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Effects of Vasopressin and Aldosterone on the Lateral Mobility of Epithelial Na^+ Channels in A6 Renal Epithelial Cells

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Abstract. We have previously demonstrated that apical Na^+ channels in A6 renal epithelial cells are associated with spectrin-based membrane cytoskeleton proteins and that the lateral mobility of these channels, as determined by fluorescence photobleach recovery (FPR) analysis, is severely restricted by this association (Smith et al., 1991. *Proc. Natl. Acad. Sci. USA* **88:**6971–6975). Recent data indicate that the actin component of the cytoskeleton may play a role in modulating Na^+ channel activity (Cantiello et al., 1991. *Am. J. Physiol.* **261:**C882–C888); however, it is unknown if the Na^+ channel's linkage to the spectrin-based membrane cytoskeleton is also involved in regulating channel activity. In this study, we have used FPR to examine if the linkage of the Na^+ channels to the membrane cytoskeleton is a site for modulation of Na^+ channel activity in filter grown A6 cells by vasopressin and aldosterone. We hypothesized that if the linkage of the Na^+ channels to the membrane cytoskeleton is a site for regulation of Na^+ channel activity by vasopressin and aldosterone, then hormone-mediated changes in either the membrane cytoskeleton or the affinity of the Na^+ channel for the membrane cytoskeleton, should be reflected in changes in the lateral mobility and/or mobile fraction of Na^+ channels on the cell surface. FPR revealed that although the rates of lateral mobility were not affected, there was a twofold increase in mobility fraction (f) of apical Na^+ channels in aldosterone-treated (16 hr) monolayers (f = 32.31 ± 5.42%) when compared to control (unstimulated) (f = 14.2 ± 0.77%) and vasopressin-treated (20 min) (f = 12.7 ± 2.4%) monolayers. The twofold increase in mobile fraction of Na^+ channels corresponds to the average increase in Na^+ transport in response to aldosterone in A6 cells. The aldosterone-induced increase in Na^+ transport and mobile fraction can be inhibited by the methylation inhibitor, 3-deazaadenosine, consistent with the hypothesis that a methylation event is involved in aldosterone induced upregulation of Na^+ transport. We propose that the membrane cytoskeleton is involved in the aldosterone-mediated activation of epithelial Na^+ channels.

Key words: Epithelial sodium channel — Fluorescence photobleach recovery — Aldosterone — Vasopressin — A6 epithelial cells

Introduction

Epithelial Na^+ channels mediate entry of Na^+ from the luminal fluid into the cells during the first stage of electrogenic Na^+ transport across Na^+ reabsorbing renal epithelia. We have previously demonstrated that these apically restricted Na^+ channels are associated with the spectrin-based membrane cytoskeleton and that the lateral mobility of these channels, as determined by fluorescence photobleach recovery (FPR), is severely restricted by this association [45]. Increasing evidence suggests that the physiological regulation of these channels is very complex. Activity of the Na^+ channels has been shown to be regulated by protein kinase A- and protein kinase C-mediated phosphorylation [24, 33], G-proteins [8, 24, 34], methylation [25, 40], arachidonic

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acid metabolites [9], and by the cytoskeleton [10]. Using the patch clamp technique, Cantiello et al. [10] have provided evidence that actin filaments modulate a 9 pS poorly selective Na^+ channel in A6 renal epithelial cells, either by affecting the membrane environment or by interacting with membrane cytoskeleton proteins associated with the channel [10]. Prat and coworkers [37, 38] have subsequently presented data demonstrating that the actin component of the cytoskeleton is involved in the vasopressin-induced upregulation of this Na^+ channel in A6 cells. Although these data indicate that actin plays a role in modulating sodium channel activity in response to vasopressin, it is unknown if the membrane cytoskeleton associated with the channels is also involved in the vasopressin-induced increase in transepithelial Na^+ transport.

Aldosterone also induces an increase in Na^+ transport across high resistance Na^+ reabsorbing epithelia, such as A6 cells [16, 28, 51]. Although the mechanism by which aldosterone activates Na^+ channels is unclear, increasing biochemical [4, 42] and electrophysiological evidence [25, 27] suggests that one mechanism by which aldosterone activates Na^+ channels is through methylation of the channel protein. The methylation inhibitor, 3-deazaadenosine, has been shown to prevent aldosterone-mediated upregulation of Na^+ transport [25, 27, 41, 50]. It is presently unknown if the membrane cytoskeleton plays a role in the aldosterone-mediated upregulation of Na^+ channel activity.

In this study, we have used the technique of FPR to examine if the spectrin-based membrane cytoskeleton is involved in the upregulation of Na^+ channel activity in filter-grown A6 renal epithelial cell monolayers by the hormones vasopressin and aldosterone. FPR has previously been used to examine the interaction of the spectrin-based membrane cytoskeleton with voltage-dependent Na^+ channel of neurons [1], Na^+, K^+ATPase of photoreceptors [32] and renal cells [35], and band 3 of erythrocytes [11, 19].

We hypothesized that if the spectrin-based membrane cytoskeleton is involved in the upregulation of Na^+ transport by vasopressin and aldosterone, then hormone-mediated changes in the membrane cytoskeleton should be reflected in changes in the lateral mobility (D_L) and/or mobile fraction (f) of Na^+ channels on the cell surface. Our results reveal that although the rates of lateral mobility were not affected, there was a twofold increase in the mobile fraction of apical Na^+ channels in aldosterone-treated monolayers when compared to control (unstimulated) and vasopressin-treated monolayers. This twofold increase in mobile fraction of Na^+ channels corresponds to the average increase in Na^+ transport in response to aldosterone by A6 cells [51]. We interpret these data to mean that the spectrin-based membrane cytoskeleton is involved in the upregulation of Na^+ channel activity by aldosterone.

