ELSEVIER

Biochimica et Biophysica Acta 1467 (2000) 54–64

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Wortmannin inhibition of forskolin-stimulated chloride secretion by T84 cells

Tom W. Ecay *, Jeffrey L. Dickson, Tracy D. Conner

Department of Physiology, James H. Quillen College of Medicine, East Tennessee State University, PO Box 70576, Johnson City, TN 37614, USA

Received 28 January 2000; received in revised form 14 March 2000; accepted 21 March 2000

Abstract

The time- and dose-dependent effects of wortmannin on transepithelial electrical resistance (R_{te}) and forskolin-stimulated chloride secretion in T84 monolayer cultures were studied. In both instances, maximal effects developed over 2 h and were stable thereafter. Inhibition of forskolin-stimulated chloride secretion, as measured by the short-circuit current (I_{sc}) technique, had an IC_{50} of 200–500 nM, which is 100-fold higher than for inhibition of phosphatidylinositol 3-kinase (PI3K), but similar to the IC_{50} for inhibition of myosin light chain kinase (MLCK) and mitogen-activated protein kinases (MAPK). Previous work demonstrated that 500 nM wortmannin did not inhibit the cAMP activation of apical membrane chloride channels. We show here that 500 nM wortmannin has no affect on basolateral Na/K/2Cl-cotransporter activity, but inhibits basolateral membrane Na/K-ATPase activity significantly. The MLCK inhibitors ML-7 and KT5926 were without affect on forskolin-stimulated I_{sc}. Similarly, the p38- and MEK-specific MAPK inhibitors SB203580 and PD98059 did not reduce forskolin-stimulated I_{sc}. In contrast, the non-specific MAPK inhibitor apigenin reduced forskolin-stimulated I_{sc} and basolateral membrane Na/K-ATPase activity similar to wortmannin. In isolated membranes from T84 cells, wortmannin did not inhibit Na/K-ATPase enzymatic activity directly. We conclude that one or more MAPK may regulate the functional expression of basolateral membrane Na/K-ATPase by controlling the abundance of enzyme molecules in the plasma membrane. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Chloride secretion; Wortmannin; Na/K-ATPase; MAP kinase inhibitor; T84 cell; Apigenin

1. Introduction

The human colonic cell line T84 is a widely used model for studies of epithelial chloride secretion [1]. These cells maintain a high degree of phenotypic differentiation and resemble colonic crypt cells in function and morphology. T84 cells grow as structurally polarized monolayers when cultured on permeable substrata and exhibit a large capacity for stimulated chloride secretion in response to vasoactive intestinal peptide (VIP), β-adrenergic and cholinergic agonists, histamine, and prostaglandin E_1 [2]. The activities and subcellular distribution of ion transporters required for vectorial movement of chloride ion across T84 monolayers conforms to the model of epithelial chloride secretion first proposed more than two decades ago [3]. As with most chloride secreting epithelia, the chloride conductive properties of the apical membrane are rate-limiting. In T84 cells, ap-

* Corresponding author. Fax: +1-423-439-7189;
E-mail: ecay@etsu.edu

0005-2736/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0005-2736(00)00204-2

ical chloride conductance is dominated by the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated chloride channel highly expressed in these cells [4,5]. Expressed at the basolateral membrane is a bumetanide-inhibitable Na/K/2Cl-cotransporter, which provides a pathway into the cell for chloride ions [6]. The energy stored in the outwardly directed sodium gradient, established by the basolateral Na/K-ATPase, drives the accumulation of intracellular chloride. Also, expressed at the basolateral membrane are potassium channels that facilitates potassium recycling across this membrane [7], thus shunting the osmotic and electrical effects of excessive potassium accumulation. The importance of each of these transporters to chloride secretion is supported by the observations that inhibitors of any one of them significantly reduces the magnitude of secretagogue-stimulated chloride secretion [8].

The chloride secretory activity of T84 cells can be influenced by an array of physiological and pharmacological agents [1,2,8]. Hormones and neurotransmitters that stimulate chloride secretion can be grouped into two broad categories; those that raise intracellular cAMP (or cGMP) and those that raise cytosolic calcium concentrations. In addition to secretory agonists, a number of secretory antagonists have been identified. These include somatostatin, epidermal growth factor (EGF), and insulin [9].

The fungal metabolite wortmannin has become a popular tool for unraveling the many roles for phosphatidylinositol 3-kinase (PI3K) and polyphosphatidylinositol lipids in practically all aspects of cellular physiology [10]. Wortmannin is a potent ($K_i = 5$ nM), selective, and fast acting inhibitor of most isoforms of PI3K [11]. The PI3K-linked effects wortmannin has include inhibition of growth factor receptor-mediated signal transduction, alterations in cytoskeletal structure, and disruption of multiple steps in membrane vesicular trafficking [10]. Wortmannin has been used to demonstrate that the inhibitory effect of EGF on T84 chloride secretion is mediated by phosphatidylinositol 3-kinase (PI3K) [12]. EGF stimulates PI3K activity in T84 cells and 50 nM wortmannin blocks effects of EGF on chloride secretion stimulated by calcium-elevating secretagogues (e.g. thapsigargin, carbachol, and histamine). EGF effects are due in part to inhibition of calcium-activated potassium channels [9]. In contrast to inhibition of calcium-stimulated chloride secretion, we reported that PI3K-inhibiting concentrations of wortmannin (i.e. ≤ 50 nM) have no significant affect on cAMP-stimulated chloride secretion by T84 cells [13]. Similarly, others have reported that wortmannin does not inhibit cAMP-stimulated chloride secretion in HT29 cells, a second human colonic line [14]. However, when we tested higher wortmannin concentrations, we observed significant inhibition of cAMP-stimulated chloride secretion, which was not due to reduced activity of apical membrane CFTR chloride channels [13]. We concluded from this study that inhibition of cAMP-stimulated chloride secretion by wortmannin above 100 nM is due to affects on ion transporters other than apical chloride channels and to inhibition of regulatory enzymes in addition to PI3K.

The aim of the present study was to characterize more fully the effects of wortmannin on the transepithelial electrical properties and cAMP-stimulated chloride secretory mechanisms in T84 cells. We have attempted to use the wortmannin dose–response relationship for cAMP-stimulated chloride secretion to make predictions as to other regulatory enzymes that are targets for wortmannin and that might regulate chloride secretion in these cells. In addition, we have assayed for effects of wortmannin on the other ion transport pathways that contribute to cAMP-stimulated chloride secretion.

