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Inhibition of Na^+-Pump Expression by Impairment of Protein Glycosylation is Independent of the Reduced Sodium Entry into the Cell

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Abstract. Previous studies indicate that inhibition of protein N-glycosylation reduces Na^+-pump activity. Since this effect is preceded by an inhibition of the entry of sodium into the cell, it is unclear whether the reduced Na^+-pump is produced by the inactivation of protein glycosylation *per se* or by the lower intracellular sodium concentration. We compared the effects of tunicamycin, which inhibits protein glycosylation, and amiloride, which inhibits the entry of sodium into the cell, on the expression of the Na^+-pump activity in A6 cells. The short-circuit current across A6 epithelia, which corresponds to sodium ions transported through the Na^+ channel and the Na^+-pump, was almost totally inhibited after 24-hr treatment with 1 μg/ml tunicamycin. The maximal Na^+-pump activity, measured after permeabilizing the apical cell membrane with amphotericin B, was only 30% inhibited. This inhibition increased to 80% after 72-hr treatment with tunicamycin. Thus, tunicamycin inhibits the activities of both the apical Na^+ channel and the basolateral Na^+-pump. However, the reduced number of Na^+-pump molecules, as well as the inhibition of the Na^+-pump activity, were not observed when the Na^+ channel was inhibited for 72-hr with amiloride. Thus, the reduced Na^+-pump expression produced by inactivation of protein glycosylation is not secondary to reduced entry of sodium into the cell.

Key words: Na^+-pump — Na^+, K^+-ATPase — Na^+-transport — Tunicamycin — Glycoprotein

Introduction

The Na^+-pump (Na^+, K^+-ATPase, EC 3.6.1.37) is responsible for the coupled active transport of Na^+ and K^+ ions across the plasma membrane of most eukaryotic cells and plays a key role in cellular ion homeostasis (Jorgensen & Anderson, 1988). In most of the epithelial cells that line the lumen of kidney tubules, the basolateral Na^+-pump provides the driving force for Na^+ entry by extruding Na^+ out of the cells and into the circulation (Katz, 1982). The minimum functional Na^+-pump molecule is composed of an α-subunit and a heavily glycosylated β-subunit (Jorgensen & Anderson, 1988; Geering, 1990). We have recently demonstrated that the α-subunit also is glycosylated with both N- and O-linked carbohydrates (Pedemonte, Sachs & Kaplan, 1990; Pedemonte & Kaplan, 1992). Several experimental observations suggest that the carbohydrate moieties of the Na^+-pump subunits do not directly participate in the catalytic activity. Thus, treatment of Na^+-pump preparations with neuraminidase (Churchill & Hokin, 1976) and chemical modification of the carbohydrate moieties (Lee & Fortes, 1985) do not impair activity. We have determined that concanavalin A and wheat germ agglutinin react with both Na^+-pump subunits without alteration of the Na^+, K^+-ATPase activity (*unpublished* results). Finally, Takeda et al. (1988) have demonstrated that nonglycosylated Na^+-pump molecules have the same affinity for ouabain as the glycosylated ones, which indicates that the N-linked carbohydrate moieties do not play any role in the binding or interaction with ouabain.

Previous reports, regarding the importance of N-glycosylation on the functional expression of the Na^+-pump, show that nonglycosylated Na^+-pumps can be delivered to the cell membrane (Tamkun & Fambrough, 1986; Zamofing, Rossier & Geering, 1989; Takeda et al., 1988) and that these molecules have the capacity to transport sodium (Zamofing et al., 1989; Takeda et al., 1988). However, important discrepancies still remain. While Zamofing, Rossier & Geering (1988), Zamofing et al. (1989) and Alboim, Bak & Sampson (1992) demonstrated that tunicamycin treatment lead to a reduced ex-

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pression of the Na^+-pump activity, Olden et al. (1979), Takeda et al. (1988), and Tamkun and Fambrough (1986) did not observe any change produced by the inhibition of protein N-glycosylation.

Zamofing et al. (1989) observed that 21-hr treatment of toad urinary bladder (TBM) cells with tunicamycin led to a 50% reduction in transepithelial sodium transport and 15% inhibition of the maximal Na^+-pump activity. The Na^+-pump inhibition increased to 50% after 42 hr of tunicamycin treatment. Then, the reduced transepithelial sodium translocation observed after 21 hr of tunicamycin treatment was caused by inhibition of the Na^+ channel. Thus, the inhibition of the Na^+-pump appears to be preceded by a reduced sodium entry into the cell via an inhibition of the Na^+ channel. Since the level of intracellular sodium is one of the major determinants of the cellular content of Na^+-pump molecules in various tissues (Brodie & Sampson, 1989; Pressley, 1988; Wolitzky & Fambrough, 1986), it is possible that the reduced expression of the Na^+-pump is not due to tunicamycin *per se* but secondary to the inhibition of the Na^+ channel. To resolve this point, we have reproduced in A6 cells the inhibition of the Na^+-pump expression by tunicamycin and compared its effects to those of amiloride which inhibits the Na^+ channel.

Materials and Methods

Chemicals and Materials

Tunicamycin; amiloride (*AM*); amphotericin B (Am-B); and antimouse antibody conjugated to agarose beads were obtained from Sigma. Proteases and Peptide N-glycosidase F were obtained from Boheringer. ^{35}S-methionine (^{35}S-Met) was obtained from Amersham and NEN. The amphibian kidney cell line A6 was obtained from the American Type Culture Collection, and cultured at 28°C (5% CO_2) in amphibian medium (Handler, 1983) supplemented with 5% fetal bovine serum (AM-5 medium). Cells were grown on plastic or permeable membrane cups (Costar) as described by Zamofing et al. (1989) and Verrey et al. (1989). An antibody that reacts with the Na^+-pump α-subunit was a kind gift from Dr. Michael Caplan (Yale University). Protein-A/G agarose was obtained from Schleicher & Schuell, and antimouse antibody conjugated to magnetic beads from Dynal and Advance Magnetics.

