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Role of the Na,K-ATPase β-Subunit in the Cellular Accumulation and Maturation of the Enzyme as Assessed by Glycosylation Inhibitors

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Summary. No functional role could yet be established for the glycosylated β-subunit of the Na,K-ATPase. In this study, we describe the intracellular processing of the β-subunit as a glycoprotein in toad bladder cells and the consequences of its structural perturbation with glycosylation inhibitors on the cellular expression of the α- and β-subunits and on the structural and functional maturation of the enzyme. Controlled trypsinolysis of homogenates from pulse-labeled cells reveals that the β-subunit is subjected to glycosylation-dependent structural rearrangements during its intracellular routing. Inhibition of correct terminal glycosylation of the β-subunit with deoxynojirimycin or swainsonine has no effect on the trypsin sensitivity of the α-subunit, its ability to perform cation-dependent conformation changes or the cellular Na,K-ATPase activity. Acquisition of core-sugars is sufficient for the enzyme to assume its catalytic functions. On the other hand, complete inhibition of glycosylation with tunicamycin leads to a destabilization of both the β- and the α-subunits as judged by their higher trypsin sensitivity. In addition, tunicamycin treatment results in a decrease of the amount of newly synthesized β- and α-subunit indicating that a glycoprotein, possibly the β-subunit itself, plays a role in the efficient accumulation of the α-subunit in the endoplasmic reticulum.

Key Words Na,K-ATPase · toad bladder cells · protein synthesis · intracellular protein processing · N-linked glycosylation

Introduction

The plasma membrane Na,K-ATPase is responsible for the maintenance of the high K^+ and low Na^+ concentrations typical for the eukaryotic cell interior. In epithelial cells the enzyme is restricted to the basolateral membrane and it generates the major driving force for net Na^+ reabsorption (for review, *see* ref. 22).

Na,K-ATPase purified in its active membrane-bound form is composed of two heterologous subunits; a catalytic α-subunit (112–114 kilodalton) and a N-linked glycoprotein of the complex type, the β-subunit [11, 22, 33, 40]. Both α- and β-subunits from different species have recently been cloned [24, 30, 31, 32, 34, 37][1] and sequence data as well as proteolytic cleavage studies on the purified enzyme have revealed that the β-subunit spans the membrane once [30, 37] and the α-subunit 6 to 8 times [24, 29, 38].

Although it is well established that the α-subunit carries the main catalytic properties of the enzyme (for review, *see* ref. 22), the minimal functional enzyme unit is an α- and β-protomer [2]. Nothing is known, however, of the precise role of the β-subunit in the catalytic cycle. This lack of information on the inherent functional properties of the β-subunit is mainly due to the fact that, so far, it was not possible to reconstitute a functionally active enzyme from solubilized subunits (for review, *see* ref. 3) or else, to selectively digest the β-subunit with proteases without concomitant hydrolysis of the α-subunit [5, 23].

The specific function of the glycomoiety of the β-subunit is also unknown. The same β-corepeptide is differently glycosylated in different tissues of the same species (for review, *see* ref. 8) and removal of terminal sialic acids with neuraminidase does not impair the enzymatic properties of the purified Na,K-ATPase [5, 6]. These observations suggest that the carbohydrates of the β-subunit might have another function than to support the catalytic cycle. The question arises whether the β-subunit and/or its carbohydrates might play a role in the membrane insertion, the maturation or the sorting of the enzyme.

In the intact cell, the α-subunit undergoes a structural rearrangement early during its intracellular transport which could be responsible for its func-

[1] F. Verrey, P. Kairuz, E. Schaerer, P. Fuentes, T.R. Kleyman, K. Geering, B.C. Rossier and J.P. Kraehenbuhl. Anti-sodium pump antibodies identify three distinct gene products in an amphibian kidney cell expression library. Primary sequence of both Na,K-ATPase subunits and characterization of an apical plasma membrane protein. (*Submitted for publication*)

tional maturation [10]. The α-subunit indeed acquires the ability to perform cation-dependent conformation changes in parallel with an increasing trypsin resistance [10].

In view of these data, we investigated whether the β-subunit and/or its glycomoiety assist the newly synthesized α-subunit to become correctly and stably expressed in the cell and whether they support the functional maturation of the enzyme. To reveal such possible functional properties, we have perturbed the structural integrity of the β-subunit with different drugs that interfere at different stages with the glycosylation processing. Controlled trypsinolysis of cell homogenates of pulse-labeled cultured cells pretreated with tunicamycin, deoxynojirimycin or swainsonine revealed that acquisition of core-sugars is essential for a correct structural maturation, not only of the β-subunit but also of the α-subunit. In addition, Na,K-ATPase activity measurements indicate that the enzyme is already functionally active when the β-subunit is only coreglycosylated.

Materials and Methods

Chemicals

Tunicamycin (TM), DNase (type I), trypsin (type XI) and soybean trypsin inhibitor were purchased from Sigma; swainsonine (SW) was from Calbiochem; L-^{35} S-methionine (specific activity > 1000 μCi/mmol) was from Amersham Corp.; ^{125}I-Protein A (2–10 μCi/μg) was from New England Nuclear; Endo H was from Seikagaku Kagyo Co. Ltd.; 1-deoxynojirimycin (dNM) was a generous gift from Prof. Dr. Schlumberger, Bayer AG, Wuppertal, FRG; glycopeptidase F was from Boehringer.

Cell Lines

TBM cells (derived from the urinary bladder of *Bufo marinus*) were obtained from J.S. Handler, National Institute of Health, Bethesda MD. Cells were cultured as described by Handler [15].