Materials and Methods

A6 Epithelial Cell Culture

The A6 cell line, clone A6 2F3, derived from the distal tubule of *Xenopus laevis* kidney, generously provided by Dr. B. Rossier, University of Lausanne, Lausanne, Switzerland was used for FPR experiments. A6 cells used for patch clamp analysis were obtained from the American Type culture collection (Rockville, MD). Cultures were maintained as previously described [40] and subcultured onto transparent permeable tissue culture inserts (25 mm Anocell filters; Wheaton). Cells were used 5–7 and 7–10 days after plating for patch clamp and FPR, respectively. Confluent monolayers were stimulated with either *d*-aldosterone (10^{-7} M; Sigma Chemical, St. Louis, MO) for 16 hr or arginine vasopressin (10^{-7} M; Sigma) for 20 min prior to FPR analysis. In some experiments, A6 cell monolayers were incubated for 16 hr in the presence of both aldosterone and 3-deazaadenosine (300 μM; Southern Research Institute, Birmingham, AL) prior to FPR to examine the role of aldosterone-mediated methylation. To determine the role of actin filaments in regulating the lateral mobility and mobile fraction of Na^+ channels on the cell surface, A6 cell monolayers were incubated in cytochalasin D (1 μM final concentration; Calbiochem, San Diego, CA) for 20 min prior to FPR. Control monolayers did not receive treatment.

Preparation of Fluorochrome Conjugated Antibody

Fifty micrograms of polyclonal anti-Na^+ channel antibody [46] were labeled with the succinidmidyl ester of 5- (and 6-) carboxytetramethylrhodamine (Molecular Probes, Eugene, OR) as described [23]. The anti-Na^+ channel antibodies were generated against a high amiloride affinity Na^+ channel purified from bovine renal papillae [6, 7] and they recognize both extracellular and intracellular epitopes of the channel complex [46]. Unbound dye was separated from the conjugate by gel filtration on a Sephadex G-50 column. For preparation of fluorophore-labeled Fab′ fragment, the column eluant was concentrated using Amicon concentrators and adsorbed to immobilized papain (Pierce, Rockford, IL); the eluant was recovered and applied to an immobilized protein A column (Pierce). Labeled Fab′ fragments were recovered in the void volume. Concentration and labeling of the Fab′ fragments were determined by measuring absorbance at 280 nm and 560 nm, the latter which measures the concentration of fluorescent dye.

Fluorescence Photobleach Recovery (FPR) Measurements

Anocell filters bearing confluent A6 cell monolayers were equilibrated with fluorescently labeled anti-Na^+ channel Fab′ (1:20 dilution) at 25°C for 30 min. In the vasopressin experiments, cells were equilibrated with labeled antibodies for 10 min prior to vassopressin addition. After being washed with Dulbecco's phosphate buffered saline (PBS), filters bearing labeled monolayers were removed from their supports and placed on the microscope stage in a chamber containing PBS. Lateral diffusion coefficients (D_L) and mobile fractions (f) of the fluorescently labeled Na^+ channels were measured by the spot photobleaching method on a previously described instrument [1]. The monitoring argon-ion laser beam (515 nm; 5 μW) was focused through the microscope to a Gaussian radius of 0.45 μm (63×, 1.2 NA water immersion objective; Zeiss). The illuminated region was bleached by a brief 50–350 msec laser pulse (5 mW) eliminating ~70% of the fluorescence. The time course of the fluorescence recovery was followed using the original, attenuated monitoring beam. Lateral diffusion coefficients

and mobile fractions were determined by curve-fitting procedures as previously described [1, 2]. Diffusion coefficients were calculated using $w^2/4t_D$ in which w is the e^{-2} radius of the beam profile and t_D is the half time of recovery. Incomplete recovery on the time scale of the experiments was interpreted as an immobile fraction of fluorophores ($D_L < 1.0 \times 10^{-12}$ cm^2/sec). The lateral mobility of apical membrane glycoproteins was measured by labeling cells with TmRhd-succinyl Concanavalin A (sCon A) (30 μg/ml; Vector Laboratories, Burlingame, CA) as described above for the Fab' fragments. Controls for nonspecific fluorescence were done by measuring areas devoid of cells or areas remote from the apical surface. All measurements were performed at room temperature.

To determine if vasopressin and aldosterone induce the insertion of new channels into the apical membrane, vasopressin and aldosterone-stimulated monolayers were labeled with anti-Na^+ channel antibodies as described above. Photon counts were measured in a 0.85 μm diameter spot on the apical surface using the monitoring laser beam of the FPR apparatus. Photon counts were compared with those obtained from untreated monolayers under identical optical conditions.

Patch Clamp Analysis

Methods for fabricating patch clamp pipettes and making seals were modified from those of Hamill et al. [21]. Pipettes were pulled from 150 μl Microcaps (Drummond Scientific, Broomall, PA) on a Brown-Flaming P-80/PC puller (Sutter Instrument, San Rafael, CA) immediately before use. The tips of the pipettes were fire polished on a Narishige Microforge (Narishige, Tokyo, Japan).

Filter grown A6 cell monolayers were placed in amphibian saline (in mM): (105 NaCl; 3.0 KCl; 2.0 $CaCl_2$; 1.25 $MgSO_4$; 1.25 KH_2PO_4; 5 HEPES (*N*-[Hydroxyethylpiperazine-N]-2-ethanesulfonic acid); 5.5 dextrose; pH 7.6; 217.5 mOsm/Kg H_2O) when patch clamping. Cells were imaged with a video camera (Javelin Electronics, Torrance, CA) and displayed on a high-resolution video monitor. To form a seal, the pipette was typically positioned over the monolayer with a mechanical manipulator. The final approach was made with a Narishige Hydraulic Micromanipulator. The data presented are from cell-attached patches. Patches with a seal resistance of less than 1.0 gΩ were discarded. Channel activity from a patch when the pipette and bathing solutions were identical and voltage-clamped to 0 mV was deemed to be from a cell-attached patch. To examine the effect of cytochalasin D on Na^+ channel activity, the bath was exchanged with saline containing either 1 μM or 10 μM cytochalasin D. Cytochalasin D (Calbiochem) was dissolved in dimethyl sulfoxide (DMSO) and the stock solution was added directly to the saline to give the desired final concentration prior to the bath exchange. The final concentration of DMSO in the bath was less than 0.1% and had no effect on Na^+ channel activity.