Cell Treatment

A6 cells were treated with tunicamycin in AM-5 medium for the time indicated in each experiment. For cells grown on permeable filters, tunicamycin was added to both the "apical" and "basolateral" media. Cells grown on plastic were released to the medium when treated with tunicamycin for more than 48 hr. Twenty-four hours later, these cells were still viable and capable of excluding Trypan Blue from their cytoplasm. Cells grown on filters remained attached after 72 hr of tunicamycin treatment. These cells produced translocation of Na^+ and generated a potential difference across the monolayer (*see* Figs. 1–3). Tunicamycin (5 mg/ml) was prepared in 12 mM NaOH. Amiloride (10 mM) and amphotericin B (5 mM) were dissolved in dimethylsulphoxide (DMSO). The small amounts (1–10 μl) of NaOH and DMSO added to the cell medium did not produce any change in the short-circuit current (SCC) of A6 cell epithelia.

Short-Circuit Current Measurements

A6 cells were seeded in permeable membrane cups which separated the medium bathing the "apical" and "basolateral" sides of the epithelium. The development of the transepithelial resistance, potential, and SCC was independently monitored. Cells reached maximum resistance (5,000 Ω cm^2) about eleven days after seeding, and at this time the tunicamycin treatment was started. The cell treatments described did not open the tight junctions as determined by the maintenance of transepithelial resistance. Measurements of SCC were conducted in filter cups containing the confluent cells. The upper (apical) and lower (basolateral) chambers were completely separated as indicated by the stable SCC across the cell layer (*see* the initial part of the results shown in Figs. 1, 2, and 3). Potential across the cells was measured with KCl/Agar bridges connected to calomel electrodes, and current with bridges connected to Ag/AgCl electrodes. The cells were continuously short-circuited by an automatic voltage clamp (WPI). SCC measurements were also performed with cells that were briefly short-circuited every minute. The same results were obtained with both protocols. The SCC was inhibited (95–100%) by addition of either amiloride to the "apical" side (to inhibit the Na^+ channel) or ouabain to the "basolateral" side (to inhibit the Na^+-pump). Therefore, the SCC measured was equivalent to the transepithelial Na^+ translocation through the Na^+ channel and the Na^+-pump (Perkins & Handler, 1981). As observed in toad urinary bladder cells (Zamofing et al., 1989), the effects of tunicamycin and amphotericin B were reversible. Thus, these reagents did not produce any major unspecified toxic effect on the A6 cells. Ouabain inhibition of the SCC was also tested after the addition of amphotericin B. The SCC measurements were repeated at least three times for control and treated samples. A typical result is shown in the figures.

Preparation of Immunobeads

Immunobeads were prepared by mixing 5 parts of anti-α-subunit antibody with 1 part of beads. Various beads were tried with similar results: antimouse antibody conjugated to agarose; protein-A/G conjugated to agarose; and antimouse antibody conjugated to magnetic beads. The antibody was coupled to beads by incubation for 30 min at room temperature in the presence of 10 mM Tris-HCl, pH 7.4, 1% sodium deoxycholate; 1% NP40; 6 mM EDTA, pH 7.4, 1% powder milk. The immunobeads were washed several times with lysis buffer by sedimentation (centrifugation or with a magnet) and resuspension, and finally were resuspended in lysis buffer to the original volume of beads. It was determined that the antibody recognized the α-subunit before and after the protein was deglycosylated with Peptide N-glycosidase F. This reaction was performed as previously described (Pedemonte & Kaplan, 1992).

Labeling and Immunoprecipitation

Cells were treated with tunicamycin for at least 24 hr before metabolic labeling. A6 cells were labeled with ^{35}S-Met (200 μCi/ml) in Met-free serum-free amphibian medium for 30 min at 28°C. Labeled cells were washed with PBS (phosphate buffer saline) containing a cocktail of protease inhibitors formed by 1 μg/ml of pepstatin, aprotinin, and leupeptin, and 0.2 mM PMSF (phenylmethylsulfonyl fluoride). Cells were solubilized with 10 mM Tris-HCl, pH 7.4, 1% sodium deoxycholate; 1% NP40; 6 mM EDTA, pH 7.4, containing the cocktail of pro-

tease inhibitors described above (lysis buffer). The suspension was sonicated twice for 30 sec in an Ultrasonic homogenizer 4710 (Cole Parmer) at 50 watts and 80% power output, and sedimented in an Eppendorf centrifuge. Aliquots of the supernatant were separated for determination of protein concentration and total protein synthesis. To facilitate the comparison of results from the various concentrations of tunicamycin used, the immunoprecipitation was made in aliquots containing an equal amount of protein. Immunobeads (20 μl/tube) were added to solubilized cells and the mixture was incubated at 4°C with rotation for 1 hr. Immunobeads were collected by centrifugation (or a magnet), and washed as described by Tamkun and Fambrough (1986) with some modifications: two washes with 0.5% (v/v) Triton X-100, 5 mM EDTA, 10 mM Tris-HCl, pH 7.4 (buffer A); one wash with 0.1% SDS, 0.1% (v/v) Triton X-100, 300 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5; one wash with 1 mM NaCl, 0.5% (v/v) Triton X-100, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5; two washes with buffer A; and one wash with 5 mM EDTA, 10 mM Tris-HCl, pH 7.5. All buffers used contained the protease inhibitor cocktail described above. Bound antigens were eluted from immunobeads with Laemmli (1970) sample buffer. The suspension was incubated in a water-bath sonicator at 40–50°C for 20 min. Immunoprecipitated proteins were analyzed by SDS-PAGE in a 7.5–15% Laemmli (1970) gel. After the gel was fixed, detection of ^{35}S-Met-labeled proteins was performed by fluorography. Usually, gels were exposed to films for several days at −70°C. For determination of total protein synthesis, aliquots were taken from cell homogenates before antibodies were added. These aliquots were analyzed by PAGE and fluorography. Gels to determine total protein synthesis were exposed to films only for a few hours. Fluorographs were quantitated by densitometry using a Sharp JX-325 scanner interfaced to a Sun Spark Classic computer. The equipment and the software "Quantity One" were obtained from "pdi Company." Gels were exposed to film for various times and quantitation was performed at nonsaturating levels of exposure.