2. Materials and methods

2.1. Materials

Cell culture medium, antibiotics, and newborn calf serum (NBCS) were from Sigma (St. Louis, MO). The permeable supports used to culture T84 cells as monolayers for electrophysiology were Millicell-HA culture plate inserts (12 mm diameter; 0.6 cm^2 surface area) from Millipore (Bedford, MA). Nystatin, forskolin, and 8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic monophosphate (cpt-cAMP) were from Sigma. Wortmannin, apigenin, ouabain, KT5926, PD98059, SB203580, and ML-7 were from Calbiochem (La Jolla, CA). All other chemicals and reagents were from Sigma or of the highest quality available.

2.2. Cell culture and transepithelial electrophysiology

Procedures for the maintenance of T84 cell stock cultures, their growth as monolayers in Millicells, and the assessment of monolayer development and integrity by transepithelial electrical resistance (R_{te}) measurements have been described elsewhere [3,15]. Measurements and computer-based data acquisition of values for basal and forskolin-stimulated short-circuit currents (I_{sc}) and monolayer conductance (G) were performed as previously described [13]. After T84 monolayers were mounted in Ussing chambers and a stable basal I_{sc} established (usually 1–3 μA/cm^2), 10 μM forskolin was added to the basolateral hemichamber. This caused the I_{sc} to rise sharply and to plateau at a new stable I_{sc} within 10–15 min [13]. The value reported as forskolin-stimulated I_{sc} is the difference between this plateau value and the basal I_{sc}.

Basolateral membrane Na/K-ATPase activity was measured in T84 monolayers permeabilized with apical nystatin by methods similar to those described previously [13,15].

2.3. Atomic absorption spectroscopy

To determine the cellular content of sodium and potassium, T84 cells were grown in either 6- or 24-well plates, respectively. The measurement of bumetanide-inhibitable potassium loss from cells cultured in potassium-free solutions is an estimate of Na/K/2Cl-cotransporter activity [16]. In experiments to determine cotransporter activity, cultures were incubated in bicarbonate-buffered T84 Ringer with wortmannin or other test compounds as indicated for 2 h and then switched to a potassium-free Ringer (composition in mM: 130 NaCl, 25 $NaHCO_3$, 1.5 $CaCl_2$, 1.5 $MgCl_2$, 5 glucose, 0.005% phenol red) for 1 h. Initial experiments showed that for cultures at approximately 70% confluence, potassium loss was linear for up to 2 h. Following the 1-h incubation in potassium-free Ringer (± test compounds), Ringer was aspirated, culture wells rinsed 6× with ice-cold 0.1 M $MgCl_2$, and culture plates air dried. For sodium determinations, confluent cultures were washed twice in T84 Ringer and cultured in T84 Ringer ± test compounds for the times indicated. Ringer was aspirated and cultures were washed in 0.1 M $MgCl_2$ and air-dried as described above. Dried cells were extracted with 2–4 ml of 1% HCl for 1 h at 20°C. Acidified cell extracts were analyzed for sodium and potassium content on a Varian Spectra 5500 atomic absorption spectrophotometer using lamp settings appropriate for each ion.

2.4. Sodium-potassium ATPase activity

Na/K-ATPase enzymatic activity was assayed in plasma membrane pellets prepared from T84 cells grown to confluence in 100- or 150-mm dishes. In some experiments, T84 cells were treated with wortmannin or apigenin prior to membrane isolation. For this, cultures were incubated in bicarbonate-buffered T84 Ringer that contained wortmannin, apigenin or DMSO (vehicle control) for 2.5 h. Culture medium or Ringer was discarded and cultures were washed twice with ice-cold phosphate-buffered saline (PBS). All subsequent steps were performed on ice unless noted otherwise. Cultures were scraped into homogenization buffer (composition in mM: 50 mannitol, 5 HEPES (pH 7.4), 0.25 $MgCl_2$, 1 ethylenediaminetetraacetic acid (EDTA), 1 phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, and 1μg/ml *N*-tosyl-l-phenylalanine-chloromethyl ketone (TPCK)), briefly disrupted with a hand-held Dounce homogenizer, and sonicated with a Micro Ultrasonic Cell Disrupter (Kontes, Vineland, NJ). Cell homogenates were centrifuged at 1500×*g* for 9 min to remove intact cells and nuclei and again at 100 000×*g* for 30 min to obtain a post-nuclear membrane pellet. This final pellet was resuspended in assay buffer (composition in mM: 70 Tris–HCl (pH 7.0), 4.2 $MgCl_2$, 84 NaCl, 14 KCl, and 0.14% Triton X-100) at 3.5–5 mg/ml protein. The BCA Protein Assay (Pierce, Rockford, IL) was utilized to determine protein content with bovine serum albumin as standard. For the enzyme activity assay, 100 μl of isolated membranes was combined with 250 μl assay buffer, 5 μl of 500 mM NaN_3, 5 μl of 300 mM ouabain or DMSO, 10.5 μl of 100 mM adenosine 5′-triphosphate (ATP). In some experiments, wortmannin and apigenin were added at this point from 1000X DMSO stocks. Assay tubes were vortexed briefly, incubated on ice for 20 min, and then at 37°C for 1 h. Enzymatic activity was stopped by chilling tubes to 4°C and addition of

35 μl of ice-cold 6 N perchloric acid (PCA). Tubes were incubated on ice for 20 min and then centrifuged at 15 000×*g* for 10 min. Duplicate 100 μl supernatant samples were combined with 1.4 ml of acetate buffer (80 mM acetic acid, 18 mM sodium acetate), 150 μl of 1% ammonium molybdate, and 150 μl of 1% ascorbic acid. Tubes were incubated at room temperature for 10 min, and their absorbance read at 700 nm (Spectronic 601, Milton Roy, Rochester, NY). Phosphate standards ranging from 5–25 μg/ml were prepared using a 10-mM Na_2HPO_4 stock.

