**Glycosylation is essential for biosynthesis of functional gastric H⁺,K⁺-ATPase in insect cells**

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The role of N-linked glycosylation in the functional properties of gastric H⁺,K⁺-ATPase has been examined with tunicamycin and 1-deoxymannojirimycin, inhibitors of glycoprotein biosynthesis and glycoprotein processing respectively. Tunicamycin completely abolished both K⁺-stimulated and 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)-imidazo[1,2a]pyridine (SCH 28080)-sensitive ATPase activity and SCH 28080-sensitive phosphorylation capacity. The expression level of both H⁺,K⁺-ATPase subunits remained unaffected. 1-Deoxymannojirimycin clearly affected the structure of the N-linked oligosaccharide moieties without affecting specific phosphorylation capacity. Purification of the functional recombinant enzyme from non-functional H⁺,K⁺-ATPase subunits coincided with purification of glycosylated β-subunits and not of non-glycosylated β-subunits. Transport of the H⁺,K⁺-ATPase β-subunit to the plasma membrane but not its ability to assemble with the α-subunit depended on N-glycosylation events. We conclude that the acquisition, but not the exact structure, of N-linked oligosaccharide moieties is essential for biosynthesis of functional gastric H⁺,K⁺-ATPase in insect cells.

**INTRODUCTION**

H⁺,K⁺-ATPase, or the gastric proton pump, is an intrinsic membrane protein. Like Na⁺,K⁺-ATPase and Ca²⁺-ATPase, it belongs to the class of the E1–E2, or P-type family of, transport ATPases. The enzyme consists of two protein subunits (designated α and β) and is therefore related more to Na⁺,K⁺-ATPase than to Ca²⁺-ATPase. Both H⁺,K⁺-ATPase subunits have been cloned and their primary structures have been elucidated from several species [1]. A major feature of the H⁺,K⁺-ATPase β-subunit is the presence of seven consensus sequences for N-linked glycosylation (six for the pig enzyme) that are all cotranslationally glycosylated [2]. Until now, the functional role of N-linked glycosylation for heterodimeric P-type ATPases has been examined for Na⁺,K⁺-ATPase only. In several reports it has been demonstrated that N-glycosylation is not essential for Na⁺,K⁺-ATPase activity [3–5]. Because H⁺,K⁺-ATPase and Na⁺,K⁺-ATPase are structurally and functionally very similar, it has been assumed that N-glycosylation is also not essential for H⁺,K⁺-ATPase activity [6]. This, however, has not been tested yet. Although H⁺,K⁺-ATPase can be completely deglycosylated in vitro by peptide N-glycosidase F, complete deglycosylation generally requires the use of detergents [7]. Because H⁺,K⁺-ATPase activity is highly sensitive to inactivation by detergents, the use of peptide N-glycosidase F is almost inevitably impaired, with a loss of catalytic activity of H⁺,K⁺-ATPase. Therefore it is virtually impossible to correlate deglycosylation studies in vitro with the activity of the gastric proton pump. We succeeded in applying the baculovirus expression system to obtain catalytically active rat gastric H⁺,K⁺-ATPase from an insect cell source [8]. This enabled us to study the characteristics of H⁺,K⁺-ATPase in a model system in vitro. We have used glycoprotein biosynthesis and processing inhibitors to study the role of N-glycosylation for functional properties of H⁺,K⁺-ATPase. Our results clearly show that the presence, but not the exact composition, of N-linked oligosaccharide units is essential to obtain a catalytically active recombinant H⁺,K⁺-ATPase.

**MATERIALS AND METHODS**

**Cells and viruses**

Sf9 cells (ATCC CRL-1711) were maintained as described previously [8]. For production of H⁺,K⁺-ATPase, Sf9 cells were grown to 1.5 × 10⁶ cells/ml in spinner flasks (Bellco, Vineland, NJ, U.S.A.), pelleted by centrifugation for 10 min at 100 g at ambient temperature and infected with DLZαAS/β viruses encoding both H⁺,K⁺-ATPase subunits and a β-galactosidase marker cassette [8] at 10⁶ cells/ml with a multiplicity of infection of 3. After 1 h of infection at 27 °C, cells were transferred to 100 ml of fresh culture medium supplemented with 1% ethanol and incubated at 27 °C with various concentrations of the glycoprotein biosynthesis inhibitor tunicamycin or the glycoprotein processing inhibitor 1-deoxymannojirimycin (dMAN) (both from Boehringer, Mannheim, Germany) as indicated. Ethanol was added to the culture medium after infecting the cells, because this leads to synthesis of higher levels of functional H⁺,K⁺-ATPase [9]. Occasionally, cells were infected as monolayer cultures in tissue culture flasks.