The patch clamp signal was monitored via an Axopatch 1-B amplifier (Axon Instruments, Burlingame, CA) equipped with a TMA-1 interface. A permanent record of experimental data was digitized (Model VR-10, Instrutech, Mineola, NY) and recorded on videotape for offline analysis. The signal was filtered to tape at 50 Hz. For analysis, data were fed into the computer at a sampling rate of 500 μsec/point and filtered at 50 Hz. Events shorter than 25 msec were discarded. p-CLAMP software (Axon Instruments) was used to analyze the data. To characterize the Na^+ channels, the chord conductance of channels was determined from the slope of the *I-V* relationship. The Na^+ channels exhibited a mean single-channel conductance of 3.42 ± 0.72 pS (n = 5) and a mean reversal potential of −116 ± 10 mV (n = 3). Clamp voltages typically varied from −20 to +120 mV pipette potential with respect to bath. The signal of an active patch was monitored for 60 to 120 sec at each voltage.

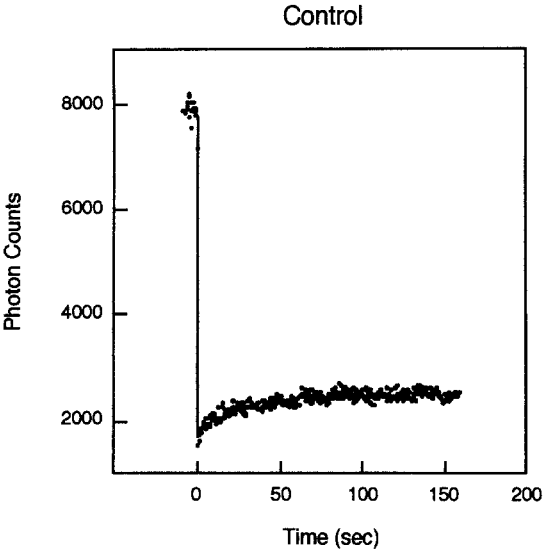

Fig. 1. Representative FPR curve of fluorescently labeled Na^+ channels on the apical surface of control (untreated) A6 monolayers demonstrating the slow mobility and low mobile fraction. Analysis of the curve yields a diffusion coefficient (D_L) of 4.82×10^{-11} cm^2/sec and a mobile fraction (f) of 14%. The time of the bleach pulse was 8.53 sec and the pulse was 340 msec in duration.

Statistics

Data are presented as mean ± SE. Student's unpaired *t*-test was used to determine significant differences between controls and the various treatments.

Results

In this study, we have examined the mobility of Na^+ channels of the apical surfaces of A6 cells grown on Anocell filters. As shown in Fig. 1, >80% of the Na^+ channels were found to be immobile on the time scale of the experiment (<10^{-12} cm^2/sec) in unstimulated A6 cell monolayers. There was a small mobile fraction (f) of Na^+ channels (14.2 ± 0.77%) that had diffusion coefficients (D_L) of (9.9 ± 1.2) × 10^{-11} cm^2/sec (Fig. 7; Table).

Effects of Vasopressin on Na^+ Channel Mobility

Hays and coworkers have shown that vasopressin induces a depolymerization of F-actin and a reorganization of spectrin underlying the apical membrane of the toad urinary bladder [12] and inner medullary collecting duct of the rat kidney [44]. These data, together with the findings of Prat et al. [37, 38] that actin-mediated activation of 9 pS Na^+ channels in coverslip grown A6 cells is dependent upon protein kinase A, indicate that vasopressin-induced upregulation of Na^+ channel activity may involve the spectrin-based membrane cytoskeleton associated with the channels. We predicted that if vaso-

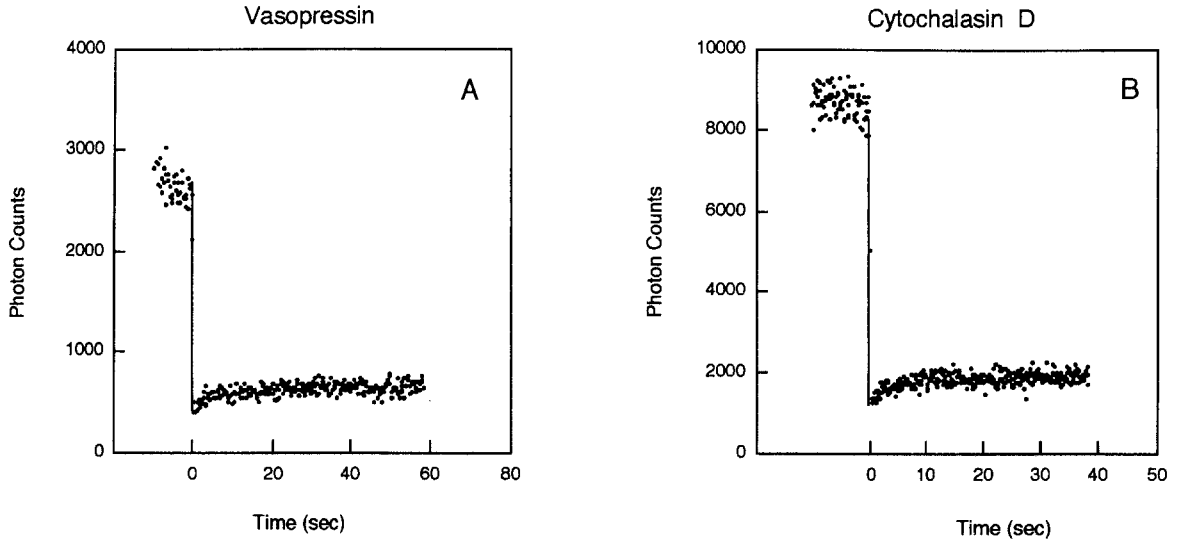

Fig. 2. Effects of vasopressin and cytochalasin D on the lateral mobility and mobile fraction of labeled Na^+ channels on A6 cells. (*A*) Representative FPR curve of labeled Na^+ channels on the surface of an A6 cell monolayer treated with vasopressin for 20 min prior to FPR. Analysis of the curve gives a diffusion coefficient (D_L) of 6.53×10^{-11} cm^2/sec and a recoverable fraction (f) of 8%. (*B*) Representative FPR curve of labeled Na^+ channels on the surface of an A6 cell monolayer incubated in the presence of cytochalasin D (1 μM final concentration) for 20 min prior to FPR. D_L and f of this curve are 6.68×10^{-11} cm^2/sec and 3%, respectively.