2.5. Measurement of cellular ATP content

T84 cells were grown to confluence in 6-well plates. Cultures were washed with bicarbonate-buffered T84 Ringer at 37°C. Cultures were incubated in Ringer that contained wortmannin or other test compounds for 2 h at 37°C. Following this, the Ringer was aspirated and 750 μl of ice-cold 6% trichloroacetic acid (TCA) was added to each well. Plates were agitated on an orbital shaking platform for 15 min at 4°C. TCA extracts were transferred to microcentrifuge tubes and spun at 10 000×*g* for 2 min. A commercial ATP assay kit (Sigma Cat. no. 366) was used to measure the ATP content of a 500-μl sample of the cleared TCA extract. The assay is based on the ATP-dependent enzymatic oxidation of nicotinamide adenine dinucleotide.

2.6. Statistical analysis

Unless noted otherwise, all values are reported as means ± standard error of the mean (S.E.M.). Statistical significance ($P \leq 0.05$) was determined using Student's *t* test.

3. Results

3.1. Wortmannin reduces basal R_{te} and inhibits forskolin-stimulated I_{sc}

We have shown previously that up to 100 nM wortmannin did not alter forskolin-stimulated I_{sc} in T84 monolayers under conditions that completely inhibited phosphatidylinositol 3-kinase dependent

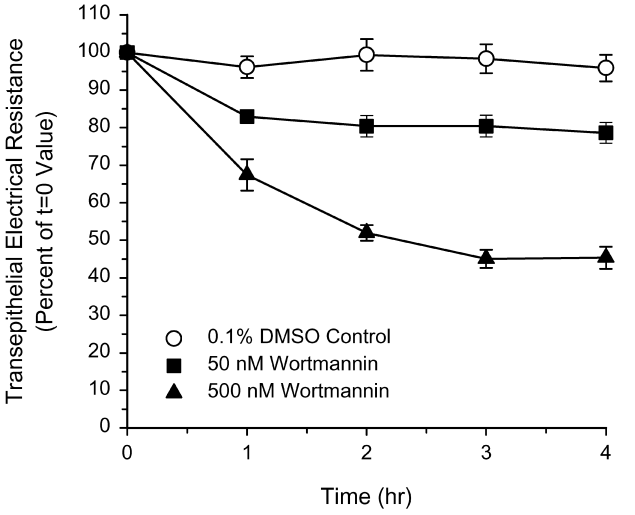

Fig. 1. Wortmannin reduces the transepithelial electrical resistance (R_{te}) of T84 monolayer cultures. Culture media bathing the apical and basolateral surfaces of confluent monolayers was removed and replaced with serum-free culture medium that contained the indicated concentrations of wortmannin or 0.1% DMSO (vehicle control). Values for R_{te} were measured with the EVOM apparatus immediately after fluid exchange ($t=0$) and at hourly intervals. Shown is a representative experiment from three replicates. Values are the mean ± S.E.M. for three monolayers in each group. The mean R_{te} value at $t=0$ for all monolayers shown was 980 ± 95 Ωcm^2.

epidermal growth factor (EGF) responses [13]. To obtain a more complete understanding of wortmannin effects on the transepithelial electrical properties of T84 monolayers, we began this study by monitoring the effects of wortmannin on transepithelial electrical resistance (R_{te}) using the EVOM apparatus. Monolayer R_{te} was measured at 1-h intervals following the addition of 50 or 500 nM wortmannin (Fig. 1). At 50 nM, wortmannin caused a 15–20% reduction in R_{te} that was stable after 1 h. At 500 nM, the reduction in R_{te} reached 50% by 2 h and was stable out to 12 h (not shown).

Brief exposure of T84 monolayers to wortmannin (500 nM for 15 min) did not affect the magnitude of a subsequent forskolin-stimulated I_{sc} responses, but longer exposures (≥ 30 min) significantly reduced forskolin-stimulated I_{sc} [13]. To access time-dependent effects of wortmannin on stimulated chloride secretion, T84 monolayers were cultured with 200 nM wortmannin for varying lengths of time prior to mounting in Ussing chambers and measurement of forskolin-stimulated I_{sc}. At 200 nM wortmannin, maximum inhibition of forskolin-stimulated I_{sc} was

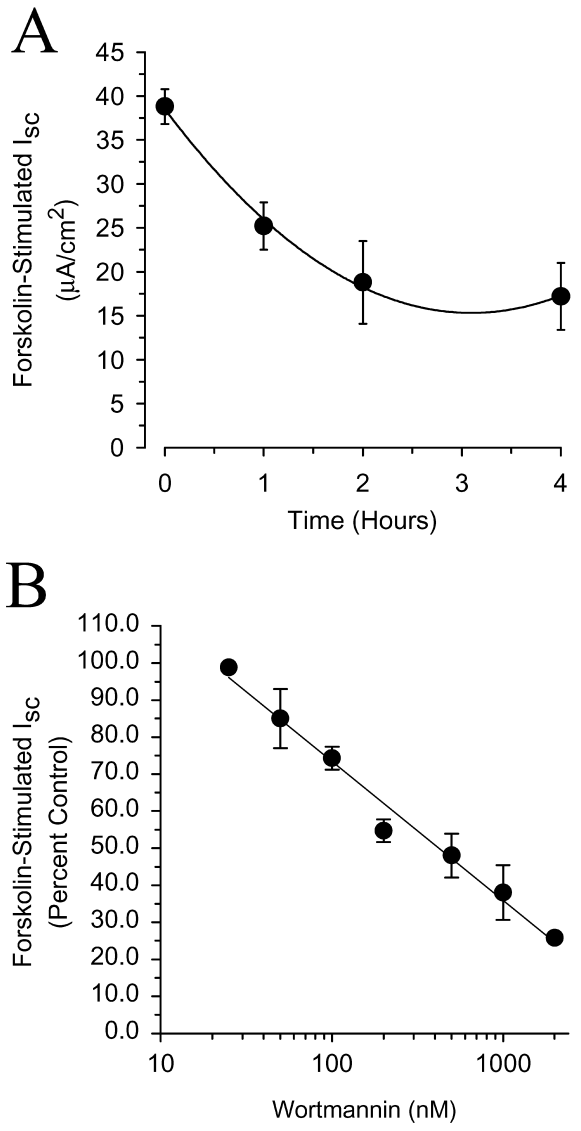

Fig. 2. Wortmannin inhibits forskolin-stimulated I_{sc}. (A) The time-dependent inhibition of forskolin-stimulated I_{sc} by 200 nM wortmannin. Monolayers were treated with either 200 nM wortmannin or 0.1% DMSO for the indicated times, mounted in Ussing chambers, and the I_{sc} response to 10^{-5} M forskolin measured. Each point is the mean (±S.E.M.) for forskolin-stimulated I_{sc} from four monolayers. (B) Dose-dependent inhibition of forskolin-stimulated I_{sc} by wortmannin. T84 monolayers were treated with increasing concentrations of wortmannin for 2 h and the I_{sc} response to 10^{-5} M forskolin measured. Each point is expressed as a percent of the vehicle control (0.1% DMSO) values and represents the mean (±S.E.M.) of 8 or more monolayers. Forskolin-stimulated I_{sc} in the 0.1% DMSO controls was 35.8 ± 1.5 μA/cm^2 (n = 8).