**Preparation of Sf9 membranes**

Infected cells at 3 days after infection were centrifuged at 2000 g for 5 min at ambient temperature. The cell pellet was frozen at −20 °C until further processing, which was done by resuspending the cell pellets at 10⁷ cells/ml in ice-cold homogenization buffer.
ATPase activity (SCH 28080-insensitive) of 100-150 nmol/h per 100000 g and 4 °C. Supernatant was centrifuged for 30 min at 100000 g and 4 °C. Pelleted membranes from this step were resuspended in 0.2 times the initial volume of storage buffer [25 mM Hpes/Tris (pH 7.0)/10 % (w/v) sucrose/2 mM EDTA/5 μg/ml leupeptin]. Membranes were disrupted by sonication with three 15 s pulses with a probe sonicator (5 mm diameter) set at 65–70 W (Branson Power Company, Danbury, CT, U.S.A.) with subsequent cooling on ice. After centrifugation for 30 min at 100000 g and 4 °C, the supernatant was centrifuged for 60 min at 100000 g and 4 °C. Pelleted membranes from this step were resuspended in 0.2 times the initial volume of storage buffer [25 mM Hpes/Tris (pH 7.0)/10 % (w/v) sucrose/2 mM EDTA]. Functional H⁺,K⁺-ATPase in this crude membrane preparation could be enriched from contaminating non-specific (auto) phosphorylating enzymes and from non-functional H⁺,K⁺-ATPase subunits by centrifuging the membranes for 1 h at 100000 g and 4 °C over a layer of 25 % (w/v) sucrose in 25 mM Hpes/Tris (pH 7.0)/2 mM EDTA on a cushion of 38 % (w/v) sucrose in the same buffer. The 25–38 % (w/v) sucrose interface was collected, diluted to 10 % (w/v) sucrose in the same buffer and pelleted by centrifugation for 1 h at 100000 g and 4 °C. The membrane pellets obtained were resuspended in storage buffer and all fractions were stored at −20 °C.

Phosphorylation assay

The functional H⁺,K⁺-ATPase content of the membrane fractions was determined by phosphorylation with [γ-32P]ATP (Amersham, Little Chalfont, Bucks., U.K.) as follows: 10–50 μg of membrane proteins was preincubated in 50 μl of 25 mM Tris/acetate acid (pH 6.0)/1 mM MgCl₂ for 1 h on ice. Next, 10 μl of 0.6 μM [γ-32P]ATP (diluted in unlabelled ATP to approx. 50–100 Ci/mmol) was added and the reaction proceeded for 10 s on ice. The reaction was stopped by adding 5 ml of ice-cold stopping solution [100 mM phosphorous acid/5 % (w/v) trichloroacetic acid]. Phosphorylated proteins were separated from free label by filtration on 0.8 μm filters (type ME-27; Schleicher and Schuell, Dassel, Germany) and repeated washing with stopping solution. Filters were analysed by liquid-scintillation analysis. The specific phosphorylation capacity of H⁺,K⁺-ATPase was determined as the difference between the phosphorylation capacity in the absence and presence of 100 μM 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)-imidazo[1,2-a]pyridine (SCH 28080) as a specific H⁺,K⁺-ATPase inhibitor [10], which was included in the preincubation mixture. Each sample was assayed in triplicate. The specific phosphorylation capacity of H⁺,K⁺-ATPase in crude membrane fractions from untreated cultures was generally 3–6 times higher than the amount of non-specific phosphorylation, with a standard error of less than 3 %.