Na^+, K^+-ATPase Assay

A6 cells were resuspended in 25 mM Imidazole, 1 mM EDTA, pH 7.5 (Buffer C) and homogenized in a glass homogenizer with a teflon pestle (20 strokes at 600 rev). The suspension was treated with 0.25 mg/ml of SDS, and after 10 min at room temperature, BSA was added to a final concentration of 0.4 mg/ml. The SDS treatment was determined to be optimum to expose latent Na^+, K^+-ATPase activity (Forbush, 1983; Pedemonte & Kaplan, 1992). SDS treatment increased by threefold the ouabain-sensitive ATPase and reduced ouabain-insensitive activity by 30% in control, tunicamycin-treated, and amiloride-treated cells.

Na^+, K^+-ATPase activity was also measured in microsome membranes. A6 cells were suspended in buffer C and sonicated twice for 10 sec in an Ultrasonic homogenizer 4710 (Cole Parmer) at 25 watts and 80% power output. The suspension was centrifuged for 5 min at 1500 × *g*. The supernatant was collected and centrifuged at 100,000 × *g* for 30 min. The final pellet was resuspended with a small volume of buffer C and used to determine Na^+, K^+-ATPase activity. The Na^+, K^+-ATPase medium (pH 7.8) contained (mM): EGTA, 0.2; NaCl, 50; KCl, 8; $MgCl_2$, 1.2; ATP, 3; Imidazole, 20. ATPase activity was determined for 30 min at 37°C as previously described (Pedemonte & Kaplan, 1986) from the difference between the ATP hydrolysis measured in the absence and presence of ouabain. Ouabain-sensitive ATPase was about 15% of the activity measured in the absence of ouabain. Na^+, K^+-ATPase activity measured in control preparations varied between 2 and 3 μmol of P_i released/mg protein/hr. Na^+, K^+-ATPase activity and the degree of inhibition of this activity by tunicamycin were the same in cell suspensions treated with SDS and microsomal membranes.

Ouabain Binding

Ouabain binding was measured in cell membranes and in entire attached cells, with similar results. These measurements were made on triplicates and repeated at least four times for each sample. A cell suspension was prepared as indicated above. The ouabain reaction medium contained 20 mM Tris-HCl pH 7.2; 10 mM $MgCl_2$; 10 mM Tris-phosphate, pH 7.2; 0.3 μM ouabain (^{3}H-ouabain); and 0.2 mg of protein. Nonspecific binding was determined in the presence of 0.3 mM ouabain. After 2 hr at room temperature, cell membranes were washed by filtration and the radioactivity content of the filters was determined. The presence of 0.2 mg/ml SDS increased the specific ouabain binding threefold. This increment was the same for control, tunicamycin-treated and amiloride-treated cells. Therefore, the degree of ouabain binding inhibition by tunicamycin was the same in the presence and absence of SDS.

For cells grown on filters, cells were washed with a K^+-free amphibian medium (Perkins & Handler, 1981). Filters were kept upside-down (the basolateral side of the cells is against the filter) and the solution containing ouabain was added on top of the filters. The reaction was performed at room temperature, in a humidified chamber, for 2 hr. Cells were scraped from the filters, washed several times with K^+-free amphibian medium, and dissolved with SDS. Aliquots were used to determine protein and radioactivity contents. The degree of ouabain-binding inhibition was the same in both cell suspension and attached cells.

Protein Determination

Protein was determined by the method of bicinchoninic acid (Pierce Chemical) with BSA as standard. For cells solubilized with detergents, proteins were precipitated with trichloroacetic acid and resuspended with formic acid. After dilution with water, protein was determined by the method indicated above.

Abbreviations

Na^+, K^+-ATPase and Na^+-pump indicates the same protein and activity (EC3.6.1.3); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; SCC, short-circuit current; AM, amiloride; AM-B, amphotericin B.

Results

Effect of Tunicamycin Treatment on the Epithelial Na^+-Translocation

In A6 cells, sodium enters into the cell through the apical Na^+ channel. The Na^+-pump, localized in the basolateral membrane, pumps Na^+ out of the cell and into the circulation (Verrey et al., 1989; Perkins & Handler, 1981). The short circuit current (SCC) across A6 epithelia corresponds to the amount of Na^+ transported from the apical to the basolateral medium (Perkins & Handler, 1981). Accordingly, we observed that 95–100% of the SCC of A6 cells grown on permeable membranes was inhibited by addition of either amiloride (which inhibits the Na^+ channel) to the "apical" medium (Figs. 1–3) or ouabain