50% and was observed at 2 h (Fig. 2A). In a subsequent series of experiments, monolayers were incubated with 200 nM wortmannin for 2 h and their response to 500 μM cpt-cAMP measured. In these experiments, wortmannin reduced cpt-cAMP-stimulated I_{sc} by 37% (46.7 ± 1.8 vs. 29.5 ± 1.8 μA/cm^2; n = 5, $P < 0.01$) demonstrating that inhibition by wortmannin is not due to effects on forskolin activation of adenyl cyclase.

Using a 2-h preincubation, a wortmannin dose–response curve for inhibition of forskolin-stimulated I_{sc} was obtained (Fig. 2B). Under these conditions, the maximum inhibition was 75% at 2 μM and the IC_{50} was in the range of 200–500 nM. This is approximately two orders of magnitude greater than the reported IC_{50} for inhibition of PI-3K [11] and phospholipase A2 [17], but is within the reported range for inhibition of myosin light chain kinase (MLCK) [18] and mitogen-activated protein (MAP) kinase [19].

3.2. *Wortmannin does not inhibit Na/K/2Cl-cotransporter activity*

The basolateral membrane Na/K/2Cl-cotransporter is required for sustained chloride secretion and a potential target for wortmannin inhibition. To determine the effect of wortmannin on the Na/K/2Cl-cotransporter we exploited its ability to catalyze transport across the plasma membrane in either direction depending on the sum of the concentration gradients for its substrate ions. When extracellular potassium is removed, the net driving force for transport is in the outward direction. Measurement of the bumetanide-inhibitable potassium loss from cells in potassium-free solutions is an estimate of cotransporter activity [16]. Fig. 3 shows that switching T84 cells to potassium-free Ringer resulted in a 33 ± 3% loss of cell potassium. Bumetanide reduced this potassium loss to 23 ± 4%. Thus, about one-third of the potassium loss in these cells was through the cotransporter (i.e. bumetanide inhibited). Inclusion of 500 nM wortmannin had no affect on potassium loss, but increased the bumetanide-sensitive component of potassium loss by a factor of two (36 ± 4.6% vs. 16.1 ± 4.5% potassium loss in the absence and presence of bumetanide, respectively). This suggests that wortmannin actually stimulates cotransporter activ-

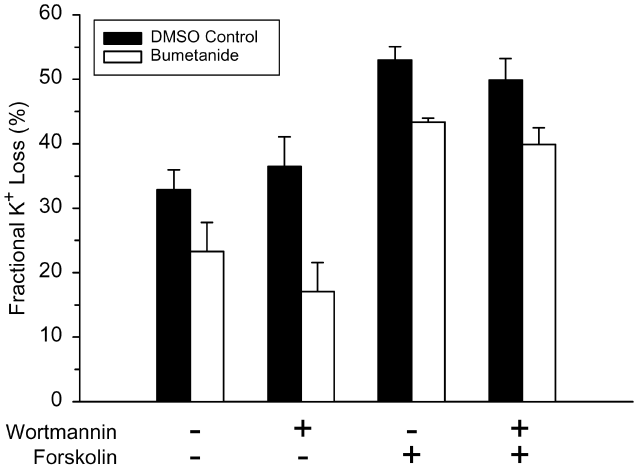

Fig. 3. Wortmannin does not inhibit Na/K/2Cl-cotransporter activity. T84 cells grown to 70% confluence in 24-well plates were treated with 500 nM wortmannin or 0.1% DMSO in HCO_3-buffered T84 Ringer for 2 h. Cultures were then switched to a potassium-free T84 Ringer that contained 500 nM wortmannin, 10^{-5} M forskolin and 10^{-4} M bumetanide as indicated and incubated for an additional 60 min at 37°C. At this point, cell potassium was measured as described in Section 2. Fractional potassium loss is the difference in potassium content between cultures in normal Ringer and potassium-free Ringer. Each bar represents the mean (±S.E.M.) of 6–10 separate culture wells from two different experiments. The difference between solid and open bars is the bumetanide-inhibitable component of potassium efflux and a measure of Na/K/2Cl-cotransport activity.

ity. When 10^{-5} M forskolin was included in the potassium-free Ringer, potassium loss was increased to 53±2% and bumetanide reduced this to 43±1%. The absolute size of the bumetanide-sensitive potassium loss was similar with and without forskolin, suggesting that the forskolin-stimulated potassium loss is not due to activation of the cotransporter under these conditions. Inclusion of 500 nM wortmannin with forskolin had no significant effect on bumetanide-sensitive potassium loss (50±3% vs. 40±3% in the absence and presence of bumetanide, respectively). These results strongly suggest that wortmannin has no inhibitory effect on cotransporter activity.

3.3. Wortmannin inhibits the Na/K-ATPase

We have used nystatin to permeabilize apical membranes of monolayer cultures and measured the resulting ouabain-inhibitable sodium current (I_{Na}) to address whether the basolateral membrane Na/K-ATPase is a target for wortmannin. Permeabilization was carried out in symmetric Ringer solutions to eliminate current flow through tight junctions or active ion channels present in basolateral membranes. As shown in Fig. 4, addition of 0.35 mg/ml nystatin caused a rapid increase in I_{Na}, which was accompanied by a gradual increase in monolayer conductance (G_{nyst}) (not shown). The increase in I_{Na} was completely reduced by 10^{-4} M ouabain (Fig. 4). The magnitude of I_{Na} following nystatin was significantly lower in wortmannin-treated cultures (Fig. 4 and Table 1). In addition, G_{nyst} was reduced in wortmannin-treated monolayers (Table 1) indicating that wortmannin inhibited the basal ion conductive prop-