ATPase activity assay

The ATPase activity of the H⁺,K⁺-ATPase produced in the membrane fractions was measured as the SCH 28080-sensitive liberation of inorganic phosphate from [γ-32P]ATP as follows: 1–5 μg of membrane proteins was incubated in 100 μl of 100 mM Tris/acetate acid (pH 7.0)/1 mM MgCl₂/1 mM NaN₃/1.5 mM KC₁/0.1 mM EGTA/10 μM [γ-32P]ATP (diluted in unlabelled ATP to approx. 200 mCi/mmol), either with or without 100 μM SCH 28080 at 37 °C for a maximum of 30 min, each performed in triplicate. Then the reaction mixture was placed on ice and mixed with 900 μl of 10 % (w/v) activated charcoal (type SX-1; Norit, Amersfoort, The Netherlands)/5.5 % (w/v) trichloroacetic acid. After centrifugation for 30 s in a Microfuge, the radioactivity in 200 μl of the clear supernatant containing the liberated inorganic phosphate was counted. In the absence of either or both MgCl₂ and KC₁, no SCH 28080-sensitive ATPase activity was detected (results not shown). A background of non-specific ATPase activity (SCH 28080-insensitive) of 100–150 nmol/h per mg was usually observed under these conditions. The specific ATPase activity of H⁺,K⁺-ATPase in crude membrane fractions from untreated cultures was generally approx. 40–80 amol/h per mg with a standard error of less than 3 %. Total ATP hydrolysis never exceeded 35 % of the ATP present in the reaction mixtures.

β-Galactosidase assay

The β-galactosidase content of infected cells was determined as described previously [9].

Confocal laser scan microscopy

Sf9 cells were grown on sterile microscope coverslides in complete growth medium and infected with a multiplicity of infection of 3 for 1 h at 27 °C. After infection, cells were incubated at 27 °C for 48 h in complete growth medium with additions as indicated. Cells were washed three times for 5 min with PBS, pH 7.4, followed by fixation in 1 % (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at room temperature. Further processing was done by permeabilization at −20 °C in 100 % methanol for 5 min. Next, the coverslides were dried at room temperature and non-specific binding sites for antibodies were blocked by incubation for 30–60 min in PBS/0.05 % polyoxyethylene sorbitan monolaurate (Tween-20)/1 % (w/v) gelatin/2 % (v/v) fetal calf serum with gentle rocking. After being washed with PBS/0.05 % Tween-20 three times for 5 min each, the cells were incubated with subunit-specific antibodies for 30–60 min in PBS/0.05 % Tween-20/1 % (w/v) gelatin/2 % (v/v) fetal bovine serum. Free antibodies were removed by washing the cells as above. Next the cells were incubated with fluorescently labelled secondary antibodies (Dako, Glostrup, Denmark) for 1 h in PBS/0.05 % Tween-20/1 % (w/v) gelatin/2 % (v/v) fetal bovine serum. From this moment onwards, the coverslides were kept in the dark as much as possible. After washing as above, the cells were deasalted by a short wash with Milli-Q water (Millipore, Bedford, MA, U.S.A.), dehydrated with methanol, dried and mounted in 10 % (w/v) Mowiol (Hoechst, Amsterdam, The Netherlands), 2.5 % (w/v) NaN₃ and 25 % (v/v) glycerol in 0.1 M Tris/HCl, pH 8.5. They were examined on a Bio-Rad MRC1000 confocal microscope. Images were averaged over eight scans.

Protein analysis and subunit quantification

Protein was determined with the modified Lowry method described by Peterson [11], with BSA as a standard. The H⁺,K⁺-ATPase subunit content of the membrane fractions was determined with a previously described quantitative ELISA [12].

Immunoblotting and immunoprecipitation

Immunoblotting was performed with subunit-specific antibodies as described previously [8]. Occasionally, staining of specific proteins on immunoblots was done with a chemiluminescence detection kit in accordance with the instructions of the manufacturer (Tropix, Bedford, MA, U.S.A.). Immunoprecipitation was performed as follows: 200 μg of crude Sf9 membrane proteins were solubilized in 0.2 ml of buffer containing 1 % (w/v) octa(ethylene glycol) monododecyl ether, 0.01 % SDS, 0.05 M Tris/HCl, pH 8.8, and 0.15 M NaCl. Monoclonal antibody 5B6 (5 μg) against the H⁺,K⁺-ATPase α-subunit [13] was added, and the entire mixture was incubated at 4 °C for 2 h. After centrifugation at 10000 g for 2 min, the supernatant was incubated with 50 μl of Protein A immobilized on agarose (KemEnTeC, Copenhagen, Denmark) and incubated for 3 h at 4 °C with...
value represent means ± S.E.M., for three to nine experiments; 100% activities are: endogenous (auto)phosphorylating enzymes; ATPase activity, 64 ± 8 nmol/h per mg for membrane tractions are plotted against the tunicamycin concentration in the culture medium.