pressin alters the membrane cytoskeleton and its interaction with the channel, this should be reflected in an increase in the mobile fraction and/or lateral mobility of the channels. To determine if vasopressin effects D_L and f of Na^+ channels, A6 cell monolayers were stimulated with vasopressin for 20 min prior to FPR analysis. Vasopressin did not have a significant effect on either D_L or f of the Na^+ channels as illustrated in Fig. 2*A*. Analysis of the FPR curves revealed a f of 12.7 ± 2.4% and a D_L of $(5.9 \pm 1.56) \times 10^{-11}$ cm^2/sec (Fig. 7, Table).

Cytochalasin D and vasopressin have been shown to depolymerize the same apical pool of actin in the toad urinary bladder [15]. Therefore, to corroborate our findings for ADH, we examined the effect of a 20-min incubation in cytochalasin D (1 μM final concentration) on D_L and f of labeled Na^+ channels. In agreement with our previous findings for cytochalasin B [45], cytochalasin D did not significantly effect either D_L ($(8.2 \pm 2.4) \times 10^{-11}$ cm^2/sec) or f (7.33 ± 2.18%) of the Na^+ channels (Figs. 2*B*; 7; Table).

To further corroborate the lack of effect on Na^+ channel mobility by cytochalasin D, we used patch clamp analysis to determine if cytochalasin D will activate 5 pS, highly selective Na^+ channel [22] which is the predominant Na^+ channel type expressed in A6 cells grown on filter supports in our laboratory. Figure 3 illustrates the effect of cytochalasin D on activity of the 5 pS Na^+ channel recorded in the cell attached patch configuration. In this experiment, three channels were present in the patch. Complete channel inactivation was observed after 5 min following application of 1 μM cytochalasin D. In all five experiments performed, three in the cell-attached configuration and two in the inside-out configuration, we were able to hold the patch long enough to observe inactivation of the 5 pS Na^+ channel by cytochalasin D. Mean time for inactivation was 10.6 ± 2.0 min after application of cytochalasin D. Application of 10 μM cytochalasin D also abolished channel activity (n = 2; *data not shown*). We did not observe activation of the 5 pS Na^+ channel in quiescent patches following application of either 1 μM or 10 μM cytochalasin D (*data not shown*).

Effects of Aldosterone on Na^+ Channel Mobility

We examined the effects of aldosterone on D_L and f of Na^+ channels to determine if modulation of the submembrane cytoskeleton associated with the Na^+ channels is involved in aldosterone-induced upregulation of Na^+ transport. A6 cell monolayers were stimulated with aldosterone for 16 hr prior to FPR to allow for the maximal change in hormonal induced Na^+ transport. Figure 4*A* illustrates a typical FPR curve for Na^+ channels under these conditions. Aldosterone did not affect D_L ($(6.5 \pm 1.64) \times 10^{-11}$ cm^2/sec) of the mobile Na^+ channels, however, it increased f approximately twofold [f = 32.31 ± 5.42% compared to control (f = 14.2 ± 0.77%) and vasopressin-treated (f = 12.7 ± 2.4%) monolayers (Fig. 7; Table)].

To determine if the aldosterone-induced increase in f is specific to the amiloride-sensitive Na^+ channels, we examined the lateral mobility and mobile fraction of Tm-Rhd-succinyl Concanavalin A (sCon A) receptors on the

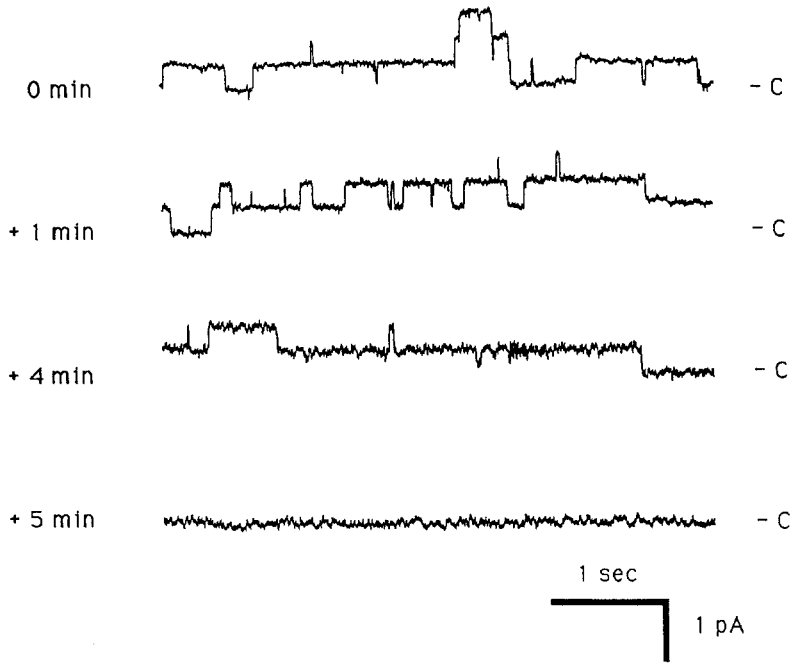

Fig. 3. Effect of 1 μM cytochalasin D on A6 cell 5 pS epithelial Na^+ channel. Representative channel recording from a cell attached patch containing 3 active channels (0 min). Channel activity within the patch is completely abolished after 5-min exposure to 1 μM cytochalasin D. Pipette holding potential was 0 mV.