Treatment of Cells with Glycosylation Inhibitors

The effects of glycosylation inhibitors on the posttranslational processing of α- and β-subunits were assessed in TBM cells grown to confluency on petri dishes (63 cm^2) by treatment with 5 μg/ml of TM for 18 hr, with 1 μg/ml of SW for 14 hr, or with 2 mM of dNM for 1 hr. These conditions, as determined in preliminary experiments, were optimal to observe the expected modifications of β-subunit glycosylation. When the effect of prolonged incubation with glycosylation inhibitors on cellular Na,K-ATPase activity was studied, TBM cells were incubated for up to 42 hr with TM, SW, or dNM.

Labeling, Homogenization and Controlled Trypsinolysis

TBM cells, pretreated or not with glycosylation inhibitors, were labeled with L-^{35} S-methionine in 5 ml culture medium (HAM, ref. 16) without serum and without methionine, at 28°C. Three pulse protocols were chosen: 15-min pulse with 400 μCi/ml, 30 min with 250 μCi/ml, and 4 hr with 100 μCi/ml L-^{35} S-methionine. In drug-pretreated cells, the inhibitors were present during the labeling period. Incorporation of radioactive precursor was stopped on ice by the addition of HAM medium containing 1 mM methionine and 20 μg/ml cycloheximide. Cells were scraped, washed twice with HAM and once with a homogenization buffer A containing DL-histidine (30 mM), EDTA (5 mM), Tris/HCl (18 mM, pH 7.4) and sucrose (200 mM). The pelleted cells were taken up in buffer B (= buffer A without sucrose) containing 0.1 mg/ml DNase, and submitted on ice to mild sonication (3 times for 3 sec in a Branson sonifier, position 4). The cell homogenates were subjected to controlled trypsinolysis as described previously [10]. Digestion proceeded for 1 hr on ice in buffer B containing 140 mM K- or Na-acetate, and in the presence of 0.1% deoxycholate (wt/vol) in order to open up all membrane vesicles produced during sonication. Trypsin-to-protein ratios (wt/wt) were between 0.06 and 0.25, and deoxycholate (wt/wt)-to-protein ratios between 0.15 and 0.62. Trypsinolysis was stopped with a fivefold excess (wt/wt) of soybean trypsin inhibitor for 10 min on ice. Samples were heated at 95°C in 3.7% SDS (final concentration) prior to immunoprecipitation. Protein [26] and specific radioactivity contents were determined on aliquots precipitated with trichloroacetic acid and resolubilized with 1 N NaOH.

Immunoprecipitation and Quantitation

Immunoprecipitation of α- and β-subunits and of their tryptic products from TBM cells was carried out with antisera prepared against purified subunits from the kidney of *Bufo marinus* [14]. Immunoprecipitation of the 30 kD polypeptide was carried out with an antiserum prepared against purified 30 kD protein. The 30 kD protein is commonly observed in SDS-purified Na,K-ATPase preparations of the kidney of *Bufo marinus* [12]. Its purification was achieved by treating microsomal fractions of toad kidney with 1.2 mg SDS/ml and subsequent centrifugation on sucrose gradients, according to the purification procedure used for Na,K-ATPase [12, 14]. With these high detergent concentrations, the α- and β-subunits of Na,K-ATPase were completely solubilized, leaving only the 30 kD protein in a membrane-bound form.

Immunoprecipitation of citrate synthase was performed with an antiserum prepared against commercially available purified citrate synthase (Sigma). Anti-30 kD- and anti-citrate-synthase sera were obtained from rabbits as previously described [14].

Immunoprecipitation protocol, SDS-PAGE and detection by fluorography were carried out as previously described [9, 11]. Quantitation of the immunoprecipitated material was performed by laser densitometry of fluorograms or by light scattering of silver grains quantitatively eluted with 1 N NaOH from the bands on the fluorograms, as described by Suissa [39]. This latter method permitted the determination of the actual content of radioactivity in bands of differently glycosylated β-subunits for which peak heights were not a reliable measure of the immunoprecipitated amount, due to their diffuse appearance on SDS-PAGE.

In order to compare the amount of fully glycosylated β-subunit synthesized in control cells to the amount of nonglycosylated β-subunit synthesized in TM-treated cells, we checked whether our polyclonal antiserum recognized the two forms to a similar extent. For this purpose, we treated SDS-denatured and acetone-precipitated cell extracts derived from 4-hr pulse-labeled cells with or without 6 U glycopeptidase F/ml for 18 hr at 37°C in 0.25 M KH_2PO_4 (pH 7.4), 50 mM EDTA, 1% β-mercaptoethanol. With these digestion conditions, 75% of the β-subunit was recovered after immunoprecipitation in its completely deglycosylated form. The signal produced of this corepeptide on autoradiographies made up 75% of the signal produced by the fully glycosylated β-subunit. This result indicates that our antiserum exclusively contained antibodies against the proteinic portion of the β-subunit.

Na,K-ATPase Enzyme Assay

TBM cells were frozen in liquid nitrogen, then homogenized with a glass-Teflon® homogenizer with 20 strokes at 2000 revolutions/min in buffer A supplemented with 2 mM PMSF. Aliquots of 20 μg of protein, frozen and thawed 3 times in liquid nitrogen were assayed for ATPase activity by measuring release of inorganic phosphate from ATP in the presence or absence of ouabain [9, 19]. During the incubation for 1 hr at 37°C, the rate of ATP hydrolysis remained constant. Na,K-ATPase activities in control cells ranged between 3 to 4 μmol P_i · mg protein^{-1} · hr^{-1}, representing about 20% of the total ATPase activity (measured in the absence of ouabain).

