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Role of F-Actin in the Activation of Na^+-K^+-Cl^- Cotransport by Forskolin and Vasopressin in Mouse Kidney Cultured Thick Ascending Limb Cells

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Abstract. The influence of microtubules and F-actin on Na^+-K^+-Cl^- cotransport was investigated in cultured cells derived from outer-medullary thick ascending limb tubules microdissected from the mouse kidney. The cultured cells contained Tamm-Horsfall protein, produced cAMP in response to dD-arginine vasopressin (dD-AVP), isoproterenol, prostaglandin E_2 and forskolin (FK), and exhibited an ouabain-resistant furosemide-sensitive (Or-Fs) component of $^{86}Rb^+$ influx mediated by the Na^+-K^+-Cl^- cotransporter. Both FK and dD-AVP stimulated the Or-Fs component of Rb^+ influx. Neither agent altered the tubulin and cytokeratin networks nor the shape of the tight junction using a specific anti-ZO-1 antibody. In contrast, they did induce a marked redistribution of F-actin to the periphery of the cells delineating the tight junctions. Preincubation of the cells with nocodazole, to disrupt microtubules, did not alter the FK- or dD-AVP-elicited Or-Fs Rb^+ influx. In contrast, phalloidin and NBD-phallicidin, which stabilize F-actin, markedly impaired the stimulation of Na^+-K^+-Cl^- cotransport by FK or dD-AVP, without affecting the Na^+-K^+ ATPase pumps and the rate constant of $^{36}Cl^-$ and $^{86}Rb^+$ efflux. These results strongly suggested that cAMP-stimulated Na^+-K^+-Cl^- cotransport is linked to F-actin in renal TAL cells.

Key words: Cytoskeleton — F-actin — Microtubules — Potassium transport — cAMP — Kidney

Introduction

The electroneutral cotransport of Na^+-K^+-Cl^- across the apical membrane domain of the renal thick ascending limb (TAL) cells of the loop of Henle is responsible for much of the transfer of chloride from the lumen to the basolateral side of the cells (Burg & Green, 1973; Rocha & Kokko, 1973). The cotransport activity that is sensitive to the so-called "loop" diuretics, furosemide and bumetanide, in several nonepithelial and epithelial cells, is modulated by a variety of effectors and hormones acting through the adenylcyclase cascade (for review, *see* Greger, 1985; O'Grady, Palfrey & Field, 1987).

The reabsorption of NaCl in renal TAL tubules can be stimulated by several hormones, particularly vasopressin (Hebert, Culpepper & Andreoli, 1981; Hebert, Friedman & Andreoli, 1984; Molony et al., 1987). However, the way this cAMP-dependent polypeptide hormone acts is still a matter of debate. The increase in NaCl reabsorption may be due to the activation of a chloride conductance followed by activation of the Na^+-K^+-Cl^- cotransport (Schlatter & Greger, 1985). Apical K^+ conductances also play a role in the function of the cotransporter (Greger & Schlatter, 1981), and some studies suggest that the hormonal stimulation of chloride reabsorption results in an increase in the recycling of K^+ across the apical membrane (Hebert et al., 1984; Molony et al., 1987; Molony, Reeves & Andreoli, 1989).

A link between the Na^+-K^+-Cl^- cotransport and cytoskeletal elements has been proposed. O'Grady et al. (1987) and Molony et al. (1987) suggested that microtubules and/or microfilaments may play a role in the transport of quiescent or newly synthesized cotransport molecules to the plasma membrane. More recently, Matthews, Awtrey and Madara (1992) used cultured human intestinal T_{84} cells to demonstrate that cAMP-elicited stimulation of Cl^- secretion was paralleled by a striking redistribution of F-actin. Moreover, stabilization of F-actin by phalloidin or the phalloidin derivative, NBD-conjugated phallicidin (Barak, Yocum & Webb, 1981), prevented the forskolin-stimulated component of rubid-

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ium influx mediated by the Na^+-K^+-Cl^- cotransport in T_{84} cells (Matthews et al., 1992), which has been shown to be on the basolateral membrane (Dharmsathaphorn et al., 1985). Thus, these results strongly suggest that the cotransport is functionally linked to elements of the cytoskeleton in intestinal cells. This association raised two questions. First, whether cAMP-dependent-stimulated Na^+-K^+-Cl^- cotransport activity is also associated with changes in the cytoskeleton components in renal TAL cells, and second, whether microfilaments and/or microtubules play a direct role in the activity of the cotransporter in this particular chloride-absorbing epithelium.

The present study investigates the effects of two activators of cAMP, forskolin (FK) and dD-arginine vasopressin (dD-AVP), on Na^+-K^+-Cl^- cotransport and cytoskeletal elements in cultured TAL cells derived from microdissected tubule of mouse kidney. The ouabain-resistant furosemide-sensitive (Or-Fs) component of rubidium influx, reflecting Na^+-K^+-Cl^- cotransport activity, was stimulated by both agents. The changes in the rate of rubidium transport were associated with a striking intracellular redistribution of F-actin. Preloading of cells with NBD-phallicidin or phalloidin prevented the increase in the Or-Fs component of rubidium uptake from cells treated with FK and dD-AVP. In contrast, nocodazole, a nonalkaloid drug that binds to tubulin (Hoebeke, Van Nijen & De Brabander, 1976), did not alter the rate of rubidium influx.

Materials and Methods

Materials

$^{86}Rb^+$ and $^{36}Cl^-$ were purchased from Amersham (Les Ulis, France). $^{22}Na^+$ was from NEN (France). Bumetanide was a gift from Hoffman LaRoche (France). Furosemide was obtained from Hoechst laboratories (Puteaux, France); dD-AVP from Ferring Pharmaceutical (Sweden), and parathormone (1–34 synthetic bovine parathormone) from Beckman (France). Forskolin was purchased from Calbiochem (San Diego, CA). Other reagents were from Sigma Chemical (St Louis, MO). Culture media (DMEM, HAM's F12) were from Flow Laboratories (Les Ulis, France). Collagenase A was from Boehringer Mannheim (Germany). NBD-phallicidin and phalloidin were from Molecular Probes (Coger, France). The rhodamine-conjugated phalloidin was purchased from Sigma. The monoclonal mouse-anti-human Tamm-Horsfall antibody was from Cedarlane Lab. (Hornby, Canada) and the monoclonal rat anti-ZO-1 antibody from Chemicon International (Temecula, CA). The other antibodies used in this study were from Sigma. The permeable filters were Transwell-COL filters (0.4 μm pore size; Costar Europe, Badhoevedorp, The Netherlands) or Millicell-CM filters (0.4 μm pore size; Millipore Continental Water systems, Bedford, MA). The cAMP radioimmunoassay was from the Institut Pasteur (kit no. 79830, Institut Pasteur, Lyon, France).

Cell Culture

TAL segments isolated from the outer medulla by microdissection (Burg et al., 1982) were used to establish cultured cells. The kidneys were removed from normal one-month-old male mice fed a standard diet and cut into thin sections. The kidney slices were incubated for 1 hr at 37°C in culture medium (DMEM: HAM's F12, 1:1 vol/vol) supplemented with 0.1% (w/vol) collagenase (type 1, Sigma). Thereafter, TAL segments were microdissected under stereomicroscopic observation at room temperature. Isolated TAL segments (0.2 to 0.5 mm long, 2-5/well) were transferred to 24-well trays coated with rat tail collagen and 1 ml fresh medium was added to each well. The tubules were maintained in a modified culture medium (DMEM: HAM's F12, 1:1 vol/vol; 60 nM sodium selenate; 5 μg/ml transferin; 2 mM glutamine; 5 μg/ml insulin; 50 nM dexamethasone; 1 nM triiodothyronine; 10 ng/ml epidermal growth factor; 2% fetal calf serum; 20 mM HEPES, pH 7.4) at 37°C in 5% CO_2-95% air atmosphere. The primary cultures reached confluency after four weeks. Cell growth accelerated after the first passage and medium was changed every four days. After three passages, cells were routinely subcultured and the medium was changed every three days. A line of mouse TAL cells could thus be subcultured for a long time (to date, over 35 passages). In the present study, experiments were performed between the 6th and 15th passages.

cAMP content

Confluent monolayers in 12-well trays were preincubated with 0.1 mM 3-isobutyl-1-methyl xanthine (IBMX) for 30 min at 37°C, and then in 1 ml culture medium containing IBMX without or with dD-AVP (10^{-6} M), parathormone (PTH 10^{-6} M), isoproterenol (ISO 10^{-6} M), prostaglandin E_2 (PGE_2 10^{-6} M) or FK (10^{-5} M) for 7 min at 37°C. The reaction was stopped by rapidly removing the medium and adding 1 ml ice-cold 95% ethanol-5% formic acid. The supernatants were evaporated to dryness and the cAMP content was measured by radioimmunoassay kit (Cartier et al., 1993).