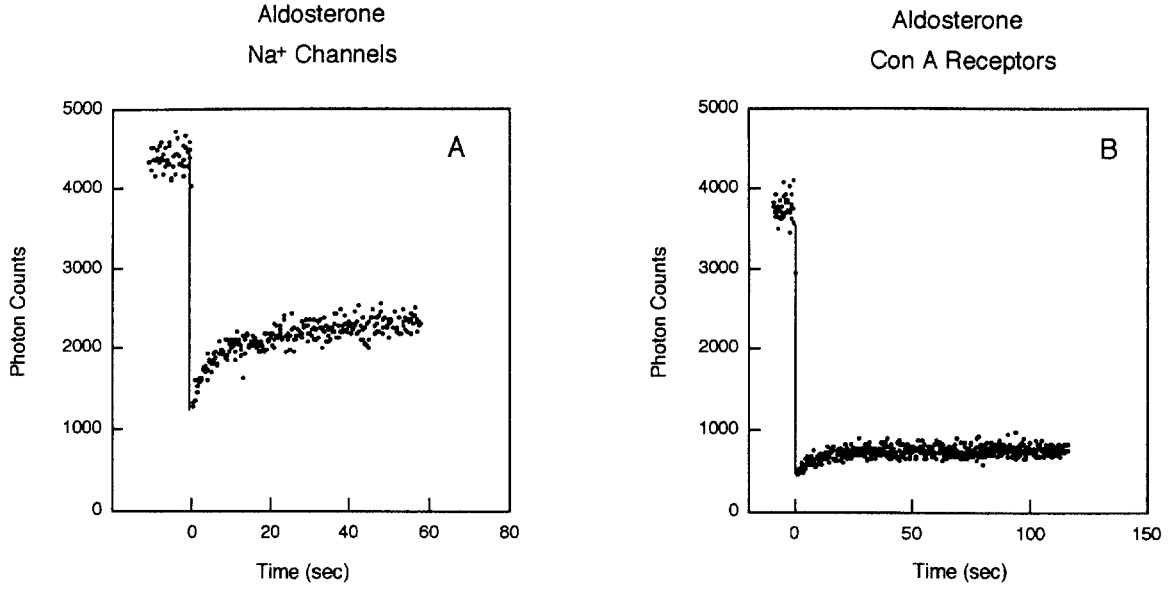

Fig. 4. Effect of aldosterone on the lateral mobility and mobile fraction of labeled Na^+ channels and sCon A receptors on the cell surface of A6 cells. (*A*) Representative FPR curve for Na^+ channels on the cell surface of an A6 cell monolayer stimulated with aldosterone for 16 hr. This curve has a diffusion coefficient of 7.7×10^{-11} cm^2/sec and a mobile fraction of 44%. Compare with Fig. 1. (*B*) Representative FPR curve for sCon A receptors on a A6 cell monolayer stimulated with aldosterone for 16 hr. Analysis of this curve yields a diffusion coefficient (D_L) of 9.81×10^{-11} cm^2/sec and a mobile fraction (f) of 10%. D_L and f of the sCon A receptors are comparable to control monolayers (*not shown*).

apical membranes of A6 cell monolayers treated with aldosterone for 16 hr. Under control conditions, sCon A receptors have limited mobility and a very low mobile fraction (10%) (*data not shown; see also* Dragsten et al. [13]). As illustrated in Fig. 4*B*, aldosterone did not effect either D_L or f of the sCon A receptors. Analysis of the FPR curves gives a D_L of $11.7 \pm 3.72 \times 10^{-11}$ cm^2/sec and an f of $7.4 \pm 1.72\%$ (n = 5) which are comparable to values from untreated monolayers.

To examine if the aldosterone-induced increase in mobile fraction is due to the insertion of newly synthesized channels into the apical membrane which are not linked to the cytoskeleton, we compared the photon counts from labeled Na^+ channels on the apical surface

Table 1. Effects of vasopressin, cytochalasin D, aldosterone, and aldosterone plus 3-deazaadenosine on the diffusion coefficients and mobile fractions of apical Na^+ channels in A6 renal epithelial cells

	Control (n = 21)	Vasopressin (20 min) (n = 13)	Cytochalasin D (n = 3)	Aldosterone (16 hr) (n = 22)	Aldosterone + 3-deazaadenosine (16 hr) (n = 6)
Diffusion coefficient (10^{-11} cm^2/sec)	9.9 ± 1.21*	5.9 ± 1.56	8.2 ± 2.4	6.5 ± 1.64	11.7 ± 4.69
Mobile fraction (%)	14.2 ± 1.2	12.7 ± 2.4	7.3 ± 2.18	32.3 ± 5.42	15.0 ± 1.14

* (SEM).

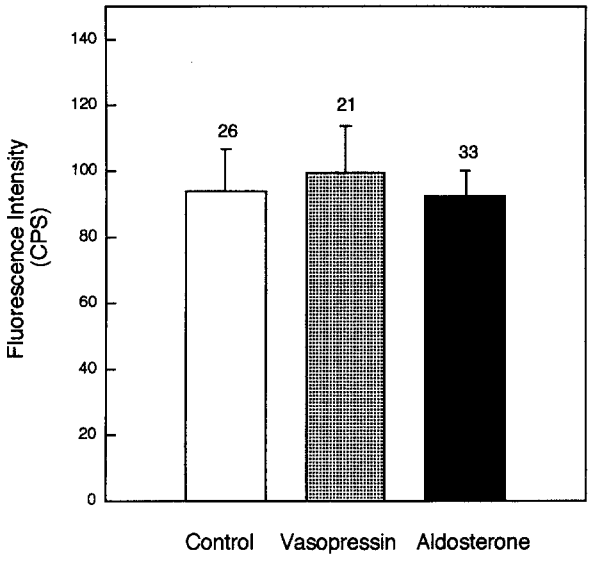

Fig. 5. Comparison of photon counts emitted from fluorescently labeled Na^+ channels on the apical surfaces of control (untreated), vasopressin (20 min), and aldosterone (16 hr) treated A6 cell monolayers. The number of measurements is shown above each error bar (SEM). CPS-photon counts per second.

of aldosterone-stimulated monolayers with the photon counts from labeled Na^+ channels on vasopressin-treated and control monolayers. As shown in Fig. 5, there was no significant difference in the photon counts between control, vasopressin, and aldosterone-treated monolayers. These data suggest that the aldosterone-induced increase in mobile fraction is not due to the insertion of newly synthesized Na^+ channels into the apical membrane.