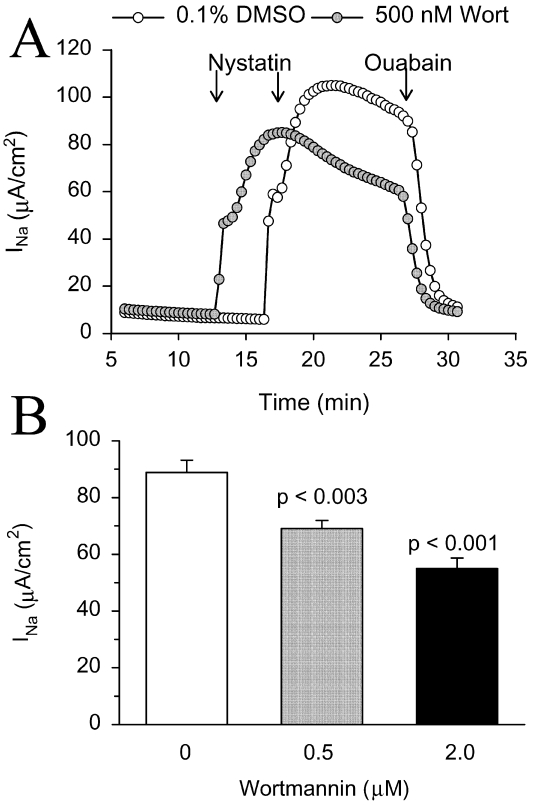

Fig. 4. Wortmannin inhibits Na/K-ATPase activity in T84 monolayers. Millicell cultures were treated with wortmannin or 0.1% DMSO (vehicle control) as indicated for 2 h prior to mounting in Ussing chambers. Addition of 0.35 μg/ml nystatin to the apical hemichamber caused an immediate increase in current (I_{Na}) that was almost completely (≥95%) inhibited by 10^{-4} M ouabain. (A) Individual monolayer recordings from a typical experiment. (B) Means (±S.E.M.) for 8–16 monolayers in each group.

Table 1
Effects of wortmannin and apigenin on I_{Na} and G_{nyst} in nystatin-permeabilized T84 monolayers

Treatment	n[a]	ΔI_{Na} (μA/cm^2)	ΔG_{nyst} (mS/cm^2)
0.1% DMSO control	17	88.3 ± 3.9	10.4 ± 0.8
500 nM Wortmannin	19	69.2 ± 2.8[b]	8.0 ± 0.6[c]
100 μM Apigenin	8	55.0 ± 3.7[b]	4.5 ± 0.6[b]

[a]Number of monolayers tested in each group.
[b]$P < 0.01$ vs. DMSO control.
[c]$P < 0.05$ vs. DMSO control.

erties of T84 basolateral membranes. Addition of forskolin (10^{-5} M) after nystatin permeabilization had no effect on I_{Na} (not shown) suggesting that wortmannin inhibition of forskolin-stimulated I_{sc} in intact cells is due to inhibition of basal Na/K-ATPase activity. Consistent with inhibition of Na/K-ATPase activity, 500 nM wortmannin caused a 30% increase in T84 cell sodium content as measured by atomic absorption spectrophotometry (Fig. 5).

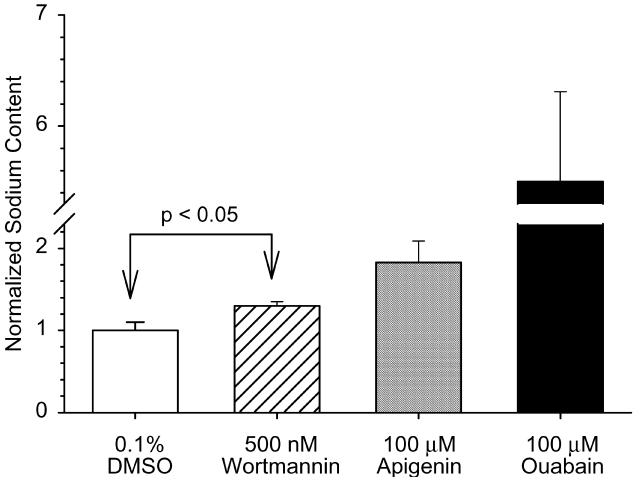

Fig. 5. Wortmannin and apigenin increase the sodium content of T84 cells. Confluent cultures of T84 cells were washed twice in T84 Ringer and treated with the indicated drugs for 2 h. Cultures were washed free of extracellular sodium, extracted in 0.1% HCl and the sodium content of cell extracts measured by atomic absorption spectrophotometry as described in Section 2. Cell extract sodium content was normalized to values measured in 0.1% DMSO control cultures. Each value represent the mean (±S.E.M.) for nine separate cultures.

3.4. Wortmannin may target a MAPK activity in T84 cells

Wortmannin inhibition of forskolin-stimulated I_{sc} has an IC_{50} of 200–500 nM, which is similar to the reported effects of wortmannin on MLCK ($IC_{50} = 200$ nM) [18] and MAP kinase ($IC_{50} = 300$ nM) [19]. To test whether either of these enzymes are targets for wortmannin, we have studied the effects of MLCK and MAP kinase inhibitors on forskolin-stimulated I_{sc}. As shown in Table 2, preincubation of T84 monolayers for 2 h with the MLCK inhibitors ML-7 and KT5926 did not alter forskolin-stimulated I_{sc}. In companion cultures, 1 μM ML-7 inhibited horseradish peroxidase endocytosis by T84 cells (Dickson and Ecay, unpublished) suggesting that the lack of ML-7 effects on forskolin-stimulated I_{sc} was not due to reduced potency of our ML-7 stocks.