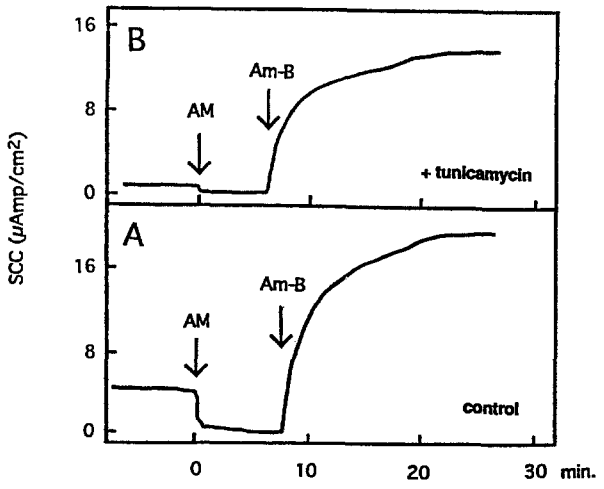

Fig. 1. Effect of 24-hr treatment with tunicamycin on the maximal Na^+-pump activity. A6 cells were grown to maximal transepithelial resistance on membrane filter cups, and then the cells were treated without (*A*) or with (*B*) 1 μg/ml tunicamycin for 24 hr. The SCC across the cell monolayer was determined and, at the indicated times, 10 μM amiloride (*AM*) and 1 μM amphotericin B (*Am-B*) were added to the "apical" bathing medium.

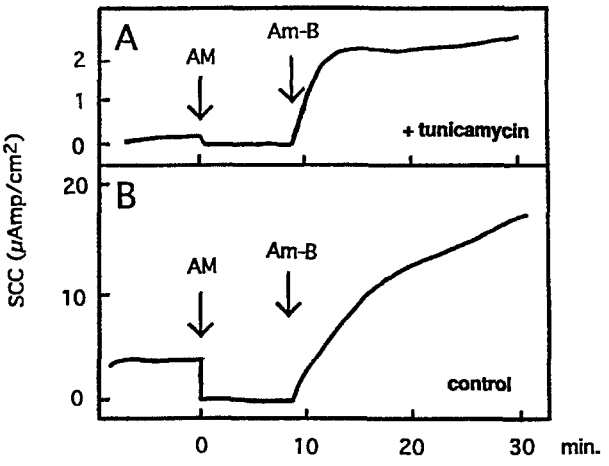

Fig. 2. Effect of 72-hr treatment with tunicamycin on the maximal Na^+-pump activity. A6 cells were grown to maximal transepithelial resistance on membrane filter cups, and then the cells were treated with (*A*) or without (*B*) 1 μg/ml tunicamycin for 72 hr. The SCC across the cell monolayer was determined and, at the indicated times, 10 μM amiloride (*AM*) and 1 μM amphotericin B (*Am-B*) were added to the "apical" bathing medium.

(which inhibits the Na^+-pump) to the "basolateral" medium (*data not shown*). This means that almost all of the sodium that is translocated from the "apical" to the "basolateral" medium goes through both the Na^+ channel and the Na^+-pump (Perkins & Handler, 1981). Amphotericin B permeabilizes the cell membrane to sodium ions (Frizzell & Schultz, 1978; Cass, Finkelstein & Krespi, 1970; Graf & Biebisch, 1979). Upon addition of this antibiotic to the cell "apical" medium, sodium entry into the cells is not limited by the influx through the Na^+ channel, and the cytosolic sodium concentration is similar to that of the external solution. Since this concentration is saturating for the Na^+-pump, the SCC under this condition corresponds to the maximal activity of the Na^+-pump. In the experiments that follow, we have used this technique to measure the maximal Na^+-pump activity.

A6 cells were seeded on permeable membrane cups. The resistance, potential, and SCC across the A6 monolayer increased with time reaching a plateau about eleven days after seeding. At this time, addition of tunicamycin (1 μg/ml) to the "apical" and "basolateral" A6 cell media reduced the SCC to a level which was barely detected 24 hr later (Fig. 1). The decrease in SCC may have been due to inhibition of the Na^+ channel, or the Na^+-pump, or both. As shown below, we established that both the Na^+ channel and Na^+-pump were inhibited by tunicamycin treatment. It should be pointed out that the amount of tunicamycin used in these experiments (1 μg/ml) did not produce a nonspecific inhibition of total protein synthesis (*shown below*).

Figure 1*A* illustrates the time course of treatment of a control A6 cell monolayer. After the SCC current had stabilized, 10 mM amiloride (AM) was added to the "apical" cell bathing medium. The inhibition of the Na^+ channel was immediate and the SCC decreased sharply to zero. Addition of 1 μM amphotericin B (Am-B) to the "apical" cell medium allowed sodium ions to enter the cell and produced a steady increase in SCC. Twenty minutes later, the SCC was 4–5 times higher than that measured before addition of amiloride (*see also* Figs. 2*B* and 3*B*). This is so because in normal A6 cells the limiting path for transepithelial Na^+ transport is the amiloride-sensitive Na^+ channel (Garty & Benos, 1988; Perkins & Handler, 1981). This experiment also illustrates the known fact that the Na^+-pump normally works at about 20% of its level for saturating sodium (compare the SCC before addition of amiloride to that measured after addition of amphotericin B in Figs. 1*A*, 2*B*, and 3*B*).

The SCC of cells treated with 1 μg/ml tunicamycin for 24 hr was close to zero (Fig. 1*B*). This corresponds to an inhibition of about 80–90% with respect to control cells (Fig. 1*A*). Addition of 10 μM amiloride to the "apical" bathing medium reduced the SCC to zero. Subsequent permeabilization of the "apical" cell surface with amphotericin B produced an increment of SCC. However, 20 min later, the SCC (13.8 μAmp/cm^2) was lower than that observed in "control" cells (19.6 μAmp/cm^2) under the same conditions (Fig. 1*A*). The difference corresponds to a 30% inhibition of the Na^+-pump activity. Since, in the absence of amphotericin B, the Na^+-pump is working at 20% of its maximal capacity, this level of inhibition in the maximal Na^+-pump activity would not