Electrophysiological Studies

The transepithelial resistance was measured on confluent cells (15 days after seeding) initially seeded on Millicell-CM filters precoated with collagen, using the Millicell Electrical Resistance System (ERS, Millipore, Philadelphia, PA) (Cartier et al., 1993).

Transport Studies

Confluent cells in 24-well trays were washed with 1 ml modified phosphate-buffered saline (PBS, in mM: NaCl 136; KCl 5; Na_2HPO_4 1; glucose 5; HEPES 10; pH 7.4) prewarmed to 37°C. The medium was removed and Rb^+ influx was initiated by addition of 1 ml PBS containing 2 μCi/ml $^{86}Rb^+$ (Vuillemin et al., 1992). The reaction was stopped by removing the solution and three rinses with 1 ml ice-cold 100 mM $MgCl_2$ solution. Cells were solubilized with 0.5 ml 1 M NaOH, and radioactivity was determined by scintillation counting. The K^+ influx was measured by incubating cells without (total influx) or with 0.5 mM ouabain or ouabain plus 10^{-4} M furosemide to determine the ouabain-sensitive influx (Os) mediated by the Na^+-K^+ ATPase pumps and the ouabain-resistant furosemide-sensitive influx (Or-Fs) mediated by the Na^+-K^+-Cl^- cotransport.

The effects of FK, dD-AVP and 8-bromoadenosine 3′:5′-cyclic monophosphate (8-br-cAMP) were analyzed after preincubating the cells with 1 ml modified PBS containing the compounds to be tested for 15 min at 37°C in a 5% CO_2 95% air atmosphere. Uptake was measured with 1 ml fresh PBS containing 2 μCi/ml $^{86}Rb^+$ plus the compounds to be tested. Cells were preincubated with 10 μg/ml nocodazole for 60 min to disrupt microtubules, and F-actin was stabilized by preincubating cells with 13 μM phalloidin or 0.5 μM NBD-phallicidin

for 2–3 hr at 37°C, following the procedure for T_{84} cells (Shapiro et al., 1991; Matthews et al., 1992). Cells were also preincubated with 2 or 20 μg/ml cytochalasin D (cyt-D), known to inhibit in vitro the association and dissociation of actin monomers (Cooper, 1987), for 30 min at 37°C. $^{86}Rb^+$ uptake was then measured. Results are expressed as nmol $^{86}Rb^+$/min/mg protein. Protein content was measured by the method of Lowry et al. (1951) with bovine serum albumin as standard.

$^{86}Rb^+$, $^{36}Cl^-$, and $^{22}Na^+$ influx studies were done on the same sets of cells at a constant external K^+ concentration (5 mM), as previously described (Vuillemin et al., 1992). For $^{36}Cl^-$ uptake studies, cells were incubated with 1 ml PBS containing 1.25 μCi/ml $^{36}Cl^-$, and influx was measured in the presence or absence of ouabain or ouabain-plus-furosemide. The incubation protocol was similar for $^{22}Na^+$ uptake studies, except that 0.5 mM amiloride was added to the PBS containing 1 μCi/ml $^{22}Na^+$. Influx was measured over 3 min at 37°C, during which uptake was linear with time. The reaction was stopped with 100 mM $MgCl_2$, cells were lyzed and the radioactivity was determined by scintillation counting.

The rate constants of $^{36}Cl^-$ and $^{86}Rb^+$ efflux were used to estimate the effects of FK and the influence of phalloidin on chloride and potassium efflux through Cl^- and K^+ channels (Venglarik, Bridges & Frizzell, 1990). Cl^- efflux studies were performed on confluent cells grown on filters (Transwell COL, 4.7 cm^2) to determine the rate constant of efflux from both apical and basal sides of the cells. After preincubation of the filters from both sides with 1.5 μCi/ml $^{36}Cl^-$, the cells were quickly rinsed five times with modified unlabeled PBS-$CaCl_2$ (apical side: 1 ml; basal side: 2.5 ml) and fresh PBS-$CaCl_2$ was added (apical side: 1 ml; basal side: 1.5 ml). A sample-and-replace technique was used to obtain 1 and 1.5 ml samples from both the apical and the basal sides of the filters, respectively, at 1 min intervals on which radioactivity was counted. Afterwards, filters were excised from their holders, incubated with 1 ml of 1 N NaOH overnight, to measure the residual radioactivity remaining in cell monolayers. To estimate the efflux from both apical and basal sides of the cells, rate constant calculations were made using the formula described by Vlengarik et al. (1990) with slight modifications. The rate constant of tracer efflux from the apical side was calculated as $[\ln(R_2) - \ln(R_1 + B)]/(t_2 - t_1)$. The rate constant of tracer efflux from the basal side was calculated as $[\ln(R_2) - \ln(R_1 + A)]/(t_2\text{-}t_1)$. R_x corresponds to the percent radioactivity remaining at time tx. A and B represent the percentage of total radioactivity counted in the apical and basal media between t_1 and t_2.

The rate constant of Rb^+ efflux from the apical side of the cells was also studied (Venglarik et al., 1990; Matthews et al., 1992) on confluent cells, grown in six-well plates, incubated for 2 hr with 2 μCi/ml $^{86}Rb^+$. The cells were quickly rinsed five times with 2 ml modified unlabeled PBS-$CaCl_2$ and 1 ml of fresh PBS-$CaCl_2$ was added. The same sample-and-replace technique was used to obtain 1 ml samples at 1 min intervals on which radioactivity was counted. In this case, the rate constant of Rb^+ efflux was calculated using the same formula previously described (Matthews et al., 1992). In all cases, results from treated cells were compared to untreated cells from the same filters or plastic dishes maintained in similar conditions.

Morphological and Immunocytochemical Studies

Confluent cells grown on petri dishes were examined under an inverted microscope (Axiovert 10, Zeiss, Germany) and photographed. For transmission electron microscopy, confluent cells grown on Transwell-COL filters were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7) for 2 hr at room temperature and processed (Cartier et al., 1993). Ultrathin sections from transversally oriented confluent monolayers were examined under a Philips electron microscope (EM 410).

Indirect immunofluorescence analyses were performed on confluent cells grown on glass coverslips and incubated for various times with the agents to be tested. Cells were fixed with ice-cold methanol at room temperature for 8 min, washed with PBS, and incubated first with monoclonal antibody (Mab) against Tamm-Horsfall protein, cytokeratin 8–18, ZO-1, α-tubulin or β-tubulin, or with rhodamine-conjugated phalloidin (10 μg/ml) and with biotinylated specific anti-mouse Ig followed by FITC-labeled streptavidin except for phalloidin. Each 30 min incubation step at room temperature was followed by extensive washing with PBS. Preparations were examined under a Zeiss microscope equipped with epifluorescence optics. Specimens labeled with rhodamine-conjugated phalloidin were also examined by confocal laser scanning microscopy (CLSM). The cell monolayer optically sectioned in vertical planes (x-z) were visualized by excitation at 468 nm and emitted light with a long-pass filter (590 nm) using a Leica TCS4D apparatus (Leica, Germany).

Data Analysis

Results are expressed as mean ± SE. Significant differences for paired or unpaired experiments were analyzed by Student's t-test.

Abbreviations

TAL = thick ascending limb; NBD = nitrobenzoxadiazole; DMEM = Dulbecco's modified Eagle's medium; FK = forskolin; dD-AVP = dD-arginine vasopressin; CLSM = confocal laser scanning microscopy; Or-Fs = ouabain-resistant furosemide-sensitive.

Results

Properties of Mouse TAL Cultured Cells

Cultured cells were derived from mouse kidney outer-medullary TAL tubules (Fig. 1*A*). Figure 1*B–F* illustrates the morphological characteristics of the cells. Confluent cells grown on petri dishes coated with collagen were of uniform cobblestone shape and formed domes when grown on plastic support (Fig. 1*B*). They contained Tamm-Horsfall protein, which is specific to TAL cells (Hoyer, Sisson & Vernier, 1979; Brunisholz et al., 1986), as shown by the predominant intracytoplasmic fluorescence obtained with an anti-Tamm-Horsfall Mab (Fig. 1*C*). Ultrastructural analysis of transverse sections of cells grown on porous filters indicated that cells were fully polarized. The cells in monolayer were epithelioid (Fig. 1*D*), with an apical domain bearing microvilli (Fig. 1*E*) separated from lateral infoldings by tight junctions (Fig. 1*E–F*), and developed a transepithelial resistance ranging between 75 and 258 $\Omega \cdot cm^2$ ($n = 20$).

The cellular cAMP content of cultured mouse TAL cells was significantly enhanced by dD-AVP, PGE_2, isoproterenol and forskolin (Table 1). The cells were still sensitive to hormones after 5–12 passages, as were cultured cells that had been kept frozen in liquid nitrogen. Cells that had been passaged over 12 times retained their dD-AVP-stimulated cAMP response, but response was smaller (2–3 folds).