We cultured T84 monolayers with several MAP kinase inhibitors to test the possibility that wortmannin might be acting through this pathway to inhibit forskolin-stimulated I_{sc}. The MAP kinase kinase or MEK-specific inhibitor PD98059 (20 μM) caused a

Table 2
Effects of MLCK and MAP kinase inhibitors on forskolin-stimulated I_{sc} in T84 monolayer cultures

Inhibitor	Target	K_i or IC_{50} (μM)	Forskolin-stimulated I_{sc} (μA/cm^2)
0.1% DMSO			20.2 ± 0.9 ($n = 10$)
1 μM ML-7	MLCK	0.3	22.1 ± 1.7 ($n = 9$)
0.1 μM KT5926	MLCK	0.018	21.4 ± 1.2 ($n = 5$)
0.1% DMSO			36.0 ± 3.9 ($n = 9$)
100 μM Apigenin	MAP Kinase	10	10.8 ± 3.9 ($n = 9$)[a]
20 μM PD98059	MAP kinase kinase (MEK)	2	36.6 ± 0.3 ($n = 4$)
10 μM SB203580	p38	0.6	42.9 ± 1.2 ($n = 4$)[b]

[a]$P < 0.01$ vs. 0.1% DMSO control.
[b]$P < 0.05$ vs. 0.1% DMSO control.

small, but insignificant, reduction in forskolin-stimulated I_{sc} (Table 2). In contrast, the p38 specific inhibitor SB203580 (10 μM) caused a small and significant increase in forskolin-stimulated I_{sc} (Table 2). Apigenin is an antiproliferative flavone that inhibits the MAP kinase signaling pathway, though the precise target of apigenin inhibition has not been identified [20]. In T84 cells, apigenin inhibited forskolin-stimulated I_{sc} strongly (Table 2), but had less of an effect on R_{te} than wortmannin. After 2 h, 100 μM apigenin reduced R_{te} by only $10 \pm 2\%$ ($n = 14$). Like wortmannin, 100 μM apigenin reduced the I_{Na} observed upon nystatin permeabilization (Table 1), and increased the sodium content of T84 cells by 80% (Fig. 5). The similar effects of apigenin and wortmannin on I_{Na} and cell sodium content suggest that one or more steps in the MAP kinase signalling cascade may be regulating Na/K-ATPase activity in T84 cells.

3.5. *Wortmannin does not inhibit Na/K-ATPase activity directly*

The reduced ouabain-inhibitable I_{Na} in wortmannin-treated T84 monolayers could result from: (1) direct inhibition of Na/K-ATPase activity by wortmannin; (2) wortmannin-stimulated degradation of the Na/K-ATPase protein; (3) a redistribution of Na/K-ATPase protein from the plasma membrane to intracellular membranes in response to wortmannin; or (4) reduced ATP levels in wortmannin-treated cells. To address these possibilities, we have measured wortmannin effects on Na/K-ATPase enzymatic activity and the ATP level in wortmannin-treated cells.

In isolated membrane preparations, 2 μM wortmannin did not reduce Na/K-ATPase activity (Fig. 6A) demonstrating that it does not inhibit the enzyme directly. In contrast, 100 μM apigenin inhibited Na/K-ATPase activity in isolated membranes by approximately 35%. To address the possibility that wortmannin promotes the degradation of the Na/K-ATPase protein, we treated T84 cells with wortmannin for 2.5 h prior to membrane isolation and enzyme assay. As shown in Fig. 6B, Na/K-ATPase activity was not reduced in membranes isolated from wortmannin-treated cells. The experiments illustrated in Fig. 6 suggest that wortmannin does not reduce

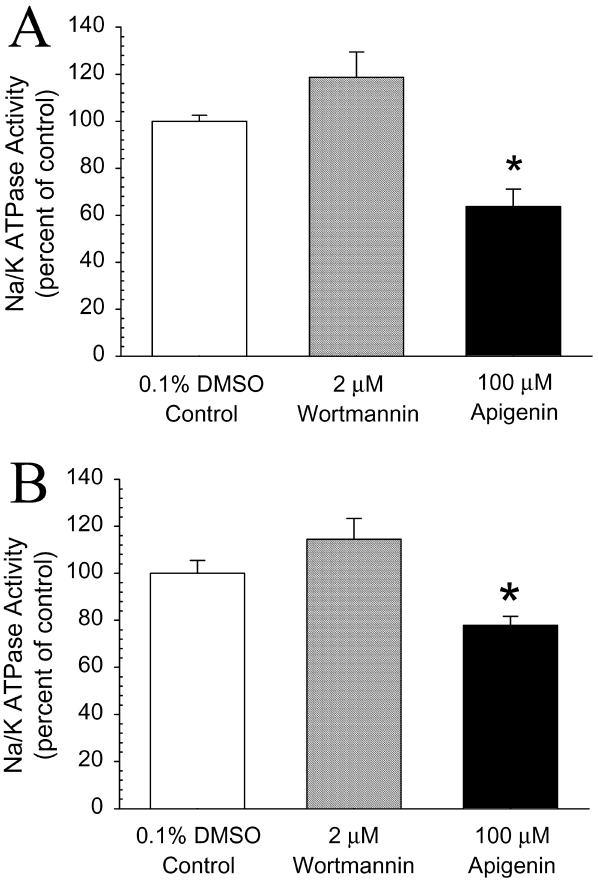

Fig. 6. Wortmannin does not inhibit the Na/K-ATPase enzymatic activity of T84 membranes. In A, T84 cells were homogenized and a crude membrane fraction prepared. Samples of the membrane fraction were treated with the indicated compounds and assayed for Na/K-ATPase activity. Values are expressed as a percent of activity in the control membranes (40–160 μg PO_4/h/mg protein) and are the mean (±S.E.M.) of four separate membrane preparations with triplicate determinations made for each group (*$P \leq 0.05$ vs. 0.1% DMSO control). In B, cultures were treated with DMSO, wortmannin, or apigenin for 2 h prior to homogenization and membrane isolation. Values are expressed as a percent of activity in the control membranes (40–190 μg PO_4/h/mg protein) and are the mean (±S.E.M.) of three separate membrane preparation with triplicate determinations made for each group.

Na/K-ATPase activity directly or reduce the total level of Na/K-ATPase activity in cells. In contrast, apigenin significantly reduced Na/K-ATPase activity in membranes isolated from treated cells (Fig. 6B). Thus, apigenin differs from wortmannin in that it causes direct inhibition of Na/K-ATPase activity and a reduction in total Na/K-ATPase activity in

Table 3
Effects of wortmannin and apigenin on T84 cell ATP content

Treatment	ATP (nmol/mg protein)
0.1% DMSO	83.1 ± 2.7 (n = 9)
2 μM Wortmannin	86.3 ± 4.0 (n = 9)
100 μM Apigenin	75.7 ± 1.9 (n = 9)[a]
500 μM Dinitrophenol	32.2 ± 2.5 (n = 3)[b]

[a] $P \leq 0.05$ vs. 0.1% DMSO control.
[b] $P \leq 0.01$ vs. 0.1% DMSO control.

treated cells. This latter effect could result from apigenin-stimulated degradation of Na/K-ATPase subunit proteins or from an inhibitory mechanism that is retained in our broken cell preparations.