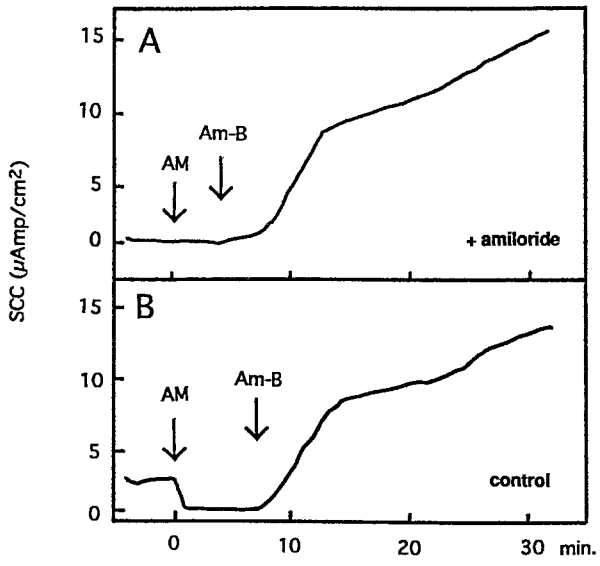

Fig. 3. Effect of 72-hr treatment with amiloride on the maximal Na^+-pump activity. A6 cells were grown to maximal transepithelial resistance on membrane filter cups, and then the cells were treated with (*A*) or without (*B*) 10 μM amiloride. The SCC across the cell monolayer was determined and, at the indicated times, 10 μM amiloride (*AM*) and 1 μM amphotericin B (*Am-B*) were added to the "apical" bathing medium.

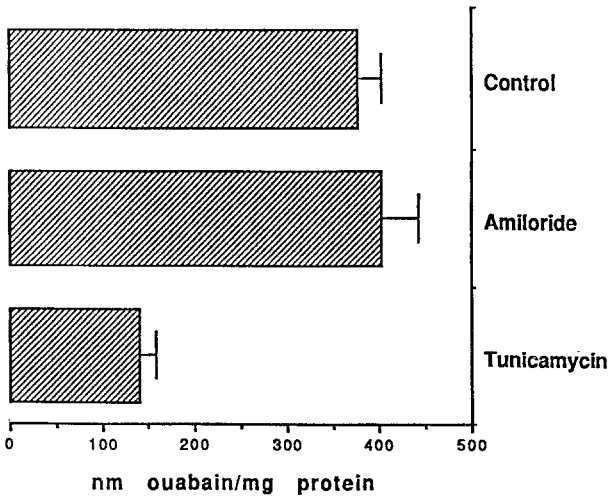

Fig. 4. Effect of tunicamycin on the number of Na^+-pump molecules. The accumulation of Na^+-pump molecules by A6 cells was assessed by the amount of ouabain-binding. A6 cells were treated with either amiloride (10 μM) or tunicamycin (1 μg/ml) for 72 hr and then ouabain binding was measured. The ouabain binding of treated cells is compared to a control from cells maintained in normal medium. Ouabain binding measurements were made in triplicates and repeated four times in control and treated samples. Bars indicate the standard error.

significantly affect the transepithelial Na^+-translocation. This observation points to a diminished Na^+ channel activity as the cause of the SCC inhibition observed in epithelia treated with tunicamycin for 24 hr (Fig. 1*B*).

A6 cells treated with tunicamycin for 72 hr showed greater inhibition of maximal Na^+-pump transport capacity. The profile of SCC of the A6 cell monolayer control (Fig. 2*B*) is very similar to the control for the 24-hr treatment (Fig. 1*A*). As previously observed for 24-hr treatment (Fig. 1*B*), the SCC of cells treated with tunicamycin for 72 hr was greatly inhibited, and addition of 10 μM amiloride to the "apical" medium reduced the SCC to zero (Fig. 2*A*). As expected, addition of amphotericin B to the "apical" medium produced an increase in Na^+-translocation, but the final level of the SCC indicates an 80% inhibition of the maximal Na^+-pump activity.

The sequential inactivation of Na^+ channel and Na^+-pump (*shown in* Figs. 1 and 2) may indicate that the reduced Na^+-pump activity is secondary to the inhibition of the Na^+ entry into the cell. This possibility was tested by measuring the Na^+-pump activity in A6 cells where the Na^+ channel had been inhibited by amiloride. Figure 3 shows that, even though the initial SCC was barely detected in cells treated with amiloride for 72 hr (Fig. 3*B*), the SCC observed after addition of amphotericin B was the same in amiloride-treated (Fig. 3*A*) as in control cells (Fig. 3*B*). This indicates that the Na^+-pump was not inhibited by amiloride treatment.

The Number of Na^+-Pump Molecules is Reduced by Tunicamycin

The reduced maximal Na^+-pump activity in tunicamycin-treated cells was very likely due to a diminished amount of functional Na^+-pump molecules at the cell membrane. The number of Na^+-pump molecules was determined by ouabain binding in entire cells grown on permeable membrane cups. Figure 4 shows that the ouabain binding per mg of protein was 70% reduced in cells treated with tunicamycin for 72 hr. This level of inhibition of ouabain binding corresponds very well with the reduced maximal Na^+-pump activity determined under the same conditions (Fig. 2).

Ouabain binding was also measured in cell extracts prepared from cells grown on plastic. The ouabain binding was 0.23 and 0.09 nmol/mg protein in control and tunicamycin-treated cells, respectively. The ouabain binding determination was also performed in presence of 0.2 mg/ml of SDS. In SDS treated extracts, ouabain binding was 0.78 and 0.29 nmol/mg protein in control and tunicamycin-treated cells, respectively. These values are the mean of two experiments performed in triplicate. Therefore, SDS treatment increased the ouabain binding threefold in both control and tunicamycin-treated cells. However, the degree of inhibition by tunicamycin was the same in the various preparations.