(Fig. 1B). Treatment with tunicamycin had no visible effect on the H+,K+-ATPase α-subunit (Figure 1A). At tunicamycin concentrations above 1 μg/ml, only non-glycosylated β-subunits were detected by Western blotting and no significant H+,K+-ATPase activity could be measured. In contrast with this inhibitory effect on the catalytic activities of H+,K+-ATPase, the level of endogenous (auto)phosphorylating enzymes (SCH 28080-insensitive) was hardly affected by tunicamycin, demonstrating that the inhibitory action is not a general effect of tunicamycin on the activities of the (auto)phosphorylating enzymes in SF9 cells. Similarly to its effect on the steady-state phosphorylation capacity, tunicamycin inhibited the K+-stimulated ATPase activity of the recombinant enzyme. To exclude direct effects of tunicamycin on H+,K+-ATPase activity, a control experiment was performed in which pig gastric H+,K+-ATPase (1 mg/ml) was incubated at 20 °C for 20 h at pH 7.0 with different concentrations of tunicamycin, whereupon the phosphorylation capacity was determined. No significant effects were found after preincubation with tunicamycin concentrations up to 10 μg/ml (results not shown). These results strongly suggest that N-glycosylation is somehow essential for H+,K+-ATPase activity.

Tunicamycin eliminates the formation of all N-linked oligosaccharides on proteins. Therefore, when using tunicamycin, we cannot establish whether the acquisition of oligosaccharides alone is sufficient for H+,K+-ATPase activity, or whether processing of the oligosaccharide precursors into complex glycosylated forms (as in native H+,K+-ATPase) is also required. Despite the fact that insect cells are poorly equipped with the enzymes to perform complex-type N-glycosylation [14], some recombinant baculovirus-infected insect-cell-expressed proteins do indeed carry complex-type oligosaccharides [15]. To assess whether or not the structure of the oligosaccharides on the H+,K'-ATPase subunits could play a role in the catalytic activity of H+,K'-ATPase, the effect of dMAN was studied. dMAN is a specific inhibitor of the α-mannosidase I-catalysed trimming of the high-mannose oligosaccharide precursor Man9GlcNAc2Asn to Man7GlcNAc2Asn. The latter form can be trimmed to Man6GlcNAc2Asn by Golgi-type α-mannosidase II and can subsequently be extended into complex-type glycosylated forms. Therefore, in the presence of dMAN, processing of the core glycosylated Glc3Man9GlcNAc2Asn is arrested at the Man6GlcNAc2Asn stage, eliminating the ability of SF9 cells to perform complex-type glycosylation.

First we studied the effect of dMAN to the culture medium on the glycosylation pattern of the H+,K'-ATPase β-subunit. In the absence of dMAN, glycosylated β-subunits appear on the Western blot as a smear of proteins with an apparent molecular mass of approx. 40–50 kDa. In the presence of at least 3 mM dMAN, the smear of the glycosylated β-subunits synthesized in vitro is decreased to two immunoreactive protein bands with apparent molecular masses of approx. 51 and 48 kDa, the upper band being predominant (Figure 2). This phenomenon was independent of the duration of the infectious process and
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with a crude membrane preparation, the purified H+,K' -ATPase fraction contained more glycosylated and almost no non-glycosylated subunits was examined on a Western blot (Figure 3). Compared to the standard crude membrane preparation could be separated continuous sucrose density gradient as described in the Materials and methods section. The extent of glycosylation of the obtained fractions was examined on a Western blot (Figure 3). Compared with a crude membrane preparation, the purified H+,K' -ATPase fraction contained more glycosylated and almost no non-glycosylated β-subunits. Because Sf9 cells are a relatively poorly studied cell type, there are no known marker enzymes from (sub)cellular organelles to specify the intracellular location of the functional H+,K' -ATPase subunits. The non-glycosylated β-subunits were more abundant in the pellet fraction, in which only little H+,K' -ATPase activity was found. This supports our conclusion that glycosylation is essential for H+,K' -ATPase activity.