To determine whether wortmannin or apigenin reduced cellular ATP levels, which could lead to diminished sodium pumping activity, we measured ATP levels in T84 cultures treated with wortmannin or apigenin for 2 h. Wortmannin treatment resulted in no change in ATP levels relative to the 0.1% DMSO control cultures, while apigenin caused a 10% reduction in ATP that was statistically significant (Table 3). The mitochondrial uncoupler dinitrophenol reduced ATP levels by 62 ± 3% in T84 cultures.

4. Discussion

Wortmannin has been shown to reduce R_{te} in MDCK monolayers and to inhibit the development of R_{te} in a rat mammary tumor cell line [21,22]. These effects are presumed to be linked to inhibition of PI3K activity by the low concentration of wortmannin required, and in the case of the mammary tumor cell study, the similar effects produced by a second PI3K inhibitor, LY294002. We have found that 50 nM wortmannin partially reduced R_{te} in T84 monolayers, which is consistent with a role for PI3K in the maintenance of T84 tight junction integrity. However, 500 nM wortmannin produced a significantly greater reduction in R_{te}, suggesting that other wortmannin-sensitive enzymes are involved in maintaining T84 cell tight junction integrity.

We have shown previously that 50 nM wortmannin produced no significant inhibition of forskolin-stimulated chloride secretion and we concluded that PI3K is not involved in this process [13]. We have now demonstrated that greater than 50 nM wortmannin produces significant inhibition of forskolin-stimulated chloride secretion, but that the full inhibitory effect of wortmannin requires 2 h to develop. At 2 h, the observed IC_{50} for inhibition of forskolin-stimulated chloride secretion was 200–500 nM. Low permeability of T84 membranes to wortmannin is unlikely to account for the time-dependent effect since complete blockade of EGF inhibition of thapsigargin-stimulated chloride secretion by 50 nM wortmannin occurs within 30 min [12,13]. In addition, we have shown that apical membrane chloride channel activity, is not affected by 500 nM wortmannin [13]. Here we show that basolateral Na/K/2Cl-cotransporter activity, as measured in a bumetanide-inhibitable potassium efflux assay, was not reduced by 500 nM wortmannin. In contrast, wortmannin reduced basolateral membrane sodium pump activity significantly. Consistent with sodium pump inhibition, wortmannin elevated T84 cell sodium content. Reduced Na/K-ATPase activity and elevated cell sodium can diminish stimulated chloride secretion in at least two ways. First, elevated sodium will reduce the driving force for chloride influx via the Na/K/2Cl-cotransporter resulting in lowered cytosolic chloride and reduced current flow through cAMP-activated chloride channels. Second, pump inhibition will depolarize the plasma membrane, again reducing the driving force for chloride exit through open chloride channels. Both of these possibilities would be expected to reduce cAMP-stimulated chloride secretion.

We have not assayed the effects of wortmannin on basolateral potassium channels directly. However, data from nystatin permeabilization studies suggests that wortmannin inhibits these as well. Wortmannin decreased R_{te} in intact monolayers, suggesting an increase in tight junction permeability. We would have predicted an increase in the conductance of nystatin-permeabilized monolayers if wortmannin affected tight junctions only. However, wortmannin reduced the conductance of nystatin-permeabilized monolayers. This observation suggests that wortmannin reduces basolateral membrane channel activity at the same time that it increases tight junction permeability. Whether these are potassium channels has not been determined, but the effect of reduced basolateral potassium channel activity would be to inhibit chloride secretion [1,7].

Another question to be addressed concerns the mechanism by which wortmannin reduces basolateral membrane Na/K-ATPase in T84 cells. Ours is at least the second report of wortmannin-sensitive Na/K-ATPase activity in epithelia. In rat proximal tubules, dopamine reduces basolateral membrane Na/K-ATPase activity by stimulating endocytosis of the Na/K-ATPase protein [23]. Wortmannin blocks dopamine-stimulated PI3K activity and Na/K-ATPase endocytosis in these cells [24]. Our results differ from those in proximal tubule cells because the 500 nM wortmannin required to inhibit Na/K-ATPase activity in T84 cells is ten times that required to fully inhibit PI3K activity [12,13] Thus, the molecular target for wortmannin must differ between the two systems. However, our observations support a similar hypothesis in which wortmannin influences Na/K-ATPase activity in basolateral membranes by altering rates of Na/K-ATPase endocytosis and exocytosis. The development of wortmannin inhibition over time in T84 cells suggests that wortmannin does not interrupt a signal transduction pathway, but affects a continuous process that runs down over time. We suggest that this process is either the delivery of new pumps to the cell surface or the endocytic recycling of pumps as part of normal membrane turnover processes. Consistent with this hypothesis, wortmannin does not inhibit Na/K-ATPase activity directly and does not reduce the total cellular content of the enzyme in treated cells. This suggests that wortmannin-treated cells possess the same amount of Na/K-ATPase activity, but that less of it is in basolateral membranes.

Wortmannin inhibition of forskolin-stimulated chloride secretion has an IC_{50} of 200–500 nM, which is similar to reported effects of wortmannin on MLCK and MAP kinase [18,19]. In T84 cells, we have ruled out MLCK inhibition by showing that the MLCK inhibitors ML-7 and KT5926 do not reduce forskolin-stimulated I_{sc}. Similarly, MEK and p38 specific MAP kinase inhibitors do not inhibit chloride secretion. On the contrary, the p38 inhibitor SB203580 produced a small, but significant, stimulation of forskolin-stimulated chloride secretion. However, the very general MAP kinase inhibitor apigenin [20] reduced forskolin-stimulated chloride secretion and Na/K-ATPase activity, and increased cell sodium content similar to wortmannin. This suggests that one or more MAP kinases regulate Na/K-ATPase activity, an observation that has not been reported in T84 or any other epithelial cell line. In addition, effects of apigenin on PI3K or MLCK have not been reported. In a human airway cell line, apigenin and other flavonoids stimulate chloride secretion at low concentrations (<30 μM) and inhibit forskolin-stimulated chloride secretion at high concentrations (>30 μM) [25].