Similar results were obtained by measuring the Na^+, K^+-ATPase activity (Fig. 5). A6 cells were treated with

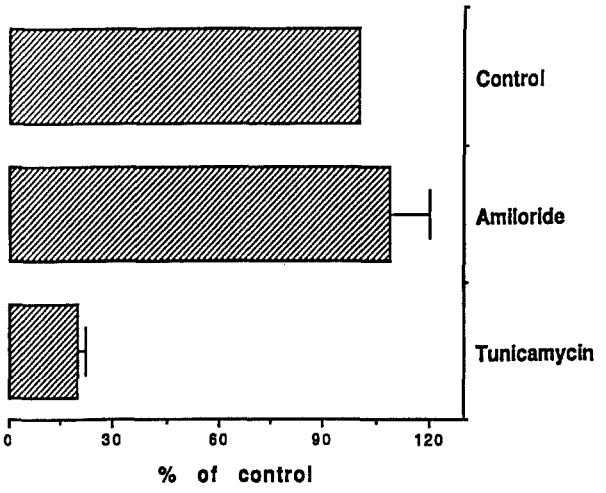

Fig. 5. Effect of tunicamycin-treatment on the Na^+, K^+-ATPase activity. A6 cells were treated with either 10 μM amiloride (*AM*) or 1 μg/ml tunicamycin (tunicamycin) for 72 hr, and the Na^+, K^+-ATPase activity was determined. The activities of treated cells are compared to control cells maintained in normal medium (control). Na^+, K^+-ATPase activity measurements were made in duplicates and repeated four times in control and treated samples. Bars indicate the maximum variance with respect to control (100% activity).

tunicamycin for 72 hr, then cell extracts were prepared and used to determine the Na^+, K^+-ATPase enzyme activity. The Na^+, K^+-ATPase activities were 1.42 and 0.34 μmols of P_i/mg of protein/hr in normal and tunicamycin-treated cells, respectively. Treatment of the membrane preparation with 0.25 mg/ml of SDS, increased Na^+, K^+-ATPase activities to 4.46 and 0.86 μmols of P_i/mg of protein/hr in normal and tunicamycin-treated cells, respectively (Fig. 5). Before and after SDS treatment, the inhibition by tunicamycin of Na^+, K^+-ATPase activity was about 80%. Treatment with SDS opens vesicles and results in the free access of ATP and cations to the catalytic sites on the Na^+, K^+-ATPase (Forbush, 1983; Pedemonte, Sachs & Kaplan, 1990). If A6 cells have an intracellular pool of Na^+, K^+-ATPase (Verrey et al., 1989), it would have been made accessible by SDS treatment. The 80% inhibition of Na^+, K^+-ATPase activity was similar to the reduction in the capacity of the Na^+-pump to transport sodium ions (Fig. 2) and the level of inhibition of ouabain binding (Fig. 4).

The Number of Na^+-Pump Molecules was Unchanged by Amiloride

A6 cells grown on permeable cell membranes were treated with amiloride for 72 hr and then ouabain binding was measured. Figure 4 shows that amiloride-treated cells had a level of ouabain binding similar to control cells. Ouabain binding was also measured in cells grown on plastic and treated with amiloride: 0.27 and 0.65 μmols of ouabain/mg protein in the absence and presence of SDS, respectively. These values are the mean of two experiments performed in triplicate. Accordingly, the Na^+, K^+-ATPase activity measured in A6 cells treated with amiloride for 72 hr was similar to that determined in control cells (Fig. 5). Thus, inhibition of the sodium entry into the cell did not reduce either the number of Na^+-pump molecules at the cell membrane or the total cellular content of Na^+-pump molecules.

Inhibition of Na^+-Pump Synthesis by Tunicamycin

The enhanced inhibition of activity with longer tunicamycin treatment suggests that reduced Na^+-pump activity may be caused by a diminished synthesis of Na^+-pump subunits. Preliminary experiments were performed to determine the best conditions to label and immunoprecipitate the newly synthesized Na^+-pump subunits. Because the antibody appeared to react effectively with the separated α-subunit, we concentrated our efforts on the immunoprecipitation of this subunit. After treatment with various amounts of tunicamycin, A6 cells were metabolically labeled with ^{35}S-Met and the Na^+-pump α-subunit was immunoprecipitated. Aliquots of cell extracts used to do the immunoprecipitation (Fig. 6*A*) contained equal amounts of protein. Figure 6*C* illustrates the relative amounts of α-subunit that were immunoprecipitated at the various concentrations of tunicamycin treatments. Lower amounts of Na^+-pump α-subunit were immunoprecipitated with increasing concentrations of tunicamycin treatment. Figure 6*B* and *C* shows that the synthesis of three proteins with apparent molecular weights of 50 KD, 75 KD, and 100 KD were not affected by tunicamycin treatment. These three proteins were chosen from the fluorography to determine total protein synthesis because they showed well-defined bands. Similarly, the incorporation of ^{35}S-Met in newly synthesized total protein was the same at the various concentrations of tunicamycin used (Fig. 6*B*). Thus, the decreased immunoprecipitation of Na^+-pump α-subunit was not due to reduced total protein synthesis.