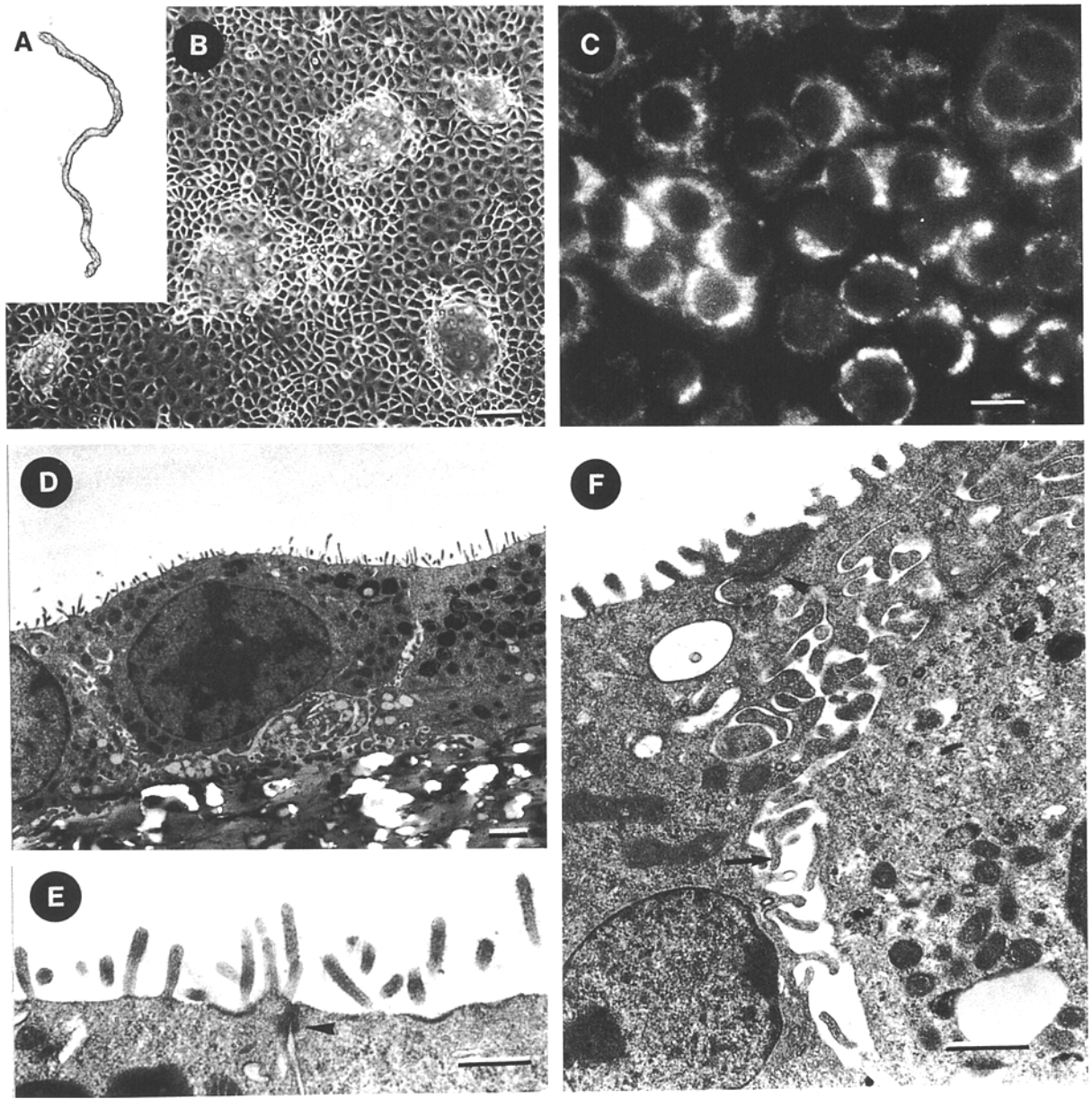

Fig. 1. Morphology of cultured mouse TAL cells. (*A*) Illustration of an outer medullary TAL microdissected tubule from which the cultured cells were derived. (*B*) Phase-contrast micrograph of confluent mouse TAL cells grown on collagen-coated plastic petri dishes. Note the presence of domes. (*C*) Indirect immunofluorescence for Tamm-Horsfall protein. Note that fluorescence is present in all cells. (*D–F*) Ultrastructure of the cells grown on a permeable filter. The cells form a regular monolayer (*D*) with numerous apical microprojections (*E*). Cells are separated by tight junctions (arrowheads, *E, F*) and have numerous lateral membrane infoldings (arrow, *F*). Bars: (*B*) 50 μm; (*C,D*) 10 μm; (*E,F*) 1 μm.

Rb^+ influx was measured to assess the Na^+-K^+-Cl^- cotransport in mouse TAL cultured cells. The influx of $^{86}Rb^+$ measured in the absence or presence of ouabain and furosemide increased with time up to 10 min and was linear for the first 3 min (Fig. 2*A*). Therefore, all further experiments used 3 min incubations. The components of rubidium influx were distinguished using specific inhibitors. Both bumetanide and furosemide inhibited the ouabain-resistant component of $^{86}Rb^+$ influx, but the former appeared to be a more potent inhibitor than furosemide (Fig. 2*B*). As shown in Table 2, ouabain inhibited the $^{86}Rb^+$ uptake by 45%, and furosemide and bumetanide inhibited the uptake by 51–52%. Ouabain plus diuretics almost completely inhibited $^{86}Rb^+$ uptake. A small fraction (12%) of Rb^+ influx was inhibited by tetraethylammonium ion (TEA), used as a K^+ channel blocking agent (Teulon et al., 1992). Results from the seven experiments given in Table 2 indicated that 49 ±

Table 1. Profile of the cAMP response to various hormones and compounds in mouse TAL cultured cells

	cAMP content (pmol/7 min/mg protein)	S/B
Basal	31.3 ± 3.0	
PTH (10^{-7} M)	34.7 ± 3.7	× 1.1
dD-AVP (10^{-6} M)	175.3 ± 27.1	× 5.6
PGE_2 (10^{-6} M)	138.4 ± 28.7	× 4.3
ISO (10^{-6} M)	525.4 ± 66.2	× 16.9
Forskolin (10^{-5} M)	715.9 ± 120.6	× 21.3

Cellular cAMP was measured on confluent cells in the absence (basal) and presence of hormones or other compounds. Values are the mean ± SE of duplicate determinations from six separate experiments. S/B represents the stimulated over basal cAMP ratio.

5% of Rb^+ uptake was mediated by ouabain-sensitive Na^+-K^+ ATPase and 40 ± 5% of the uptake was via the ouabain-resistant furosemide-sensitive component of Rb^+ influx reflecting Na^+-K^+-Cl^- cotransport activity. The stoichiometry of the cotransport was checked in uptake studies on the same sets of cultured cells at constant external K^+ concentration (5 mM). The results indicate a stoichiometry close to 1 Na^+:1 K^+:2 Cl^- [$^{22}Na^+$, 7.8 ± 1.4; $^{86}Rb^+$, 11.0 ± 1.6; $^{36}Cl^-$, 17.8 ± 1.1 nmol · min^{-1} · mg $protein^{-1}$ (n = 4)].

Stimulation of Na^+-K^+-Cl^- Cotransport by dD-AVP and Forskolin

Forskolin, used as a nonspecific adenylcyclase effector, and dD-AVP significantly enhanced cAMP production by cultured mouse TAL cells (Table 1). In addition, the transepithelial resistance from mouse TAL cells grown on filters decreases by 67% after the addition of forskolin (10^{-5} M) for 5 min (control: 203 ± 28; + FK: 68 ± 12 Ω · cm^2, n = 4). Confluent mouse TAL cells were incubated with FK (10^{-5} M) and dD-AVP (10^{-6} M) for various times before Rb^+ influx was measured to define the kinetics of Os and Or-Fs components of Rb^+ influx. Neither FK nor dD-AVP significantly altered the Os component of Rb^+ influx (Fig. 3, *upper panels*). In contrast, the Or-Fs component of Rb^+ influx was significantly increased by a 10 min incubation with either FK or dD-AVP (Fig. 3, *lower panels*). Maximal fluxes were obtained after 10–15 min. The increase in the Or-Fs component of Rb^+ influx remained at a plateau for up to 30 min when uptake was measured in the presence of 0.1 mM IBMX (Fig. 3, *lower panels*). Thus, the Na^+-K^+-Cl^- cotransport in cultured mouse TAL cultured cells was activated after cAMP stimulation by vasopressin. The action of dD-AVP was delayed as was that of calcitonin in another model of SV40-transformed rabbit cortical TAL cultured cells (Vuillemin et al., 1992).

