However, methylation of proteins that control activity of the protein almost always occurs only at a very restrictive consensus sequence, the so-called “CAAAX” box (a cysteine residue followed by two aliphatic residues, followed by any residue at the C-terminal end of the protein). None of the ENaC sequences contain any cysteine residues that meet the criteria for methylation. However, there are other possible cellular targets for aldosterone-induced methylation of proteins that regulate sodium channels, but based on the present work, the target for methylation, if it is not a channel subunit itself, must be a membrane-associated protein in close proximity to the channels.

**Methylation and the Mechanism of Aldosterone Action**—The experiments we report here paint an interesting picture of some of the steps that lead from the induction of aldosterone-induced proteins to an increase in sodium channel open probability. We have demonstrated at a single channel level that methylation is essential for significant channel activity and that aldosterone promotes methylation in A6 cells. We have also demonstrated that the enzyme responsible for aldosterone-induced methylation is membrane-associated and that it is present both in the presence and absence of aldosterone. Therefore, the methyltransferase is essential for the action of aldosterone but is not an aldosterone-induced protein. In other recent work (65), we have also demonstrated by direct biochemical experiments that O-carboxymethyltransferase is not an aldosterone-induced protein. However, methylation reactions are required for normal sodium channel activity; therefore, methylation is necessary but not sufficient to account for aldosterone-induced increases in ENaC activity.

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Methylation Increases the Open Probability of the Epithelial Sodium Channel in A6 Epithelia
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