Determination of the Cellular Pool of α-Subunit

In order to correlate Na,K-ATPase activities measured in cell homogenates with the cellular content of catalytic α-subunit, aliquots of 12 μg of protein were run on 5–13% SDS polyacrylamide gels and transferred onto nitrocellulose filters [41]. After 1 hr incubation in phosphate-buffered saline (PBS) containing 5% (wt/vol) milk powder [20], the blots were incubated overnight with anti α-subunit serum diluted 1:50 in the same solution. After several washes with 0.5% (vol/vol) Tween 20 in PBS containing 5% milk powder, bound antibodies were overlayed for 1 hr with ^{125}I-Protein A at 0.2 μCi/ml. The blots were then washed as above, rinsed with water, dried and exposed for autoradiography. The revealed bands were quantitated by laser densitometry.

Results

Postranslational Processing of the β-Subunit of Na,K-ATPase

Normal and Perturbed N-Linked Glycosylation of the β-Subunit

The first issue of this study was to elucidate the normal processing of the β-subunit as a glycoprotein during its intracellular routing and to test the action of glycosylation inhibitors in view of their use as experimental tools to perturb the structural integrity of the β-subunit.

N-linked glycosylation processing in eukaryotic cells starts with the transfer of a core-sugar (Glc_3 Man_9 $GlcNAc_3$) from a dolichol precursor to susceptible asparagine residues on the nascent polypeptide in the ER. This high-mannose form which is susceptible to endoglycosidase H (Endo H) digestion rapidly loses the three terminal glucose residues by the action of glucosidases I and II. During the transport through the ER and the Golgi, the glycoprotein is further trimmed by ER and Golgi mannosidases until it reaches medial or trans-Golgi compartments where complex sugars are added to the glycoprotein. This mature form is insensitive to Endo H digestion (for review, *see* refs. 25 and 35). Recently, several drugs have been described which interfere at different stages with this glycosylation process and which thus became useful tools to study the functional role of the carbohydrate moiety of glycoproteins (for review, *see* ref. 7).

In the present study, we have used 1) tunicamycin (TM), which inhibits the synthesis of the dolichol precursor and thus results in the synthesis of a nonglycosylated corepeptide (for review, *see* ref. 7), 2) deoxynojirimycin (dNM), which inhibits glucosidases I and II and thereby prevents trimming and complex glycosylation [34, 36], and 3) swainsonine (SW), which inhibits mannosidase II and which prevents trimming of one of the mannose branches [42]. Complex type glycosylation can in this case only occur on the second fully trimmed mannose branch which leads to hybrid glycosylated forms (for review, *see* ref. 7).

Figure 1 illustrates the results obtained for the N-linked glycosylation processing of the β-subunit in control and in TM, dNM- or SW-treated cells labeled with ^{35}S-methionine for various times. In control cells, the immunoprecipitated β-subunit was recovered on SDS-PAGE as a 42 kD protein up to 30 min of pulse (Fig. 1, lanes 2 and 5). On the other hand, after a 4-hr pulse, most of the β-subunit was immunoprecipitated as a large band of about 60 kD and small amounts as a 41 kD band (Fig. 1, lane 7). In contrast to the 60 kD form which was resistant to Endo H digestion (Fig. 1, lane 8), the 41 kD form detected after a 4-hr pulse (Fig. 1, lanes 7 and 8) and the 42 kD form detected up to 30 min after synthesis (Fig. 1, lanes 5 and 6) were cleaved by Endo H into a 32 kD peptide. From these data we conclude that the 42 kD and the 41 kD species are the core-glycosylated and trimmed forms, respectively, while the 60 kD is the mature complex-glycosylated β-subunit. The 32 kD form of the β-subunit obtained by Endo H treatment of the 41 and 42 kD species (Fig. 1, lanes 6 and 8) was very similar to

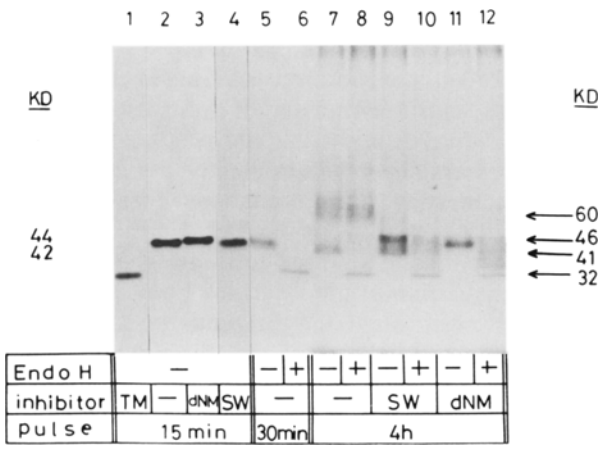

Fig. 1. Processing of the β-subunit with N-linked sugars. TBM cells in culture treated or not with tunicamycin (TM, 5 μg/ml for 18 hr), 1-deoxynojirimycin (dNM, 2 mM for 1 hr), or swainsonine (SW, 1 μg/ml for 14 hr) were labeled with ^{35}S-methionine for 15 min, 30 min or 4 hr. Cell extracts were prepared, immunoprecipitated with anti β-serum, treated or not with Endo H (10 mU/ml, 18 hr at 37°C) and resolved by SDS-PAGE and fluorography as described in Materials and Methods. The molecular masses indicated are mean values of two to seven experiments and were determined with respect to protein standards of known molecular mass; 60 kD: 60,000 dalton, for example