Discussion

Impairment of Protein Glycosylation Inhibits the Expression of the Na^+-Pump

Treatment of A6 cells with tunicamycin for 72 hr produced an 80% reduction of both maximal Na^+ transport in apical permeabilized cells and Na^+, K^+-ATPase activity in broken membranes. The reduced activities correlated with a decreased number of Na^+-pump molecules measured by ouabain binding. Na^+, K^+-ATPase activity and ouabain binding were determined under conditions where the total cellular pool of Na^+, K^+-ATPase was exposed. Therefore, the decreased ouabain binding and Na^+-pump activity measured by SCC in tunicamycin-

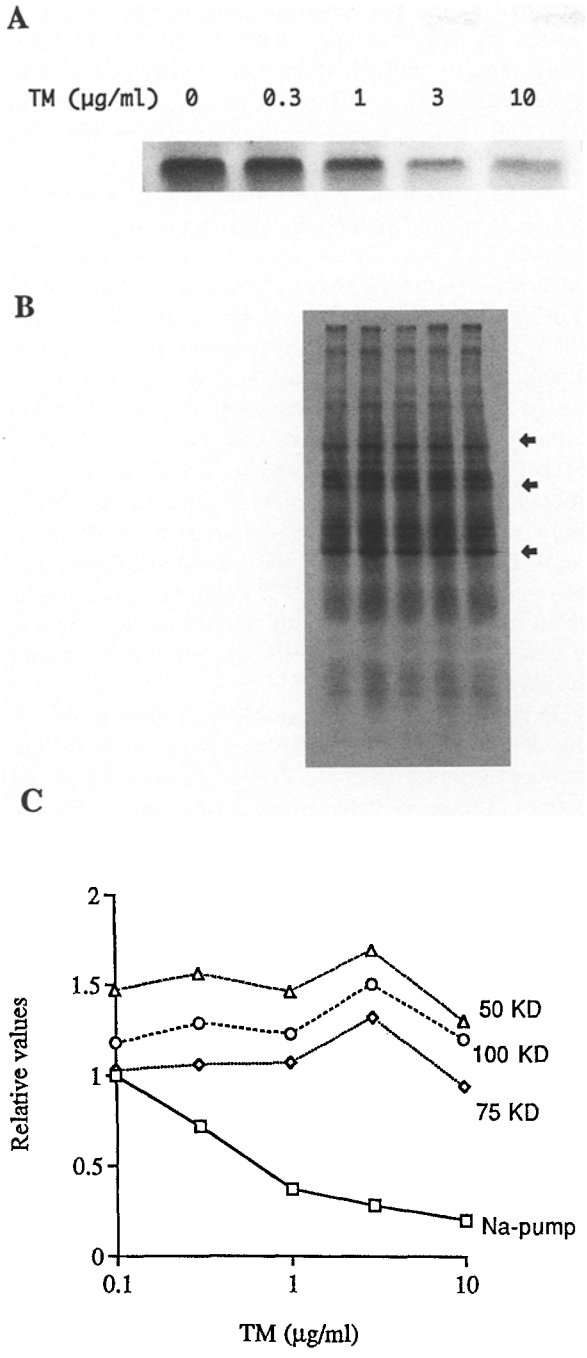

Fig. 6. Effect of various tunicamycin concentrations on the Na^+-pump α-subunit synthesis. (*A*) A6 cells were treated for 18 hr with the indicated concentrations of tunicamycin (*TM*). Then, cells were metabolically labeled with ^{35}S-Met for 30 min, in the presence of tunicamycin. The Na^+-pump α-subunit was immunoprecipitated and analyzed by PAGE and fluorography. To facilitate the comparison of the results, the immunoprecipitation mixture for the various tunicamycin concentrations contained equal amounts of protein. (*B*) Total protein synthesis at various tunicamycin concentrations. To determine total protein synthesis, aliquots were taken from the metabolically labeled cell homogenates before the antibodies were added. The samples were analyzed by PAGE and fluorography. Lanes correspond to the concentrations of tunicamycin indicated above. Arrows indicate the protein bands that were quantitated. (*C*) The fluorography illustrated in Fig. 5*A* was quantitated in a light densitometer and the amounts of Na^+-pump α-subunit immunoprecipitated are shown as relative values (Na-pump). Three proteins (50 KD, 75 KD and 100 KD) that displayed discrete bands in the fluorography of total protein synthesis, as indicated by arrows in Fig. 6*B*, were quantitated. The values shown are related to their optical density.

treated cells was due to a lower cellular content of Na^+-pump molecules and not to an internalization of Na^+-pump molecules from the cell membrane.

Inhibition of protein glycosylation by tunicamycin produced a reduced synthesis of the Na^+-pump α-subunit (Fig. 6). We have previously shown that this subunit has a molecule of N-acetyl glucosamine attached to an amino acid residue, very likely an asparagine (Pedemonte & Kaplan, 1992). We do not know whether tunicamycin inhibits this kind of protein glycosylation. However, we have determined that the antibody recognized the α-subunit before and after deglycosylation with Peptide N-glycosidase F. Thus, the reduced precipitation of the α-subunit after tunicamycin-treatment was not due to an inability of the antibody to recognize the nonglycosylated α-subunit.

It has been shown that the amount of α-subunit synthesized in A6 cells corresponds to the accumulation of Na^+-pump molecules (Geering et al., 1985; Zamofing et al., 1989). It follows that the reduced α-subunit synthesis produced by tunicamycin would result in decreased cellular content of Na^+-pump molecules. Thus, the general pattern of reduced Na^+, K^+-ATPase activity and lower accumulation of Na^+-pump molecules appears to be the same in cultured skeletal muscle and fibroblasts (Alboim et al., 1992), as well as toad urinary bladder (Zamofing et al., 1988, Zamofing et al., 1989), and A6 cells (*present results*).

The Inhibition of Na^+-Pump Expression by Tunicamycin is not due to a Reduced Na^+ Channel Activity

In A6 cells treated with tunicamycin, the SCC was barely detected after 24 hr whereas the Na^+-pump was only 30% inhibited under this condition (Fig. 1*B*). Since the Na^+-pump normally works at about 20% of its maximum capacity (Fig. 1*A*), a 30% inhibition would not significantly affect the SCC. Thus, the SCC inhibition after 24 hr tunicamycin treatment must be exclusively due to an impairment of Na^+ channel activity. Since treatment with tunicamycin for 72 hr further reduced the Na^+-pump activity, the effects of tunicamycin on the Na^+-channel and the Na^+-pump appear to be sequential. It is conceivable, therefore, that a halt of Na^+ entry due to inhibition of the Na^+ channel by tunicamycin could result in a reduced intracellular Na^+ concentration, leading to a down regulation of the Na^+-pump.