Effects of dD-AVP and Forskolin on Cytoskeleton Elements

The influence of cAMP activators on cytokeratin, microtubules, F-actin and ZO-1 was examined by indirect immunofluorescence of cultured mouse TAL cells treated with FK or dD-AVP. All confluent cells expressed the cytokeratin revealed by an antibody directed against cytokeratin 8–18 (Fig. 4*A*). After 5 and 15 min exposure to FK (10^{-5} M), cells were enlarged, but the cytokeratin network did not appear altered (Fig. 4*B–C*). An anti-α-tubulin Mab showed generally similar patterns of fluorescence under basal condition (Fig. 4*D*) and exposure to FK (Fig. 4*E–F*). Incubation with dD-AVP gave the same results, and the pattern of fluorescence was identical after incubation with a Mab against β-tubulin (*not shown*).

Since FK induced an important decrease in transepithelial resistance from TAL cultured cells, indirect immunofluorescence were performed using a Mab directed against ZO-1, a protein associated to the tight junctions (Stevenson et al., 1986), to see whether FK altered the shape of tight junctions. In the basal state, the localization of ZO-1 was typically localized at the junctional region of the cell periphery (Fig. 5*A*). No major differences could be evidenced in the ZO-1 staining after 5 min (Fig. 5*B*) and 20 min (Fig. 5*C*) incubation with FK.

Fluorescence studies with rhodamine-conjugated phalloidin indicated that FK induced changes in the organization of F-actin (Fig. 5*D–J*). Unstimulated cells were only faintly labeled with rhodamine-conjugated phalloidin, with flocculated fluorescence around the nuclei (Fig. 5*D*). Incubation with FK for 5 min produced an intense labeling at the periphery of the cells delineating the lateral cell membrane areas (Fig. 5*F*). The same pattern of fluorescence was observed after 20 min incubation with FK, together with arrays of cytoplasmic fluorescence (Fig. 5*H*). Cells also exhibited apical labeling, which may correspond to microvillus-associated F-actin and F-actin underlying the apical membrane (Fig. 5*I*). Identical results were obtained with dD-AVP (*not shown*). CLSM analyses in the *x-z* axis showed that F-actin was randomly distributed around the nuclei of untreated cells (Fig. 5*E*). Cells treated with FK for 5 min became flattened, and the F-actin was concentrated in the regions of tight junctions (Fig. 5*G*). The same pattern of labeling was observed after 20 min exposure to FK (Fig. 5*J*). Taken together, these results confirmed that mouse cultured TAL cells expressed cytokeratin, exhibited tight junctions and that forskolin both stimulated Na^+-K^+-Cl^- cotransport and altered the distribution of F-actin.

Influence of Microtubule Disruption and Stabilization of F-Actin

The influence of the cytoskeleton on FK-activated Na^+-K^+-Cl^- cotransport was investigated on confluent mouse

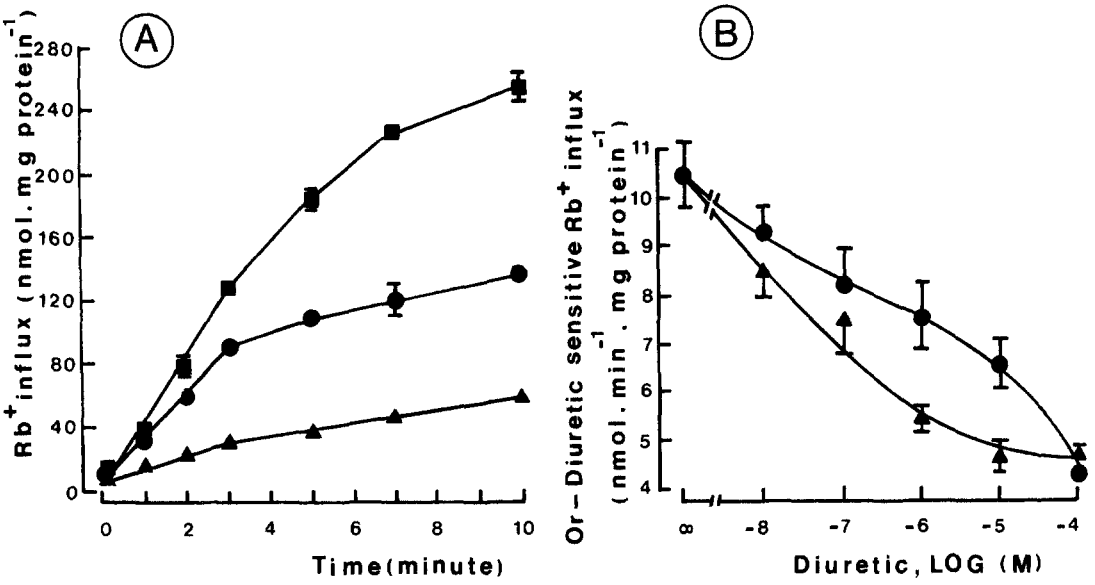

Fig. 2. Kinetics of Rb^+ influx in mouse TAL cultured cells. (*A*) Time course of Rb^+ influx performed in the absence (squares) or presence of ouabain (circles) and ouabain plus furosemide (triangles). (*B*) Dose-response inhibition of the ouabain-resistant component of Rb^+ influx by furosemide (circles) and bumatanide (triangles). Values are the mean ± SE from four (*A*) and three (*B*) experiments.

Table 2. Influence of effectors on $^{86}Rb^+$ influx in mouse TAL cultured cells

Effector	$^{86}Rb^+$ influx (nmol · min^{-1} · mg $protein^{-1}$)
Control	28.9 ± 2.6
+ Ouabain (0.5 mM)	15.9 ± 2.4
+ Furosemide (10^{-4} M)	14.1 ± 1.7
+ Bumetanide (10^{-4} M)	14.3 ± 1.9
+ Ouabain + furosemide	5.3 ± 0.4
+ Ouabain + bumetanide	2.9 ± 0.4
+ TEA (10 mM)	25.5 ± 1.9

$^{86}Rb^+$ influx was measured on confluent cells in the absence (control) or presence of various effectors or inhibitors. Values are the mean ± SE of duplicate determinations from seven separate experiments.

TAL cells preloaded with nocodazole to disrupt microtubules (Hoebeke et al., 1976), or with phalloidin and NBD-phallicidin to prevent changes in F-actin (Barak et al., 1981; Shapiro et al., 1991). Compared to untreated cells (Fig. 6*A*), incubation with 10 μg/ml nocodazole for 60 min completely disrupted the tubulin network (Fig. 6*B–C*). Incubation with FK for 5 or 20 min resulted in more intense, diffuse labeling of the rhodamine-conjugated phalloidin in TAL cells preloaded with 13 μM phalloidin (Fig. 6*E–F*) or 0.5 μM NBD-phallicidin (Fig. 6*H–I*) than in controls (Fig. 6*D,G*) and only induced a modest increase in condensation of F-actin at the lateral cell border of the NBD-phallicidin-loaded cells (Fig. 6*H–I*). Thus, as for intestinal T_{84} cells (Shapiro et al., 1991), incubation of mouse TAL cells with phalloidin or NBD-phallicidin allowed the probes to enter into cytoplasm, where they hampered the action of FK on F-actin redistribution. Electrical resistance measurements on cells grown on filters indicated that preincubation with NBD-phallicidin for 3 hr did not significantly alter the transepithelial resistance (control: 99 ± 7 Ω · cm^2; plus NBD-phallicidin; 90 ± 4 Ω · cm^2, $n = 8$), hence the probe had no major cytotoxic effect on mouse TAL cells, as it had for T_{84} cells (Shapiro et al., 1991). To test whether NBD-phallicidin could affect the levels of cellular cAMP following hormonal stimulation, cAMP was measured on TAL cells preincubated or not with 0.5 μM NBD-phallicidin. The results are illustrated in Table 3. Under these conditions, NBD-phallicidin did not significantly alter the rise in cAMP induced by dD-AVP and FK, indicating that the probe did not alter AVP receptors and the adenylcyclase enzyme. Moreover, preloading of the cells with 13 μM phalloidin did not influence the stimulatory effect of FK on cAMP content (FK: 734.9 ± 83.7; phalloidin plus FK: 682.5 ± 186.6 pmol · 7 min^{-1} · mg $protein^{-1}$, $n = 5$).