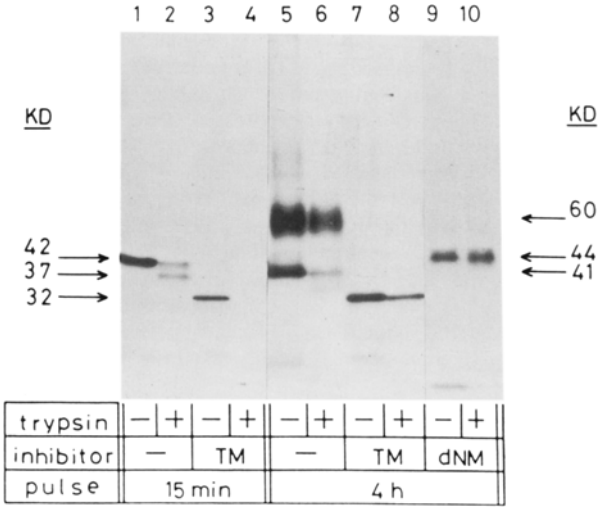

Fig. 2. Structural rearrangement of β-subunit during intracellular routing. TBM cells were treated with TM and dNM and labeled with ^{35}S-methionine as in Fig. 1. Cell extracts were subjected to controlled trypsinolysis at a trypsin-to-protein ratio of 0.2 and in the presence of 0.1% deoxycholate. Detection of immunoprecipitated material was as described in Fig. 1 and Materials and Methods

the form immunoprecipitated from cells pretreated for 18 hr with TM (Fig. 1, lane 1). This form thus represents the corepeptide of the β-subunit.

From dNM-treated cells, labeled for 15 min or 4 hr, a 44 kD species was immunoprecipitated (Fig. 1, lanes 3 and 11) which was Endo H sensitive (Fig. 1, lane 12). This form was slightly bigger than the core-glycosylated form detected in control cells after a 15-min pulse (compared Fig. 1, lanes 2 + 3) or even after a 5-min pulse (*data not shown*), which indicates that in control cells the terminal glucose residues are rapidly cleaved from the core-sugars either during or soon after synthesis of the polypeptide.

Finally, two differently glycosylated species were recovered from SW-treated cells labeled for 4 hr; a 41 kD form, similar to the minor form detected in control cells (compare Fig. 1, lanes 7 and 9) and a 46 kD form (Fig. 1, lane 9), which was only partially sensitive to Endo H digestion (Fig. 1, lane 10). Thus, this latter form probably represents a partially trimmed and incompletely reglycosylated species. As shown in Fig. 1, lane 4, a normal core-glycosylated form was recovered from SW-treated cells labeled for 15 min. This indicates that the newly synthesized β-subunit had not yet reached the site of action of SW in a *cis* or medial Golgi compartment.

Structural Rearrangement of the β-Subunit During Intracellular Transport

One potential role of the sugar moiety of membrane glycoproteins is to confer stability to the protein structure (for review, *see* ref. 31). We have checked whether addition or modifications of carbohydrates are involved in a structural rearrangement of the β-subunit during its intracellular transport. To probe the native conformation of the β-subunit in different cellular compartments and in differently glycosylated states, TBM cells were pretreated or not with TM, dNM or SW. The cells were labeled for different times with ^{35}S-methionine and controlled trypsinolysis was performed on cell homogenates.

Figure 2 shows that the β-subunit is subjected to glycosylation-dependent structural reorganizations during its intracellular routing. Indeed, trypsin-resistance appeared to increase with increasing complexity of glycosylation. In control cells, the core-glycosylated β-subunit analyzed after a 15-min pulse was fairly trypsin sensitive and gave rise to two proteolytic fragments (Fig. 2, lanes 1 and 2). The 40 kD fragment was similar to the tryptic fragment obtained from the β-subunit synthesized in vitro in the presence of rough microsomes [11] and is most likely produced by cleavage of the short N-terminus exposed to the cytoplasmic side of the