Recent evidence suggests that aldosterone may activate Na^+ channels via methylation of the channel or an associated regulatory protein [4, 25, 27, 42]. We, therefore, asked if the methylation inhibitor 3-deazaadenosine would prevent the aldosterone-induced increase in mobile fraction of Na^+ channels. A6 monolayers were incubated in aldosterone plus 3-deazaadenosine (300 μm) for 16 hr prior to FPR. It has previously been demonstrated in TB-6c cells that there is a comparable inhibition of aldosterone-stimulated Na^+ transport following a 6-, 12- or 18-hr incubation in aldosterone plus 300 μm 3-deazaadenosine [50]. A typical recovery curve for an A6 monolayer treated with aldosterone in the presence of 3-deazaadenosine for 16 hr is shown in Fig. 6. D_L and f are (11.7 ± 4.69) × 10^{-11} cm^2/sec and 15 ± 1.14%, respectively, which are comparable to control values (Fig. 7; Table).

Figure 7 compares D_L of labeled Na^+ channels on the cell surfaces of control monolayers with monolayers treated with vasopressin, cytochalasin D, aldosterone, or aldosterone plus 3-deazaadenosine. As shown, only aldosterone treatment resulted in a statistically significant increase ($P < .05$) in the mobile fraction of Na^+ channels when compared to control monolayers.

Discussion

The actin component of the cytoskeleton has been suggested to play a role in the regulation of renal Na^+, K^+, and Cl^- channels [10, 37, 38, 43, 47, 49], neuronal voltage dependent Ca^{2+} channels [26], and hippocampal *N*-methyl-*D*-aspartate receptor channels [39]. Using patch clamp methodology, these studies have shown that disruption of actin through cytochalasins B and D, either results in the activation or inactivation of these channels. Although these studies provide compelling evidence for submembrane actin being a component of the regulatory pathway of these channels, a direct interaction between actin and ion channels has not been demonstrated. Rather ion channels, such as voltage-dependent sodium channels and epithelial Na^+ channels have been shown to be linked to the membrane cytoskeleton through ankyrin and spectrin, or through spectrin alone [*see* 3]. Although channel-membrane cytoskeleton linkages are involved in maintaining the distribution of channels within distinct membrane domains, it is unknown if they are also involved in regulating channel activity. We have previously demonstrated that epithelial Na^+ channels expressed in bovine papillary collecting ducts and A6 renal epithelial cells are linked to the spectrin-based membrane cytoskeleton and that this association severely restricts the lateral mobility of the Na^+ channels [45]. In this study, we have used the technique of FPR to gain

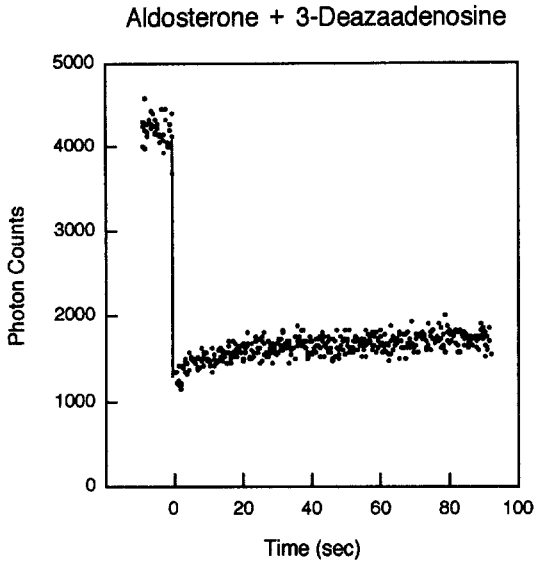

Fig. 6. Effect of the methylation blocker, 3-deazaadenosine, on the aldosterone-induced increase in mobile fraction of Na^+ channels on the surface of A6 cells. A6 monolayers were incubated in aldosterone plus 3-deazaadenosine (300 μM) for 16 hr prior to FPR analysis. This representative curve has a diffusion coefficient of 9.53×10^{-11} cm^2/sec and a mobile fraction of 15%.

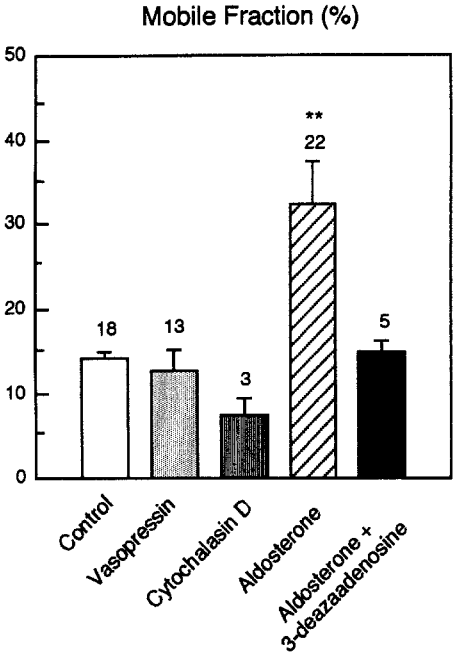

Fig. 7. Comparison of the effects of vasopressin, cytochalasin D, aldosterone, and aldosterone plus 3-deazaadenosine, on the mobilities of labeled Na^+ channels on the apical surface of A6 monolayers. Control monolayers did not receive treatment. Mobile fractions were determined by curve-fitting procedures (2). The number of measurements is shown above each error bar (SEM); **significantly different ($P < .05$) from control (unpaired t-test).

insight as to whether the linkage between the epithelial Na^+ channels and the membrane cytoskeleton is involved in the vasopressin and aldosterone-induced upregulation of Na^+ transport across A6 cell monolayers.