The loading conditions described above were used to study the influence of microtubules and F-actin on the kinetics of Rb^+ influx. Preincubation with nocodazole for 60 min did not alter the kinetics of Or-Fs Rb^+ influx following stimulation by FK (Fig. 7*A*) and the Os component of Rb^+ influx (FK: 12.8 ± 0.6; FK plus nocodazole: 13.7 ± 0.3 nmol · min^{-1} · mg $protein^{-1}$, $n = 6$). By contrast, preloading the cells with phalloidin or NBD-phallicidin almost completely prevented stimulation of the Or-Fs component of Rb^+ influx (Fig. 7*B*). The inhibitory effect was slightly greater with phalloidin than

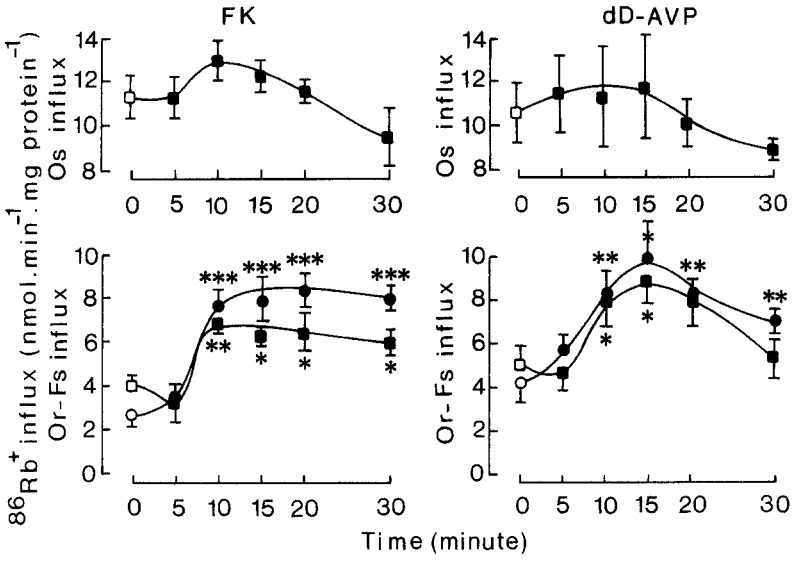

Fig. 3. Effects of forskolin and dD-AVP on ouabain-sensitive (*Os*) and ouabain-resistant furosemide-sensitive (*Or-Fs*) $^{86}Rb^+$ influx in cultured mouse TAL cells. Confluent cells were incubated (0–30 min) with 1 ml fresh medium containing 10^{-5} M FK or 10^{-6} M dD-AVP (filled symbols) in the absence (■) or presence (●) of 0.1 mM IBMX before adding $^{86}Rb^+$. Open symbols (□, ○) represent the influx measured in the absence of effectors. Values are the mean ± SE of duplicate determinations from four separate experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ represent statistical differences from control values.

NBD-phallicidin. To directly assess the effect of a non-hydrolyzable analogue of cAMP on phalloidin-treated cells, the effect of 8-br-cAMP (10^{-4} M) for 15 min on the Or-Fs component of Rb^+ influx was measured on TAL cells preloaded or not with NBD-phallicidin or phalloidin. Similar to that observed with dD-AVP and FK, both NBD-phallicidin and phalloidin prevented the stimulated rate of Rb^+ influx induced by 8-br-cAMP (control: 4.4 ± 0.2; 8-br-cAMP: 10.7 ± 0.7, $P < 0.001$ *vs.* control; 8-br-cAMP plus NBD-phallicidin: 6.0 ± 0.2; 8-br-cAMP plus phalloidin: 4.7 ± 0.4 nmol · min^{-1} · mg $protein^{-1}$, $n = 4$). Again the inhibitory action was more pronounced with phalloidin than NBD-phallicidin. Preloading the cells with phalloidin reduced by half the V_{max} of Na^+-K^+-Cl^- cotransport, from 13.2 ± 1.7 to 7.9 ± 0.5 nmol · min^{-1} · mg $protein^{-1}$, without affecting the K_m for K^+ (FK: 2.6 ± 0.5 mM; FK plus phalloidin: 2.1 ± 0.5 mM, $n = 4$) when Rb^+ influx was measured with various concentrations of K^+ from 1 to 20 mM in the incubation medium after 15 min exposure to FK. Taken together, these results confirmed the link existing between cAMP and F-actin reordering (Lamb et al., 1988; Shapiro et al., 1991).

One can question whether other agents affecting the depolymerization-polymerization of actin such as cytochalasin D (Cooper, 1987) could also directly influence the electroneutral Na^+-K^+-Cl^- cotransport activity. To test this hypothesis, Rb^+ influx was measured on TAL cells preloaded with 2 μg/ml or 20 μg/ml cytochalasin D (cyt-D) for 30 min at 37°C. At both concentrations, cyt-D significantly enhanced ($P < 0.01$) the rate of the Or-Fs component of Rb^+ influx (control: 4.9 ± 0.4; 2 μg/ml cyt-D: 10.8 ± 0.4; 20 μg/ml cyt-D: 8.3 ± 0.5 nmol · min^{-1} · mg $protein^{-1}$, $n = 6$). Thus, these results showed that cyt-D can mimic the effect of dD-AVP and FK and supported that reordering of F-actin may directly affect the Na^+-K^+-Cl^- cotransport activity.

The capacity of phalloidin to interfere with Na^+-K^+ ATPase pumps was tested by measuring the Os and Or-Fs components of Rb^+ influx in the same sets of cells incubated in different conditions. Phalloidin preloading did not significantly affect the Os component of Rb^+ influx, but reduced the FK- and dD-AVP-stimulated rates of the Or-Fs component of Rb^+ influx by 31.5 and 49%, respectively, to almost basal state values (Fig. 8). Thus, the stabilization of F-actin with phalloidin and NBD-phallicidin impaired the cAMP-activated Na^+-K^+-Cl^- cotransport activity without altering the Na^+-K^+ ATPase pumps.

Previous studies indicated that the activation of basolateral chloride conductance preceded the activation of the Na^+-K^+-Cl^- cotransport in TAL tubules (Schlatter & Greger, 1985). We showed that apical K^+ recycling through K^+ conductance could regulate the cAMP-stimulated rates of rubidium influx mediated by the cotransport activity in SV40-transformed TAL rabbit kidney cells (Vuillemin et al., 1992). We therefore examined whether phalloidin altered the rates of Cl^- and Rb^+ efflux of mouse TAL cells exposed to FK. To analyze the basolateral efflux of chloride, experiments were performed on cells grown on filters. Neither FK nor phalloidin modified the rate constant of $^{36}Cl^-$ efflux from the apical side of the cells (Fig. 9*A*). The rate constant for apical $^{36}Cl^-$ efflux remained constant and even decreased after addition of FK. As already emphasized by Venglarik et al. (1990), this result could be consistent with depletion of an extracellular compartment resulting, in the case of mouse TAL cells, from a trapping of Cl^- at the level of apical microvilli (*see* Fig. 1). In contrast, FK induced an increase in the rate constant of $^{36}Cl^-$ efflux from the basal side of the cells with maximal values achieved after 2–6 min exposure to FK (Fig. 9*B*). Phalloidin neither affected the rate constant of $^{36}Cl^-$ efflux