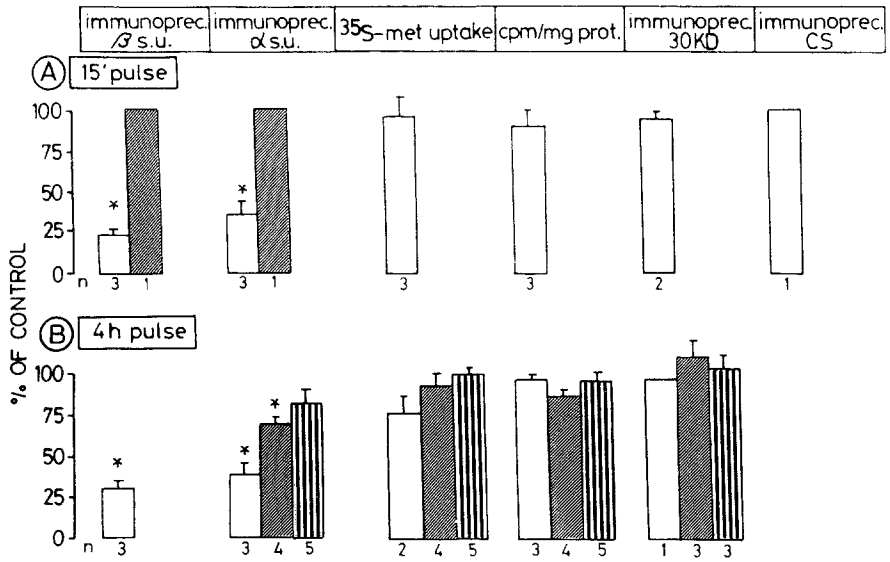

Fig. 3. Effects of TM, dNM and SW on protein synthesis and on the cellular accumulation of β- and α-subunits of Na,K-ATPase. TBM cells were treated or not with TM (□), dNM (▨) or SW (▥) as in Fig. 1 and labeled for 15 min (*A*) or 4 hr (*B*) with ^{35}S-methionine. The difference in the number of counts in the medium before and after the pulse is referred to as ^{35}S-methionine uptake in the Figure. Cell extracts were prepared and total TCA precipitable counts and protein content were determined. From these data, we calculated the specific radioactivity (cpm/mg protein) as a measure of total protein synthesis. β-subunit (β s.u.), α-subunit (α s.u.), 30 kD protein (30 kD) and citrate synthase (CS) were immunoprecipitated by using the same amount of proteins of control and drug-treated material. Quantitation of immunoprecipitated material was performed as described in Materials and Methods. Shown are single values or mean values of two to five experiments ± SE representing the percentage of immunoprecipitated material compared to untreated controls. $^*P < 0.05$, Student's *t*-test: Drug-treated samples *vs.* control

membrane. The 37 kD fragment probably results from cleavage of a tryptic site in the large extracytoplasmic part of the β-subunit which was made accessible in the present study by the opening of membrane vesicles with small amounts of detergent added during trypsinolysis. On the other hand, the fully glycosylated 60 kD β-subunits assessed after a 4-hr pulse was much more trypsin resistant and proteolytic fragments could no longer be produced (Fig. 2, lanes 5 and 6). In addition, inhibition of glycosylation with TM rendered the polypeptide extremely trypsin sensitive. Under the experimental conditions used, the β-subunit was completely digested, at least when the polypeptide was assessed after a 15-min pulse (Fig. 2, lanes 3 and 4) or a 30-min pulse (*data not shown*). After a 4-hr pulse, however, the nonglycosylated β-subunit became trypsin resistant (Fig. 2, lanes 7 and 8). This result could indicate that the β-subunit had been subjected to structural rearrangements during the 4-hr pulse which are independent of its glycosylation. This inference would be supported by the fact that the 44 kD core-glycosylated form produced in the presence of dNM and assessed in a 4-hr pulse was more trypsin resistant (Fig. 2, lanes 9 and 10) than the very similar core-glycosylated form from control cells assessed after 15 min (Fig. 2, lanes 1 and 2).

Effects of Glycosylation Inhibitors on the Cellular Accumulation of α- and β-Subunits of Na,K-ATPase

To assess the importance of the β-subunit for an efficient cellular accumulation of the Na,K-ATPase, we perturbed the structural integrity of the β-subunit with glycosylation inhibitors and measured the amounts of β- and α-subunits immunoprecipitable from cells labeled for 15 min or 4 hr.

As can be seen in Fig. 3(*A*), in cells treated with TM and labeled for 15 min, the immunoprecipitable amount of both the nonglycosylated β-subunit *and* the α-subunit is decreased by about 70% compared to nontreated control cells. This reduction was not due to an overall inhibition of protein synthesis since 1) the uptake of labeled precursor into TM-treated cells was comparable to nontreated cells (Fig. 3*A*) and 2) the incorporation of ^{35}S-methionine into total proteins (Fig. 3*A*) or into two specific polypeptides, a 30 kD nonglycosylated membrane protein and the mitochondrial citrate synthase, was the same in TM-treated and control cells (Fig. 3*A*). After a 4-hr pulse, α-subunit synthesized in the presence of TM still represented about 30% of controls (Fig. 3*B*) indicating that no further degradation of the polypeptide had occurred during intra-

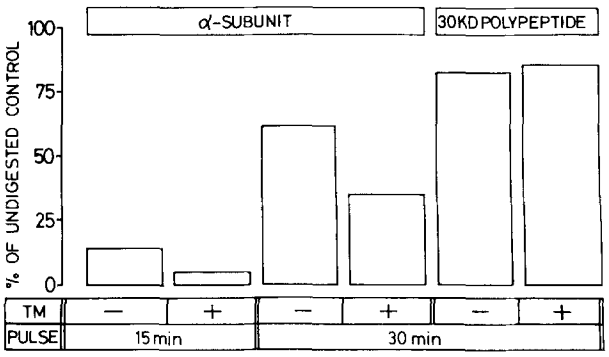

Fig. 4. Effect of TM on the structural maturation of the α-subunit. TBM cells were treated or not with TM and labeled for 15 or 30 min, with ^{35}S-methionine as described in Materials and Methods. Cell extracts were prepared and submitted to controlled trypsinolysis at a trypsin-to-protein ratio of 0.06 and in the presence of 0.1% deoxycholate. Tryptic products were recovered and quantitated on fluorograms as described in Materials and Methods. Shown is the percentage of α-subunit related material or of 30 kD polypeptide resisting trypsinolysis compared to undigested controls

cellular transport and that the effect of TM on the accumulation of α- and β-subunits must occur during or rapidly after protein synthesis. SW had no significant effect on any of the parameters measured, after a 15-min (*data not shown*) or a 4-hr pulse (Fig. 3*B*). On the other hand, dNM slightly but consistently reduced the accumulation of newly synthesized α-subunit measured after a 4-hr pulse (Fig. 3*B*). Since the amount of newly synthesized α- and β-subunits assessed after a 15-min pulse was not affected by dNM treatment (Fig. 3*A*), this result suggests that, in contrast to the α-subunit associated to nonglycosylated β-subunit (Fig. 3*A* and *B*), the α-subunit associated to the coreglycosylated β-subunit is subjected to partial degradation.