The FPR technique has been widely used to measure the lateral mobility of membrane proteins [52], including a variety of membrane transport proteins [1, 11, 32, 35]. In the FPR technique, the signal from fluorescently labeled membrane proteins is depleted within a defined spot on the cell surface by photobleaching with a brief, intense laser pulse [52]. Typically, the membrane proteins are labeled with fluorescently conjugated antibodies [52]. Analysis of the kinetics of fluorescence recovery into the bleached area gives a diffusion coefficient for the labeled protein and a mobile fraction which represents the percentage of unbleached molecules which diffuse into the bleached area during the time course of the experiment [52]. Recently, single particle tracking (SPT) microscopy has been used to examine the lateral mobility of membrane proteins. In contrast to FPR, which represents the average measurement calculated from hundreds to thousands of labeled molecules in the micrometer-sized bleached region, SPT follows the movements of individual or small groups of molecules labeled with gold conjugated antibodies over nanometer distances on the cell surface [52]. Direct comparisons of SPT and FPR measurements of membrane proteins indicate that FPR yields a smaller diffusion coefficient and a larger immobile fraction than SPT [30, 52]. The observed differences between the two techniques have been equated to the differences in scale, with FPR determining micron scale diffusion coefficients and SPT determining nanometer scale diffusion coefficients [52]. It has been proposed that the immobile fraction recognized by FPR may include a subpopulation of proteins undergoing constrained slow percolative diffusion [52]. Thus, in the present study we cannot unequivocally discern between labeled Na^+ channels which are immobile and labeled Na^+ channels were are undergoing slow percolative diffusion.

Vasopressin and Na^+ Channel Lateral Mobility

Two hypotheses have been proposed to explain the vasopressin-stimulated increase in sodium transport across high electrical resistance Na^+ reabsorbing renal epithelia. Because the vasopressin-induced increase in Na^+ transport is correlated with an increase in adenylate cyclase activity and intracellular cAMP levels, it has been hypothesized that cAMP dependent protein kinase (protein kinase A) activated quiescent Na^+ channels within the apical membrane through the phosphorylation of a channel subunit [*see* 5]. The alternative hypothesis proposes that vasopressin may cause the insertion of new channels

or a regulatory protein into the apical membrane from an intracellular pool [*see* 5]. Recent studies have presented evidence supporting both hypotheses, thereby suggesting that both mechanisms may contribute to vasopressin induced upregulation of transepithelial Na^+ transport [29, 33].

Prat and coworkers [37, 38] have presented data demonstrating that vasopressin induces a PKA-mediated phosphorylation of actin which results in actin depolymerization and activation of a 9 pS amiloride-sensitive Na^+ channel expressed in coverslip grown A6 cells. Disruption of the endogenous actin by pretreatment with cytochalasin D prevents the PKA-mediated activation of this Na^+ channel. These authors postulated that the observed effects of actin on activity of the 9 pS channel were mediated through membrane cytoskeletal proteins directly associated with the channel [37].

Vasopressin has also been shown to induce a transient depolymerization of apical actin in both the toad urinary bladder and the inner medullary collecting duct that may permit the fusion of vesicles containing water channels with the apical membrane [12, 15, 44]. This actin pool is also sensitive to cytochalasin D [15]. Simon et al. [44] have used immunogold electron microscopy to demonstrate that in response to vasopressin there is a significant reduction in actin in both the intervillar spaces and the microvilli of the principal cells of the rat inner medullary collecting duct in response to vasopressin. Vasopressin also alters the distribution of spectrin and ankyrin associated with the apical membranes [17, 18]. Using immunogold electron microscopy, vasopressin treatment was shown to decrease the amount of ankyrin and spectrin associated with the intervillar membranes. However, no significant change in the density of either of these proteins within the microvilli, the sites of Na^+ channel localization [45], were observed [17, 18].

In the present FPR study, we found that vasopressin did not significantly increase the mobile fraction or rate of lateral mobility of Na^+ channels on the apical surface of filter grown A6 cell monolayers. We interpret this observation to mean that the interaction between the spectrin-based membrane cytoskeleton and Na^+ channels is not directly involved in the natriuretic response of vasopressin. Our findings are corroborated by Qao and coworkers [17, 18] who report that vasopressin, at least at the electron microscopic level, does not alter the distribution of spectrin and ankyrin within the microvilli of Na^+ reabsorbing renal epithelia.

Is actin involved in the natriuretic response to vasopressin? The lack of effects of cytochalasin D and vasopressin on Na^+ channel mobility strongly suggest that actin is not directly associated with the Na^+ channels and does not play a direct role in restricting their mobility in A6 cells. Cytochalasins have been shown to have no effect on the lateral mobility or mobile fraction of transport proteins linked directly to ankyrin and spectrin, such as band 3 [19] and the voltage-dependent Na^+ channel [1]. Because actin is not directly involved in restricting channel mobility, FPR does not allow us to determine if actin is directly involved in vasopressin-mediated upregulation of Na^+ channel activity in filter grown A6 cells. However, our patch clamp analysis has demonstrated that the actin component of the cytoskeleton is involved in the modulation of a 5 pS, highly selective Na^+ channel expressed in filter grown A6 cells. In contrast to the findings of Cantiello et al. [10] for the 9 pS, moderately selective Na^+ channel expressed in coverslip grown A6 cells, cytochalasin D was found to inactivate the 5 pS channel.

At present, it is unclear as to whether the 9 pS channel and the 5 pS channel are distinct entities or if they represent different manifestations of the same channel under different growth conditions. However, because the 5 pS channel is expressed in native renal epithelia, it is thought to be the principal Na^+ channel involved in Na^+ reabsorption by the kidney [36]. Our data suggest that the role of actin in channel regulation differs between the 9 pS and 5 pS channels. It is thus conceivable that actin is not directly involved in the activation of the 5 pS channel by vasopressin. Rather vasopressin-mediated activation of the 5 pS channel may be through a PKA-catalyzed phosphorylation of an accessory protein associated with the channel, as demonstrated by Oh et al. [33] for the renal high amiloride affinity epithelial Na^+ channel biochemically purified from A6 cells and bovine papillary collecting ducts. Interestingly, apical K^+ secretory channels in the principal cells of the mammalian collecting duct, which are activated by PKA, are also inhibited by depolymerization of actin by cytochalasin D [49]. This is consistent with the idea proposed by Oh et al. [33], that regulation of apically situated Na^+ reabsorptive and K^+ secretory channels in renal principal cells can be modulated simultaneously by common signaling pathways.