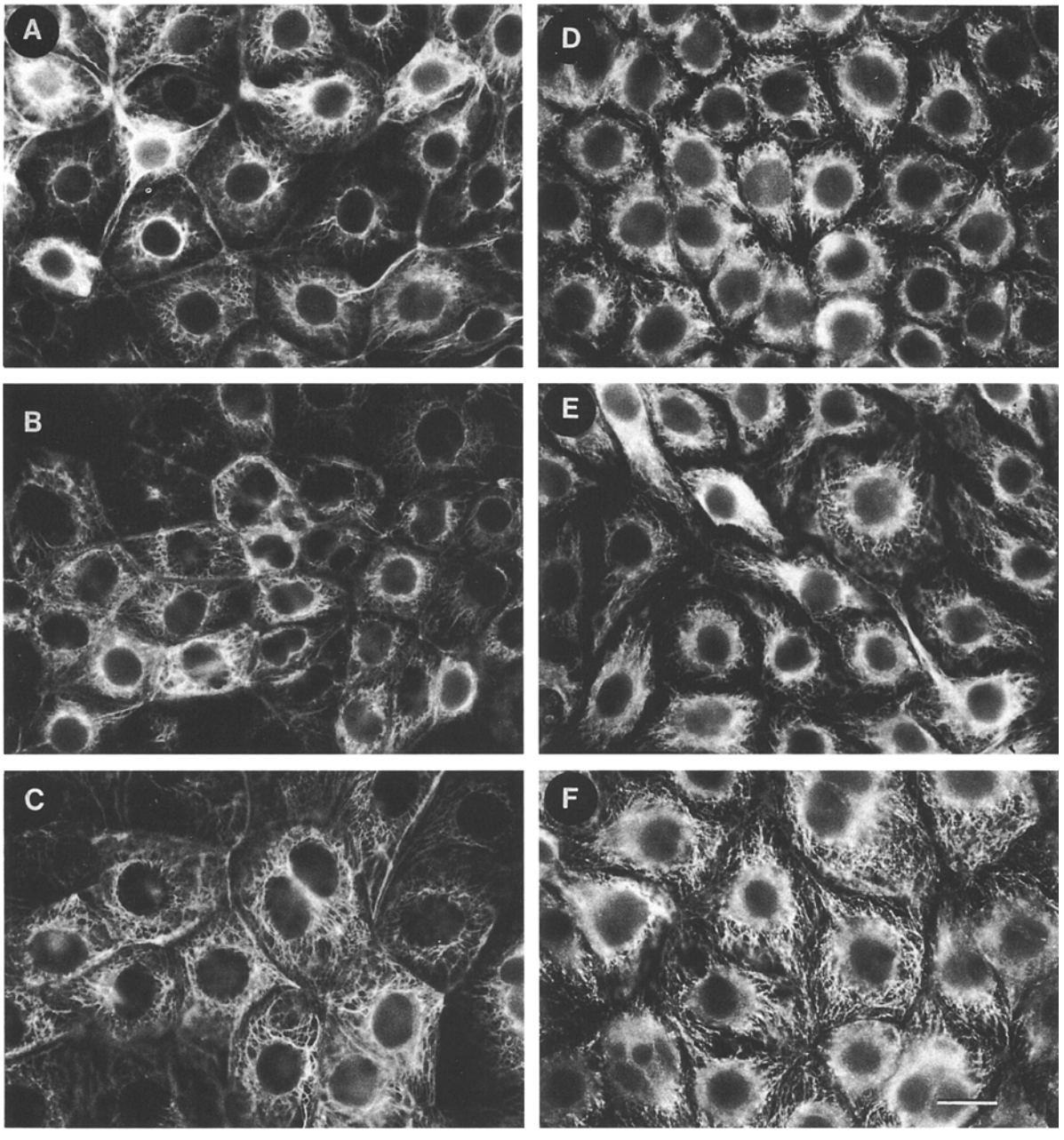

Fig. 4. Influence of forskolin on cytokeratin and microtubules in cultured mouse TAL cells. Confluent cells were incubated in medium without (*A, D*) or with 10^{-5} M FK for 5 min (*B, E*) and 20 min (*C, F*) at 37°C. Cells were then fixed with methanol, rinsed and incubated with Mabs against cytokeratin 8–18 (*A–C*) and α-tubulin (*D–F*). There were no major differences in the labeling of cytokeratin and α-tubulin in untreated (*A, D*) and FK-treated cells (*B, C, E, F*) except that FK-treated cells were enlarged after 20 min (*C, F*). Bar: 10 μm.

nor impaired the effect of FK on this parameter. These results indicated that FK induced an increase in Cl^- efflux from the basal side of the cells which preceded the observed increase in the ouabain-resistant furosemide-sensitive component of Rb^+ uptake mediated by the Na^+-K^+-Cl^- cotransporter (*see* Fig. 3). Similar to that previously reported by Schlatter and Greger (1985) on isolated perfused medullary thick ascending limbs from the mouse kidney, FK had no significant effect on the rate constant of $^{86}Rb^+$ efflux (Fig. 9*C*). Moreover, phalloidin did not affect the rate constant of $^{86}Rb^+$ efflux from cultured mouse TAL cells grown on plastic, under either basal conditions or after incubation with forskolin (Fig. 9*C*). These results were in agreement with data on T_{84}

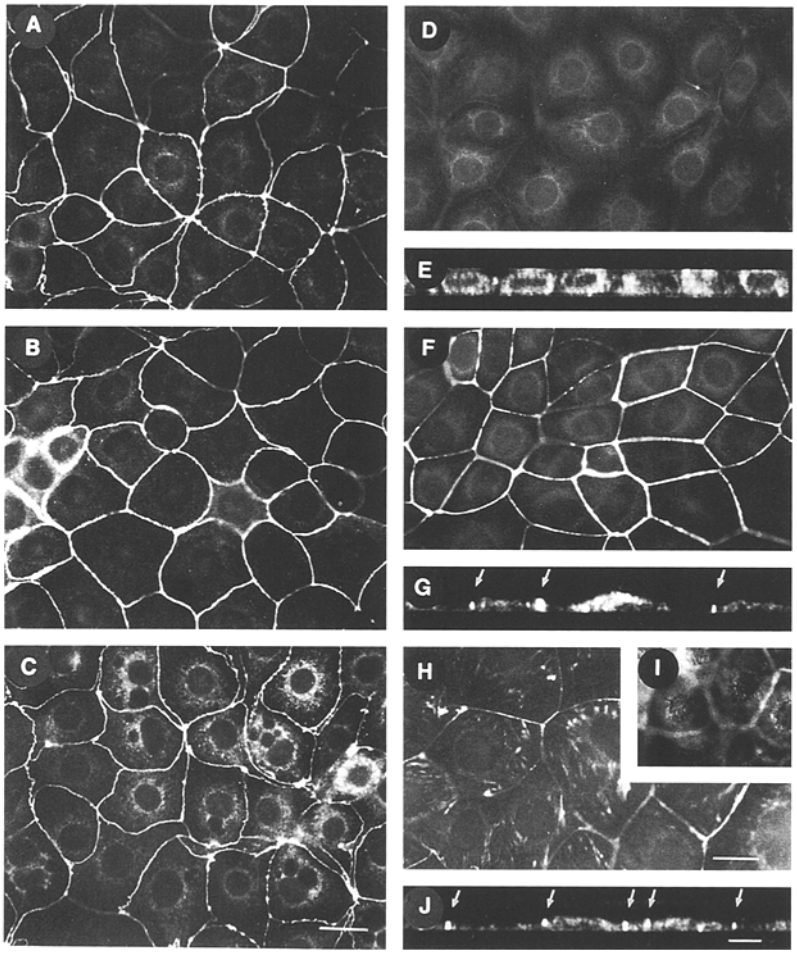

Fig. 5. Influence of forskolin on distribution of ZO-1 and F-actin in cultured mouse TAL cells. Confluent cells were incubated in medium without (*A, D, E*) or with 10^{-5} M FK for 5 min (*B, F, G*) and 20 min (*C, H–J*) at 37°C. Untreated cells exhibited a regular ZO-1 staining at the periphery of the cells (*A*). No differences could be detected at the optical level after incubation with FK for 5 min (*B*) and 20 min (*C*). On cells fixed with methanol, the labeling of F-actin is weak and mainly around the nuclei in untreated cells (*D*), whereas a 5 min incubation with FK caused intense labeling at the periphery of the cells (*F*). The pattern of fluorescence was similar after 20 min, with arrays of cytoplasmic labeling (*H*) and a fine apical staining in some, but not all, cells (*I*). CLSM analysis (*x-z* sections) of untreated cells showed that F-actin is distributed around the nuclei (*E*). Incubation with FK for 5 min (*G*) and 20 min (*J*) caused the cells to flatten and have intense labeling near the junctions (arrows). Bars: 10 μm.

cells and confirmed the lack of nonspecific toxic effects of micromolar concentrations of phalloidin (Matthews et al., 1992). They also suggested that stabilization of F-actin did not interfere with either the basolateral Cl^- or the apical K^+ membrane conductances.

Discussion

Cultured TAL cells retain the principal morphological and functional features of the parental mTAL cells and appear polarized. The evidence for the presence of the cotransporter system in these cells is the inhibition of Rb^+ influx by bumetanide and furosemide and the apparent stoichiometry of about 1 Na^+:1 K^+:2 Cl^- for the ouabain-resistant furosemide-sensitive influx in the absence of any effectors. The rates of stimulated Or-Fs components of Rb^+ influx by FK and dD-AVP were also close to that measured with calcitonin on rabbit SV40-transformed RC-SV2 cells having features of the cortical TAL cells (Vuillemin et al., 1992). Unlike a previous study of Sun et al. (1991), which showed that AVP switched a Na^+-Cl^- into Na^+-K^+-Cl^- cotransport in mouse medullary TAL cells, we found no differences in the apparent stoichiometry of the cotransporter measured either in basal state or AVP-stimulated conditions. This apparent discrepancy could be simply due to the Na^+-K^+-Cl^- cotransporter being present in many growing nonepithelial and epithelial cells (*see, for review,* O'Grady et al., 1987) and appearing to be dependent on the growth status of the cells (Paris & Pouyssegur, 1986; Vandewalle et al., 1993).

There are now several lines of evidence indicating that the actin-based cytoskeleton plays an important role in cell polarity and influences ion transport processes (Duffey et al., 1981; Madara et al., 1988; Cantiello et al., 1991; Prat et al., 1993). Many studies have been devoted to the effects of stimulated cAMP on ionic fluxes in TAL tubules, but the role of the cytoskeleton has not been investigated. Such studies have been performed on T_{84} cells that have features of intestinal cells (Shapiro et al., 1991; Matthews et al., 1992). In these cells, the cAMP-elicited Cl^- secretion can be inhibited by stabilizing F-actin by phalloidin and NBD-phallicidin without altering the cells, since NBD-phallicidin does not affect cell viability or electrical resistance (Shapiro et al., 1991).