The intracellular sodium level is a major regulatory factor of the cellular content of Na^+-pump molecules (Pressley, 1988; Wolitzky & Fambrough, 1986), and Brodie and Sampson (1989) have shown that reduction of intracellular sodium in rat skeletal muscle decreased the number of Na^+-pump molecules. The possibility that a similar situation occurred in tunicamycin-treated cells was explored by treating A6 cells with amiloride. Even though the transepithelial Na^+-transport of amiloride treated cells was exceedingly low, the SCC measured in the presence of amphotericin B (maximal Na^+-pump activity) was at the same level as control A6 epithelia. Accordingly, Na^+, K^+-ATPase and ouabain binding of amiloride-treated cells were the same as those of control cells. It should be taken into account that the results of the experiment illustrated in Fig. 3 indicate that the permeability of tight junctions has not been increased by the amiloride treatment, and that sodium was not entering into the cell through any alternative pathway. Thus, the decreased Na^+-pump activity produced by tunicamycin was not secondary to inhibition of the sodium entry into the cell.

The Role of Protein Glycosylation in the Expression of the Na^+-Pump

There have been some conflicting reports regarding the importance of N-glycosylation on the functional expression of the Na^+-pump. On the one hand, some studies suggest that N-glycosylation is not essential for the functional expression of the Na-pump. Thus, Olden et al. (1979) have shown that inhibition of protein glycosylation did not affect Na^+, K^+-ATPase activity in chick embryo fibroblasts. In the same line, tunicamycin treatment of chick sensory neurons did not alter the Na^+-pump subunit assembly, sorting, membrane delivery, and degradation (Tamkun & Fambrough, 1986). Despite the fact that tunicamycin treatment decreased total protein synthesis by greater than 60%, Tamkun and Fambrough (1986) found that the rates at which individual subunits appeared at the cell membrane were identical for both the glycosylated and nonglycosylated Na^+-pump. These authors did not determine Na^+-pump activity in tunicamycin-treated cells. However, it can be speculated that no inhibition should have been observed, taking into account that (i) the kinetics of Na^+-pump membrane delivery and retrieval were unchanged by tunicamycin; and (ii) the nonglycosylated molecules delivered to the membrane were as active as the glycosylated ones (as described below). That nonglycosylated Na^+-pump molecules are fully active was demonstrated by Takeda et al. (1988). These authors expressed Torpedo californica Na^+-pump α- and β-subunit mRNAs in tunicamycin-treated *Xenopus laevis* oocytes and observed that the "oligosaccharide-deficient ATPase thus synthesized was transported to the oocyte plasma membrane, where it exhibited virtually the same ATPase activity, ouabain-binding capacity and Rb^+-transport activity as the fully glycosylated enzyme." Thus, the results of Tamkun and Fambrough (1986) are very much in agreement with those of Takeda et al. (1988).

On the other hand, a 70% inhibition of the newly synthesized α- and β-subunits accompanied by a 50% decreased Na^+, K^+-ATPase activity was determined in tunicamycin-treated TBM cells (Zamofing et al., 1986; Zamofing et al., 1989). These authors also observed that the lack of carbohydrates affects the interaction of α- and β-subunits since the nonglycosylated α-subunit is more sensitive to trypsin degradation than in fully glycosylated Na^+-pump molecules. Nevertheless, some nonglycosylated Na^+-pump molecules were delivered to the cell membrane where they contributed to the total Na^+-pump activity. The inhibition of Na^+-pump expression by tunicamycin does not appear to be restricted to epithelial cells since Alboim et al. (1992) determined a reduced ouabain binding and Na^+-pump activity in both embryonic chick skeletal muscle cells and fibroblasts treated with tunicamycin.

Geering (1990) has suggested that the results of Tamkun and Fambrough (1986) are very much in line with those of Zamofing et al. (1989). However, there are important differences between these two reports. By assuming that (i) the turnover of the α- and β-subunits are the same, and (ii) the half-life of both subunits is not affected by tunicamycin, Zamofing et al. (1989) calculated a half-life of 17 hr for the Na^+-pump and a delay of 7 hr between the addition of tunicamycin and the time that nonglycosylated Na^+-pump subunits would start to be produced. Then, the level of nonglycosylated β-subunits should have been 43, 61, and 76% of the total polypeptide at the cell membrane after 21 hr, 30 hr and 42 hr of tunicamycin treatment, respectively. However, Fig. 5*B* of Zamofing et al. (1989) illustrates that only 14, 20, and 47% of nonglycosylated β-subunits were detected at those times. These values are more consistent with a $t_{1/2}$ of 45–70 hr. Thus, it is very likely that the Na^+-pump molecules have a longer half-life at the membrane in tunicamycin-treated cells. It is important to notice that this is a major difference between the results of Zamofing et al. (1989) and Tamkun & Fambrough (1986), who observed that the kinetics of Na^+-pump membrane delivery and retrieval were not affected at all by tunicamycin.

In conclusion, the importance of protein glycosylation in the expression of the Na^+-pump activity is still an open question, and more studies would be necessary to resolve the differences. One of the points that emerges from the present investigation is that the inhibition of the Na^+-pump expression produced by impairment of protein glycosylation in epithelial cells is likely a direct effect and not due to reduced entry of sodium into the cell.

Thus, the level of protein glycosylation appears to affect the normal accumulation of Na^+-pump molecules in epithelial cells and may be a mechanism by which cells regulate the optimal number of Na^+-pump molecules that are synthesized and directed to the cell membrane.

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