Effects of Glycosylation Inhibitors on the Structural and Functional Maturation of the α-Subunit

Tamkun and Fambrough [40] have recently shown in chick neurons that inhibition of core-glycosylation of the β-subunit does not impede its association to the α-subunit or its transport to the plasma membrane. We wondered whether in TBM cells the α-subunit synthesized concurrently with nonglycosylated or aberrantly glycosylated β-subunit is structurally perturbed or not and whether it acquires functional properties.

To assess the structural properties of the α-subunit, we subjected cell homogenates to controlled trypsinolysis before immunoprecipitation and compared the recovery of the α-subunit related, trypsin resistant material from control and TM-treated cells.

In control cells labeled for 15 min, the α-subunit was highly trypsin sensitive. Only about 12% resisted proteolytic cleavage in the experimental conditions used (Fig. 4). With longer pulses, the α-subunit became more trypsin resistant, e.g. after a 30-min pulse, about 60% of the α-subunit was recovered after trypsinolysis. The α-subunit synthesized in the presence of TM also acquired partial trypsin resistance with longer pulses but it always remained lower than that of the α-subunit of control cells (Fig. 4). In contrast, the 30 kD membrane protein showed the same trypsin resistance when synthesized in the presence or absence of TM. Thus, our data indicate that inhibition of core-sugars of the β-subunit affects the structural organization of the α-subunit either since assembly of the two subunits is retarded and/or since association of the two subunits is less stringent.

To assess whether the α-subunit synthesized in parallel with non- or aberrantly glycosylated β-subunit acquires functional properties, we checked the ability of the α-subunit to perform cation-dependent conformation changes [21] and measured Na^+- and K^+-dependent ATPase activity in TM-, dNM- and SW-treated cells.

Recently, we have shown that, soon after synthesis, the α-subunit becomes able to change its conformation in response to cations as reflected by the production of a different tryptic pattern in the presence of Na^+ and K^+ [10]. In this study, we tested this functional property after a 4-hr pulse in control and drug-treated cells. As expected, in control cells the α-subunit was preferentially cleaved into an 83-kD fragment in the K^+ form but never in the Na^+ form (Fig. 5, lanes 1–3). With higher trypsin concentrations, the Na^+ form produced exclusively a 73-kD fragment (*data not shown*).

The α-subunit synthesized in the presence of TM produced similar tryptic cleavage products in the presence of K^+ or Na^+ as in controls (Fig. 5, lanes 4–6). However, about 50% less α-subunit related material could be recovered in these tryptic fragments than in those of controls (compare Fig. 5, lanes 5 and 6 to lanes 2 and 3). These data indicate that the α-subunit from TM-treated cells is more trypsin sensitive than that from control cells, even after a 4-hr pulse and thus probably in a structurally relaxed form. Finally, in SW- and dNM-treated cells, the α-subunit was the same as in controls with respect to both its trypsin resistance and its cation-specific tryptic fragmentation (Fig. 5, lanes 7–12).

In a further attempt to assess the role of glycosylation of the β-subunit in the catalytic cycle of the Na,K-ATPase, we incubated TBM cells for 21 to 42

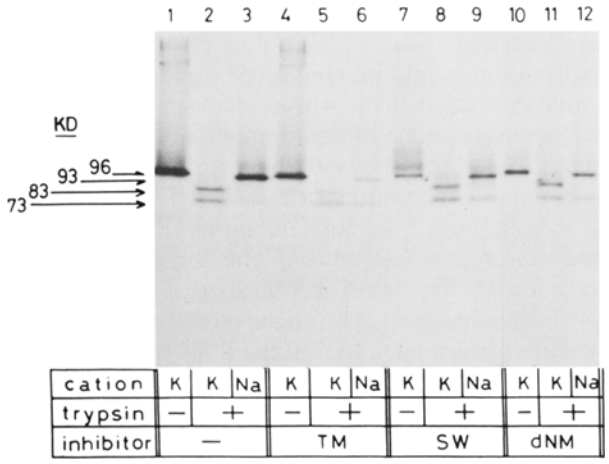

Fig. 5. Conformational changes of the α-subunit is synthesized in the presence of TM, SW or dNM. TBM cells were treated or not with TM, SW or dNM and labeled for 4 hr with ^{35}S-methionine as described in Materials and Methods. Cell extracts were submitted to controlled trypsinolysis, in the presence of 140 mM K- or Na-acetate, at a trypsin-to-protein ratio of 0.2 in the presence of 0.1% deoxycholate. Tryptic products were immunoprecipitated with anti-α serum and revealed by SDS-PAGE and fluorography. Lanes 1–6: 130 μg protein were immunoprecipitated; lane 7: 60 μg; lanes 8–12: 120 μg