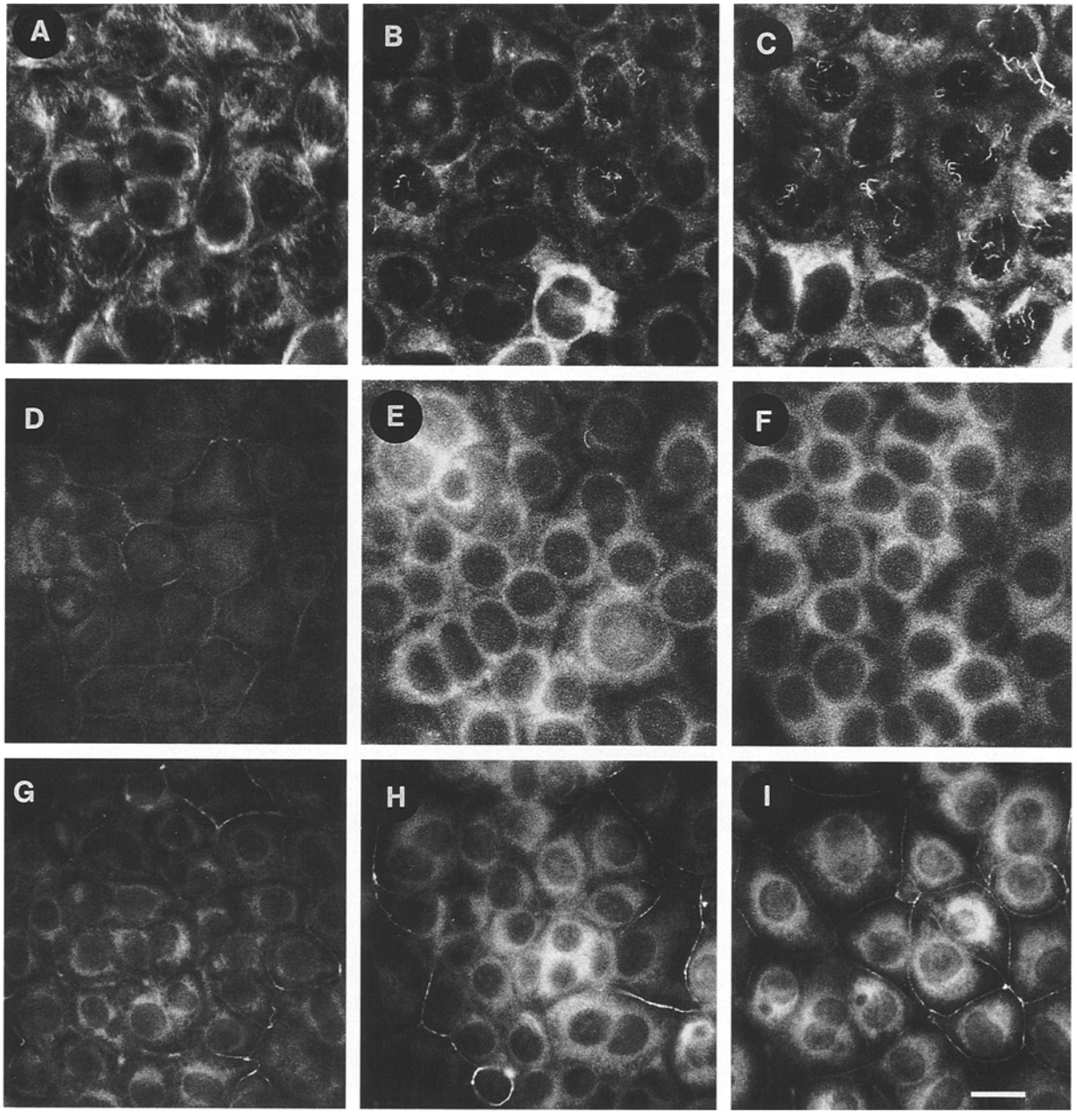

Fig. 6. Effects of nocodazole, phalloidin and NBD-phallicidin on tubulin and F-actin in cultured mouse TAL cells. Confluent cells were first incubated with 10 μg/ml nocodazole for 60 min (*A–C*), or with 13 μM phalloidin (*D–F*) or 0.5 μM NBD-phallicidin (*G–I*) for 3 hr. Cells were then incubated without (*A, D, G*) or with FK for 5 min (*B, E, H*) and 20 min (*C, F, I*), fixed and treated for indirect immunofluorescence with anti-α-tubulin antibody (*A–C*) or rhodamine-conjugated phalloidin (*D-I*). The distribution of tubulin in untreated cells is shown in *A*. Nocodazole completely disrupted the organization of tubulin (*B–C*). FK induced a more intense cytoplasmic labeling of the rhodamine-conjugated phalloidin (*E, F, H, I*) in phalloidin and NBD-phallicidin-treated cells than in control cells (*D, G*) without any clear redistribution of F-actin. Bar: 10 μm.

Previous studies showed that the increased reabsorption of NaCl by medullary TAL tubules induced by vasopressin is associated with an increase in the active transport potential and a decrease in the transepithelial resistance (Hall & Varney, 1980; Hebert et al., 1981; Schlatter & Greger, 1985). In mouse TAL cultured cells, cAMP also causes a profound decrease in transepithelial resistance. At the optical level, our immunocytochemical studies clearly show that FK does not modify the shape of the tight junction but induces major changes in the distribution of F-actin from confluent mouse TAL cells. Forskolin causes F-actin to become condensed in

Table 3. Influence of NBD-phallicidin on dD-AVP- and forskolin-stimulated cAMP in mouse TAL cultured cells

	cAMP content (pmol/7 min/mg protein)	
	−NBD phallicidin	+ NBD phallicidin
Control	31.3 ± 2.5	32.9 ± 5.9
dD-AVP (10^{-6} M)	149.9 ± 13.4	132.0 ± 9.7
Forskolin (10^{-5} M)	735.0 ± 83.7	738.2 ± 65.5

The effects of dD-AVP and FK on cellular cAMP were measured on a set of untreated cells (−NBD) and cells preloaded with 0.5 μM NBD-phallicidin (+NBD). Values are the mean ± SE of duplicate determinations from six experiments.

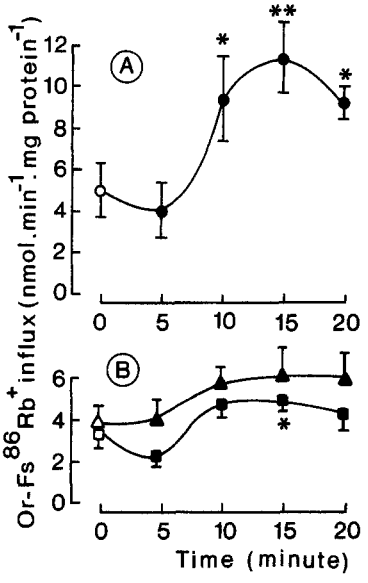

Fig. 7. Influence of nocodazole, phalloidin and NBD-phallicidin on ouabain-resistant furosemide-sensitive $^{86}Rb^+$ influx in cultured mouse TAL cells. (*A*) Confluent cells were first incubated with nocodazole (○, ●) as described in the legend of Fig. 6. (*B*) Confluent cells were first incubated with phalloidin (□, ■) or NBD-phallicidin (△, ▲) as described in the legend of Fig. 6. Cells were then incubated for 0–30 min without (open symbols) or with 10^{-5} M forskolin (filled symbols) before adding $^{86}Rb^+$. Values of Or-Fs influx are means ± SE of duplicate determinations from five experiments. $*P < 0.05$, $**P < 0.01$ represent statistical differences of treated *vs.* control values.

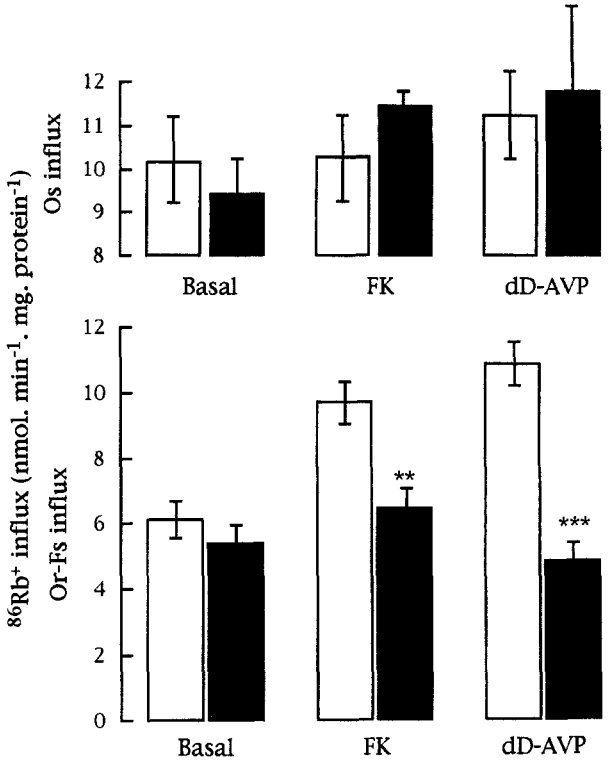

Fig. 8. Influence of phalloidin preloading on forskolin-stimulated and dD-AVP-stimulated $^{86}Rb^+$ influx mediated by Na^+-K^+ ATPase and Na^+-K^+-Cl^- cotransport in cultured mouse TAL cells. Confluent cells were first incubated without (open bars) or with phalloidin (filled bars) for 3 hr (*see* legend of Fig. 6). Cells were then incubated for 15 min without or with 10^{-5} M FK or with 10^{-6} M dD-AVP before adding $^{86}Rb^+$. Values are the mean ± SE of duplicate determinations from five to six experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ represent statistical differences between groups.