Aldosterone and Na^+ Channel Lateral Mobility

Aldosterone increases transepithelial Na^+ transport 2–4-fold across Na^+ reabsorbing renal epithelia. The aldosterone increase in Na^+ permeability has been postulated to be due to the synthesis and/or the insertion of new Na^+ channels or a regulatory subunit into the apical membrane or, to the activation of quiescent channels preexisting in the apical membrane [*see* 5, 16 for review]. Recent biochemical [28] and electrophysiological studies [25, 27] have provided support for the latter hypothesis. However, the mechanism by which aldosterone activates quiescent channels is unclear. In this report, we show that stimulation with aldosterone increases the mobile fraction of Na^+ channels on the apical surface of A6 cells 2-fold when compared to untreated control and vaso-

pressin-stimulated monolayers. Aldosterone was not found to alter the diffusion coefficient of the mobile Na^+ channels. This 2-fold increase in mobile fraction of Na^+ channels corresponds to the average increase in Na^+ transport in response to aldosterone in A6 cell monolayers [51], indicating that the increase in mobile fraction is correlated with the increase in Na^+ transport. It is possible that release of the Na^+ channels from the lateral constraints imposed by the spectrin-based membrane cytoskeleton activates channels, or that recruitment to specific domains on the cell surface serves to promote channel activity.

We interpret the increased mobile fraction of Na^+ channels as resulting from aldosterone-mediated changes in either the affinity of the Na^+ channel for the membrane cytoskeleton or in the spectrin-based membrane cytoskeleton itself. Although epithelial Na^+ channels in A6 cells are linked directly to the spectrin-based membrane cytoskeleton [45], it is possible that the membrane cytoskeleton also forms a corral-like barrier that restricts the mobility of the channels. This corral-like barrier may be modulated by aldosterone. As pointed out by Edidin and Stroynowski [14], bleaching of a membrane protein in a spot greater than the dimension of the barrier formed by the spectrin-based membrane cytoskeleton would make the protein appear immobile. The experiments presented in this report cannot unequivocally allow us to determine whether the aldosterone-associated increase in mobile fraction of Na^+ channels is due to an altered affinity of the Na^+ channels for the membrane cytoskeleton or if aldosterone alters a barrier to channel mobility imposed by the membrane cytoskeleton. Nevertheless, they do indicate that the spectrin-based membrane may be involved in aldosterone mediated upregulation of Na^+ channels.

The A6 cell monolayers in this study were stimulated with aldosterone for 16 hr to achieve maximal rates of Na^+ transport. Because aldosterone induces protein synthesis [16], it is conceivable that the increase in mobile fraction of Na^+ channels is due to the insertion of newly synthesized channels into the apical membrane that are not linked to the membrane cytoskeleton. However, two lines of evidence argue against this interpretation: (i) we have been unable to detect a significant difference in the binding of fluorescently labeled anti-Na^+ channel antibodies, as determined by photon counting, between control, vasopressin and aldosterone-treated monolayers (Fig. 5) and [2] Kleyman and coworkers [28] have demonstrated biochemically that aldosterone does not alter the apical expression of epithelial Na^+ channels in A6 cells following 16 hr of stimulation with aldosterone.

An alternative explanation for the increase in mobile fraction of Na^+ channels is that it is the result of an aldosterone-mediated modification of the apical membrane lipid composition. It has been demonstrated, in the toad urinary bladder, that aldosterone stimulates fatty acid synthesis and phospholipid deacylation, and that these modifications in the membrane phospholipids are involved in the aldosterone mediated increase in Na^+ transport [20, 31]. If the increase in mobile fraction of the labeled Na^+ channels was due to a modification of the membrane lipid environment, then we would predict that other transmembrane proteins should exhibit similar increases in their mobile fraction. To test this, we examined the effect of aldosterone on the mobility of labeled sCon A receptors. sCon A receptors have restricted rates of lateral mobility and small mobile fractions (10%) on the apical surfaces of A6 cell monolayers [this study; 13]. Following aldosterone treatment, we did not observe an increase in the mobile fraction of fluorescently labeled sCon A receptors. This indicates that the increase in mobile fraction is specific to the Na^+ channels and that it is due to aldosterone-associated changes in the cytoskeleton and/or affinity of the Na^+ channels for the membrane cytoskeleton, rather than changes in the membrane lipid composition.

In light of increasing evidence that suggests that methylation of the Na^+ channel is the mechanism whereby aldosterone activates Na^+ channels [4, 25, 27, 41, 42], we examined whether the methylation blocker 3-deazaadenosine would inhibit the increased mobile fraction of Na^+ channels associated with aldosterone. 3-Deazaadenosine was found to prevent the statistically significant increase in mobile fraction associated with aldosterone. Although not statistically significant, the mobile fraction of Na^+ channels was slightly higher in the 3-deazaadenosine plus aldosterone monolayers ($f = 15 \pm 1.4\%$) when compared to either control ($f = 14.2 \pm 0.77\%$) or vasopressin treated ($f = 12.7 \pm 2.4\%$) monolayers. Although we interpret these data to mean that a methylation event is involved in the aldosterone-associated increase in mobile fraction of Na^+ channels in A6 cells, one potential caveat of these experiments is that 3-deazaadenosine may also have altered DNA transcription, because methylation of histone proteins is required for transcription [27]. Further experiments are necessary to elucidate the biochemical mechanisms underlying the aldosterone-associated increase in mobile fraction of Na^+ channels that we have observed.

In summary, our FPR results reveal that there is a 2-fold increase in the mobile fraction of Na^+ channels on the apical membrane of aldosterone-treated A6 cell monolayers when compared to control and vasopressin-treated monolayers. This 2-fold increase in mobile fraction corresponds to the average increase in Na^+ transport in response to aldosterone. We interpret the increased mobile fraction of Na^+ channels to be a result of the aldosterone associated changes in the spectrin-based membrane cytoskeleton and/or the affinity of the Na^+ channels for the membrane cytoskeleton. We propose that the membrane cytoskeleton is involved in the aldo-

sterone-mediated activation of epithelial Na^+ channels in A6 cells.

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