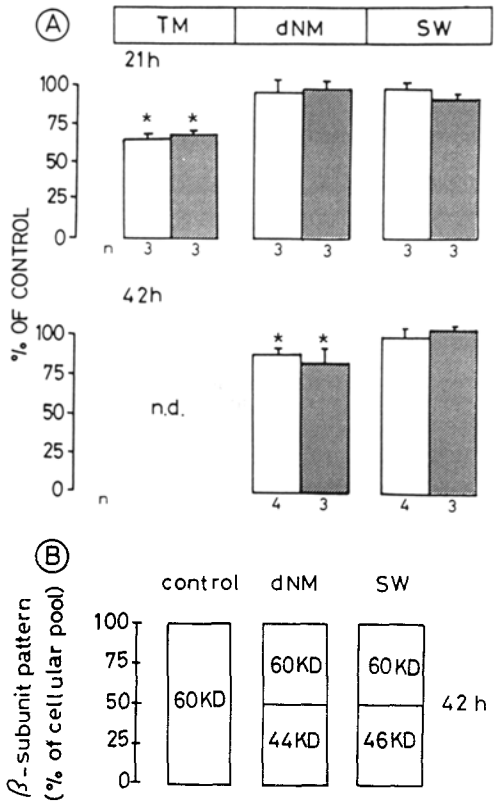

Fig. 6. Effects of TM, SW and dNM on the cellular Na,K-ATPase pool and activity. (*A*) Cellular α-subunit pool and Na,K-ATPase activity. TBM cells were treated with TM (5 μg/ml), dNM (2 mM) or SW (1 μg/ml) for 21 or 42 hr. Cells were collected, homogenized and both the cellular α-subunit pool (▨) and Na,K-ATPase activity (□) were determined as described in Materials and Methods. Shown are mean values (% of untreated controls) of three to four experiments, ± SE. Absolute values for Na,K-ATPase activity were comprised between 3 to 4 μmol P_i · mg protein^{-1} · hr^{-1}. n.d. = not determined. $^*P < 0.05$, Student's *t*-test: Drug-treated samples *vs.* control. (*B*) Effect of dNM and SW on the glycosylation pattern of the cellular β-subunit pool. TBM cells were labeled with ^{35}S-methionine for 42 hr, and then labeled for a further 42 hr in the presence of dNM (2 mM) or SW (1 μg/ml). Cell extracts were prepared, immunoprecipitated with anti-β- serum and revealed by SDS-PAGE and fluorography. The distribution of the radioactivity between the different forms of the β-subunit (expressed as a percentage of the total cellular pool) was calculated after light scattering of silver grains eluted from fluorograms. 60 kD = 60,000 dalton polypeptide, for example

hr with TM, SW and dNM to permit a substantial replacement of the cellular Na,K-ATPase pool with non- or aberrantly glycosylated species of the Na,K-ATPase. In these pretreated cells, we measured Na^+- and K^+-dependent ouabain-inhibitable ATPase activity and compared it to the total cellular content of the α-subunit and to the relative proportions present of normally and abnormally glycosylated β-subunit species.

In cells treated for 21 hr with TM, the total cellular content of the α-subunit determined by immunoblots of cell homogenates was decreased by about 35%, compared to controls (Fig. 6). This decrease probably reflects the inhibitory effect of TM on the cellular accumulation of α-subunit as assessed by a 15-min pulse (Fig. 3). The specific Na,K-ATPase activity was reduced to a similar extent as the α-subunit pool in these cells (Fig. 6*A*). Unfortunately, cells could not be exposed for 42 hr to TM since this long treatment led to detachment of the cells from the plastic dish. In cells pretreated with dNM or SW which had no general effects on cell metabolism up to 42 hr the Na,K-ATPase activity corresponded also to the measured α-subunit pool size (Fig. 6*A*).

In order to determine the proportion of aberrantly glycosylated β-subunits associated to the measured α-subunit pool in drug-treated cells, we pulsed TBM cells for 42 hr with ^{35}S-methionine, in order to label the total cellular Na,K-ATPase pool, and continued the pulse for an additional 42 hr in the presence of SW or dNM. After immunoprecipitation of cell homogenates with anti-β-serum, we determined the relative amounts of differently glycosylated β-subunit species by light scattering of eluted silver grains from fluorograms. Figure 6(*B*) shows that, in contrast to control cells in which the β-subunit was exclusively recovered in its fully gly-

cosylated form of 60 kD, in dNM- and SW-treated cells, about 50% of the immunoprecipitated material was represented by the core-glycosylated (44 kD) and the hybrid type (46 kD) species of the β-subunit, respectively (Fig. 6*B*). Thus, by exposing cells for 42 hr to dNM and SW, it was possible to fill up half of the cellular Na,K-ATPase pool with aberrantly glycosylated β-subunits. Since specific Na,K-ATPase activity remained comparable to control cells (Fig. 6*A*), we conclude from these data that β-subunit trimming and complex-type glycosylation are not required for the enzyme to perform its catalytic cycle.

Discussion

In the present study, we have made use of glycosylation inhibitors to examine the intracellular processing of the β-subunit of Na,K-ATPase and to assess the functional role of this subunit and its glycomoiety in the structural and functional maturation of the enzyme in TBM cells.

N-Linked Glycosylation in the Structural Maturation of the β-Subunit

Previous studies have shown that the β-subunit of Na,K-ATPase is a N-linked glycoprotein which is processed from a core-glycosylated to a complex-type glycosylated form within 60 min after synthesis [10, 40]. In this study, we have examined whether glycoprotein processing is implicated in the structural maturation of the β-subunit. Our data indicate that the β-subunit is subjected to glycosylation-dependent structural reorganizations during its intracellular transport. The nonglycosylated β-subunit synthesized in the presence of TM was much more trypsin sensitive than the core-glycosylated or the fully glycosylated form (Fig. 2). Thus, it seems that acquisition of core-sugars is important for a correct folding of the β-chain, or else shields the protein from proteolytic attack as has been suggested for other glycoproteins (for review, *see* refs. 7, 13). Interestingly, however, when the β-subunit in TM-treated cells was assessed after long pulses, even the nonglycosylated peptide became trypsin resistant (Fig. 2). One explanation for this result is that the trypsin resistance is conferred to the β-subunit by the assembly to the α-subunit. In TM-treated cells, this event might be retarded leading to the observed delay in the acquisition of trypsin resistance of the nonglycosylated β-subunit. Another explanation would be that the nonglycosylated β-subunit had moved to another cellular compartment which permitted a glycosylation-independent structural rearrangement of the polypeptide. Such an interpretation would be supported by the finding that nonglycosylated β-subunit in chick neurons indeed reaches the plasma membrane [40] and implies that sugars do not serve as signals to direct the β-subunit to its final localization.