the area of the perijunctional actin-myosin ring at the level of the tight junction. Changes in F-actin distribution have been also observed in T_{84} cells stimulated by cAMP (Shapiro et al., 1991). However, in this model of intestinal cultured cells, cAMP induces the formation of thickened bundles of F-actin at the basal pole of T_{84} cells. No definite explanation can be advanced for the differences observed between T_{84} cells and mouse TAL cells. One cannot exclude that these differences are due to changes in the structural organization of the junctional complexes of T_{84} cells, which possess numerous desmosomes and have high transepithelial resistance (Dharmsathaphorn et al., 1985), and mouse TAL cells which have no visible desmosome and a much lower electrical resistance. Nevertheless, the relocation of F-actin following stimulation by FK suggests that this cytoskeletal element plays a role in some membrane ion transport process. With the rearrangement of F-actin, FK and dD-AVP both stimulate Rb^+ influx via the cotransport. The rise in Na^+-K^+-Cl^- cotransport activity was almost completely prevented by stabilizing F-actin with phalloidin or NBD-phallicidin. In addition, we found that cytochalasin D can induce *per se* a rise in the Na^+-K^+-Cl^- cotransport activity. Recently, Stevenson and Begg (1994) showed that incubation of MDCK cells with low concentration of cytochalasin D alters both the apical peripheral ring of actin and the basal actin filament bundles, whereas increasing concentrations caused gradually less perturbation of the apical actin ring. Although one cannot exclude that the action of cytochalasin D on Na^+-K^+-Cl^- cotransport is related to cell volume changes, the

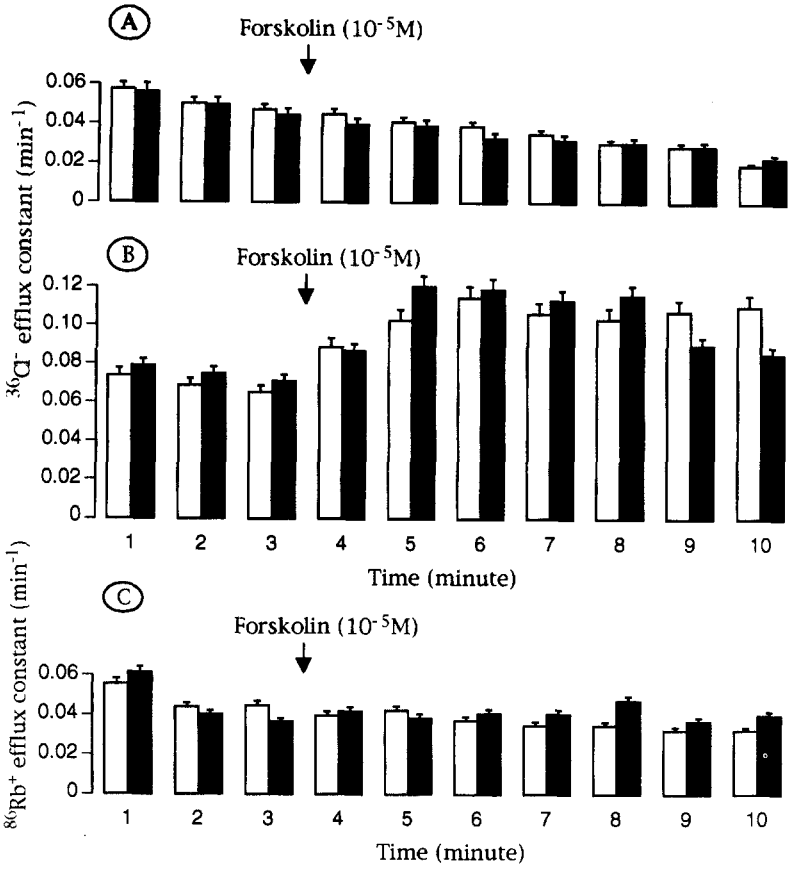

Fig. 9. Kinetics of $^{36}Cl^-$ and $^{86}Rb^+$ efflux following phalloidin preloading of cultured mouse TAL cells. (*A–B*) Sequential 1 min rate constants of $^{36}Cl^-$ efflux from the apical side (*A*) and basal side (*B*) of confluent mouse TAL cells grown on filters. (*C*) Sequential 1 min rate constants of $^{86}Rb^+$ efflux measured on confluent mouse TAL cells grown on plastic dishes (*C*). FK stimulated the Cl^- efflux from the basal side without altering the apical efflux. The rates of $^{36}Cl^-$ efflux and $^{86}Rb^+$ efflux before and after addition of FK remain identical in untreated cells (open bars) and in cells preloaded with phalloidin (filled bars). Values are the mean ± SE from six to ten experiments.

fact that loading TAL cells with different concentrations of cytochalasin D stimulates the Or-Fs component of Rb^+ influx could suggest that changes (polymerization-depolymerization) in F-actin directly affected the activity of the Na^+-K^+-Cl^- cotransporter.

Microtubules play a role in the secretion of proteins (Parczyk, Haase & Kondor-Koch, 1980), the vesicular traffic of polarized epithelial renal cells (Breifeld, McKimmon & Mostov, 1990), and cAMP-mediated antidiuretic response of the bladder (Valenti, Hugon & Bourget, 1988). However, disruption of microtubules by nocodazole has no effect on the stimulated rate of Rb^+ influx in cultured mouse TAL cells. Thus, microtubules have no great influence on cotransport, whereas F-actin is essential for activation of the cotransporter in response to cAMP. Phalloidin and NBD-phallicidin appear to interfere specifically with the cotransport activity without altering Na^+-K^+ ATPase pumps. As already discussed by Matthews et al. (1992), the question arises as to whether stabilization of F-actin alters the chloride conductance. Electrophysiological studies on TAL tubules have suggested that the stimulation of Na^+-K^+-Cl^- cotransport by polypeptide hormones or cAMP is associated with modulation of basolateral Cl^- conductance (Schlatter & Greger, 1985). A cAMP-activated chloride channel has also been demonstrated by patch-clamping in the basolateral plasma membrane of mouse TAL cells (Paulais & Teulon, 1990). Results from Cl^- efflux studies showed that FK induces an increase in rate constant of Cl^- efflux from the basal side of the cells without affecting the rate constant of apical Cl^- efflux. Moreover, the increase in Cl^- efflux was maximum after 2–6 min following FK and preceded the activation of the Or-Fs component of Rb^+ influx mediated by the Na^+-K^+-Cl^- cotransporter. Preloading with phalloidin did not impair the increased rate constant by FK, thus suggesting that phalloidin had no significant action on the chloride conductance.

Apical K^+ recycling through K^+ membrane channels also appears to be an essential modulator of cotransport-mediated ion fluxes stimulated by hormones (Hebert et al., 1984; Molony et al., 1987, 1989). Phalloidin and NBD-phallicidin do not affect the kinetics of Rb^+ efflux in mouse TAL cells, with or without FK, thus suggesting that F-actin stabilization does not alter K^+ channel conductance.

Results from kinetic studies indicate that phalloidin is responsible for a decrease in V_{max} of the Or-Fs component of Rb^+ influx from mouse TAL cells stimulated by FK. This could suggest that stabilization of the F-actin cytoskeleton interferes in processes involved in regulation of functional cotransport units. The exact mech-

anism controlling the upregulation of cotransporter activity remains unknown. Previous studies showed that increased or decreased Na^+-K^+-Cl^- activity via different processes results in similar variations in the number (^3H)-bumetanide binding (Haas & Forbush, 1986; O'Donnel, 1989; Haas, Johnson & Boucher, 1990), suggesting changes in the number of functional cotransporter or changes in conformation of cotransporters on the plasma membrane. There is still no direct evidence that cAMP effectively stimulates the insertion of functional cotransporters within the plasma membrane. However, our present results and the previous studies on T_{84} cells show that stabilization of F-actin may play an important role in this process. As emphasized by Matthews et al. (1992), blockade of depolymerization of microfilaments by phalloidin or NBD-phallicidin (Barak et al., 1980) could interfere with protein kinase A-dependent phosphorylation processes and thereby alter the activation of the Na^+-K^+-Cl^- cotransporter following cAMP-dependent stimulation.

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