It is important to note that the effects of wortmannin and apigenin are not the result of generalized cytotoxicity in T84 cells. Wortmannin did not reduce ATP levels in T84 cells. Although apigenin caused a 10% reduction in ATP levels, this is not sufficient to account for its 70% reduction in forskolin-stimulated I_{sc}. In addition, apigenin was not as effective as wortmannin at reducing R_{te} values. Finally, the caveats associated with any inhibitor study must be applied here. The effect of wortmannin on Na/K-ATPase activity is relatively selective as it did not inhibit cAMP activation of apical membrane CFTR chloride channels [13] or activity of the basolateral membrane Na/K/2Cl-cotransporter. However, at the concentrations used here, wortmannin may be inhibiting other important regulatory proteins in addition to or different from MAP kinases.

A link between Na/K-ATPase activity and the MAP kinase signaling pathway has been reported in cardiac myocytes and HeLa cells [26,27]. Ouabain inhibition of ATPase activity causes activation of MEK in both cell types. In cardiac myocytes, ouabain alters gene expression patterns similar to hypertrophic stimuli [26]. In MDCK cells, ouabain activates protein tyrosine phosphorylation and promotes the redistribution of cell adhesion molecules and detachment of cells from the substrata [28]. While these studies demonstrate that Na/K-ATPase can regulate MAP kinases and related pathways, our results suggest that these pathways can regulate the functional expression of Na/K-ATPase in epithelial cells. We hypothesize that cells maintain a feedback loop in which activated MAP kinases promote the insertion or retention of Na/K-ATPase proteins in the plasma membrane. When Na/K-ATPase levels in the plasma membrane rise sufficiently, its activity can turn off or turn down activated MAP kinases. If Na/K-ATPase activity in the plasma membrane falls, analogous to ouabain treatment, MAP

kinases are stimulated to, among other things, boost Na/K-ATPase levels in the plasma membrane.

Acknowledgements

We are grateful to Jim Napier and Dr. Philip Scheuerman for assistance with atomic absorption spectrophotometer measurements and to Drs W.L. Joyner and B.B. Bush for critical reading of a draft manuscript. This work was supported by a grant from the American Heart Association (TN97N74) to T.W.E. A portion of this work has been published in abstract form (Mol. Biol. Cell 9 (1998) 120a).

References

[1] K.E. Barrett, Am. J. Physiol. 272 (1997) C1069–C1076.
[2] K. Dharmsathaphorn, J.A. McRoberts, K.G. Mandel, L.D. Tisdale, H. Masui, Am. J. Physiol. 246 (1984) G204–G208.
[3] P. Silva, J.S. Stoff, M. Field, L. Fine, J. Forrest, F.H. Epstein, Am. J. Physiol. 233 (1977) F298–F306.
[4] M.P. Anderson, M.J. Welsh, Proc. Natl. Acad. Sci. USA 88 (1991) 6003–6007.
[5] J.A. Cohn, A.C. Nairn, C.R. Marino, O. Melhus, J. Kole, Proc. Natl. Acad. Sci. USA 89 (1992) 2340–2344.
[6] M. Haas, Am. J. Physiol. 267 (1994) C869–C885.
[7] D.C. Devor, R.A. Frizzell, Am. J. Physiol. 265 (1993) C1271–C1280.
[8] J.A. McRoberts, K.E. Barret, in: K.S. Matlin, J. D. Valentich (Eds.), Functional Epithelial Cells in Culture, 1989, Alan R. Liss., Inc, New York, pp. 235–265.
[9] K.E. Barrett, J. Smitham, A. Traynor-Kaplan, J.M. Uribe, Am. J. Physiol. 274 (1998) C958–C965.
[10] A. Toker, L.C. Cantley, Nature 387 (1997) 673–676.
[11] G. Powis, R. Bonjouklian, M.M. Berggren, A. Gallegos, R. Abraham, C. Ashendel, L. Zalkow, W.F. Matter, J. Dodge, G. Grindey, C.J. Vlahos, Cancer Res. 54 (1994) 2419–2423.
[12] J.M. Uribe, S.J. Keely, A.E. Traynor-Kaplan, K.E. Barrett, J. Biol. Chem. 271 (1996) 26588–26595.
[13] J.L. Dickson, T.D. Conner, T.W. Ecay, Am. J. Physiol., in press.
[14] D. Merlin, X. Guo, K. Martin, C. Laboisse, D. Landis, G. Dubyak, U. Hopfer, Am. J. Physiol. 271 (1996) C612–C619.
[15] T.W. Ecay, J.D. Valentich, Am. J. Physiol. 265 (1993) C422–C433.
[16] D.W. Jayme, C.W. Slayman, E.A. Adelberg, J. Cell Physiol. 120 (1984) 41–48.
[17] M.J. Cross, A. Stewart, M.N. Hodgkin, D.J. Kerr, M.J.O. Wakelam, J. Biol. Chem. 270 (1995) 25352–25355.
[18] S. Nakanishi, K.J. Catt, T. Balla, J. Biol. Chem. 269 (1994) 6528–6535.
[19] I.M. Ferby, I. Waga, M. Hoshino, K. Kume, T. Shimizu, J. Biol. Chem. 271 (1996) 11684–11688.
[20] M.L. Kuo, N.C. Yang, Biochem. Biophys. Res. Commun. 212 (1995) 767–775.
[21] P. Nybom, K.E. Magnusson, Biosci. Rep. 16 (1996) 265–272.
[22] P. Kometiani, J. Li, L. Gnudi, B.B. Kahn, A. Askari, Z. Xie, J. Biol. Chem. 273 (1998) 15249–15256.
[23] A.V. Chibalin, A.I. Katz, P.-O. Berggren, A.M. Bertorello, Am. J. Physiol. 273 (1998) C1458–C1465.
[24] A.V. Chibalin, J.R. Zierath, A.I. Katz, P.-O. Berggren, A.M. Bertorello, Mol. Biol. Cell 9 (1998) 1209–1220.
[25] B. Illek, H. Fischer, Am. J. Physiol. 275 (1998) L902–L910.
[26] P.L. Woo, D. Ching, Y. Guan, G.L. Firestone, J. Biol. Chem. 274 (1999) 32818–32828.
[27] S. Li, E.V. Wattenberg, Toxicol. Appl. Pharmacol. 151 (1998) 377–384.
[28] R.G. Contreras, L. Shoshani, C. Flores-Maldonado, A. Lázaro, M. Cereijido, J. Cell Sci. 112 (1999) 4223–4232.