Though our results support the idea that acquisition of core-sugars permits a stable membrane integration of the newly synthesized β-subunit, the role of the complex-type sugars remains open. Further stabilization of the intracellularly transported polypeptide might in fact not be necessary. Most of the mass of the β-subunit is indeed exposed on the luminal side of the intracellular compartment [30, 37] and thus protected from cellular proteases. On the other hand, an extensive protection might again become important when the polypeptide is expressed in the plasma membrane and exposes major domains to the extracellular medium. Complex type sugars might assume this role. Further studies on the half-life of nonglycosylated or core-glycosylated β-subunit expressed in the plasma membrane are needed to support this hypothesis.

Inhibition of Core-Glycosylation Prevents Efficient Cellular Accumulation of α- and β-Subunits of Na,K-ATPase

In the absence of any significant effects of total protein, or on specific nonglycosylated protein synthesis, TM treatment of TBM cells led to a decrease in the amount of newly synthesized β-subunit (Fig. 3). This result might be explained by a rapid degradation of the polypeptide after its synthesis since, as discussed above, the nonglycosylated β-subunit is indeed highly sensitive to proteolysis. Alternatively, a regulatory link might exist between glycosylation and protein synthesis as has been suggested for certain other glycoproteins [17, 27, 28]. In any case, this result supports the hypothesis that core-glycosylation has an essential role in the correct and stable accumulation of the β-peptide.

Interestingly, the amount of α-subunit, which is not glycosylated, was decreased to a similar extent as the β-subunit (Fig. 3). At present, we have no definite explanation for this phenomenon but the result suggests that an efficient cellular accumulation of the α-subunit depends in some way on the accumulation of the β-subunit. Tamkun and Fambrough [40] have shown that the α- and the β-subunits assemble during or at least soon after their synthesis. Since the β-subunit is inefficiently synthesized or degraded in TM-treated cells, it might be that the concomitantly synthesized α-subunit can

no longer be stabilized in the membrane through the assembly to the β-subunit and is for its turn rapidly degraded. Alternatively, a yet undefined mechanism might exist which coordinates the synthesis of the two subunits. The linking factor could be the β-subunit itself, or else, another glycoprotein, the accumulation of which could be decreased by TM treatment.

Our data which point to an intimate relation between α- and β-subunit accumulation in the ER of intact cells at first seem in contradiction to our observations made in vitro that synthesis and membrane insertion of the two subunits can occur independently of each other [11]. We should, however, keep in mind that the efficiency of the in vitro expression cannot easily be estimated and might be very low compared to the in vivo situation. On the other hand, it could be that the α-subunit becomes associated to another protein before its assembly to the β-subunit occurs. Support for such a hypothesis are data on Ig heavy chains which associate to heavy chain-binding proteins (BIP) before assembling to light chains [1]. BIP is thought to prevent the secretion of unassembled heavy chains [18]. A similar regulatory protein might be involved in the stable expression of the α-subunit of Na,K-ATPase [4]. In rough microsomes used in in vitro systems, such a protein might be present in sufficient amounts while in TM-treated cells, its expression might be decreased. Association of the α-subunit to the protein would be impeded, leading to a destabilization and consequent degradation of the α-subunit.

Tamkun and Fambrough [40] who studied the effect of TM treatment in chick sensory neurons did not observe a specific effect of this drug on the accumulation of Na,K-ATPase. We do not know the reasons for these contradictory results but it has to be noted that in their system, TM treatment produced a 60% decrease in total protein synthesis. This situation might be unfavorable to assess effects on the expression of specific proteins.

Glycosylation of the β-Subunit and its Implication on the Functional Maturation of the Na,K-ATPase

Recent data obtained in chick neurons suggest that nonglycosylated β-subunit assembles to the α-subunit and that the nonglycosylated complex is transported to the plasma membrane [40]. In this study, we asked the question, whether non- or aberrantly glycosylated Na,K-ATPase indeed becomes functionally active. Two tests were used to probe the functional integrity of the Na,K-ATPase made in the presence of TM, dNM or SW, namely its ability to perform cation-dependent conformational changes and to hydrolyse ATP in a ouabain-inhibitable fashion. Recently, we have shown that the α-subunit becomes able to respond to Na^+ and K^+ by a conformational change soon after synthesis [10]. This property is thus a good measure for an early posttranslational maturation process. Since it can be assessed by controlled trypsinolysis as originally described by Jørgensen [21] on the purified enzyme, information can be gained at the same time on the structural stability of the polypeptide. We could indeed show that the α-subunit synthesized in the presence of TM (about 30% of controls) and thus of nonglycosylated β-subunit was more trypsin sensitive than the α-subunit concurrently expressed with core- or fully glycosylated β-subunit (Fig. 4). The lack of sugars on the β-subunit thus seems to influence the structural organization of the associated α-subunit. Interestingly, however, this relaxed form of the α-subunit is still able to change its conformation as reflected by a differential tryptic pattern in the presence of Na^+ and K^+. We do not yet know whether the nonglycosylated Na,K-ATPase can hydrolyze ATP. The inhibitory effect of TM on the accumulation of the two enzyme subunits did not permit to sufficiently fill up the cellular pool of Na,K-ATPase with nonglycosylated enzyme to study this property. On the other hand, in the presence of dNM which maintains the β-subunit in a core-glycosylated form and in the presence of SW, which perturbs complex-type glycosylation, the α-subunit became trypsin resistant and able to change its conformation and the enzyme expressed ATPase activity (Figs. 5 and 6). These data indicate that acquisition of core-sugars is sufficient for the enzyme to acquire its functional state.

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