**Glycosylated β-subunits co-purify with functional H+,K' -ATPase**

Additional results demonstrating the need of H+,K' -ATPase to be N-glycosylated to perform catalytic activities was obtained from purification experiments. Only a relatively small fraction of the H+,K' -ATPase subunits synthesized in vitro possessed catalytic activity [8,9]. Functional H+,K' -ATPase subunits in the standard crude membrane preparation could be separated from non-functional H+,K' -ATPase subunits by using a discontinuous sucrose density gradient as described in the Materials and methods section. The extent of glycosylation of the obtained fractions was examined on a Western blot (Figure 3). Compared with a crude membrane preparation, the purified H+,K' -ATPase fraction contained more glycosylated and almost no non-glycosylated β-subunits. Because Sf9 cells are a relatively poorly studied cell type, there are no known marker enzymes from (sub)cellular organelles to specify the intracellular location of the functional H+,K' -ATPase subunits. The non-glycosylated β-subunits were more abundant in the pellet fraction, in which only little H+,K' -ATPase activity was found. This supports our conclusion that glycosylation is essential for H+,K' -ATPase activity.

**N-Glycosylation is essential for targeting the β-subunit to the plasma membrane**

The intracellular distribution of the H+,K' -ATPase subunits in recombinant-baculovirus-infected Sf9 cells was examined by confocal image processing. As shown in Figure 4(A), the H+,K' -ATPase α-subunit is found exclusively in intracellular membranous structures. No detectable levels of the α-subunit were found in the plasma membrane, which is consistent with observations by others [17]. This means that the catalytically active H+,K' -ATPase fraction also originates from an intracellular source.

The H+,K' -ATPase β-subunit (Figure 4B) is partly targeted to the plasma membrane and partly localized in intracellular membranous structures. In the presence of 5 μg/ml tunicamycin, the non-glycosylated β-subunit is localized entirely in intracellular membranous structures (Figure 4C). Apparently, proper processing of the H+,K' -ATPase β-subunit to the plasma membrane depends on the presence of N-linked oligosaccharides on this subunit, although other explanations cannot be excluded. These results are in contrast with observations on the Na+,K' -ATPase β-subunit by others [3–5]. It was demonstrated in several different cell types that the Na+,K' -ATPase β-subunit does not need to be N-glycosylated to be transported to the plasma membrane. However, such a study has not yet been reported for the Na+,K' -ATPase expressed in insect cells. Because the H+,K' -ATPase α-subunit is detected exclusively in intracellular membranous structures, processing of the H+,K' -ATPase β-subunit to the plasma membrane does not seem to be essential for synthesis of a functional H+,K' -ATPase in insect cells.

**N-Glycosylation is not essential for subunit assembly**

In order to express H+,K' -ATPase functionally, we showed the need for both H+,K' -ATPase subunits to be co-expressed in individual Sf9 cells [8]. This means that both H+,K' -ATPase subunits must be engaged in the formation of a heteroduplex molecule, or a higher-order structure, to establish catalytic
activity. This kind of protein interaction has often been studied with immunoprecipitation procedures. We therefore used this method to study the role of N-glycosylation in the assembly of H\+,K\+-ATPase subunits. The result is shown in Figure 5. In immunoprecipitates from untreated cultures, both glycosylated and non-glycosylated H\+,K\+-ATPase \( \beta \)-subunits were precipitated with the anti-\( \alpha \)-subunit antibody (Figure 5, lane 1). This means that both forms of the \( \beta \)-subunit must be engaged with the \( \alpha \)-subunit in a detergent-resistant complex and are therefore tightly associated. No glycosylated \( \beta \)-subunits were produced in tunicamycin-treated cultures. As a result, the quantity of nonglycosylated \( \beta \)-subunits was increased relative to untreated cultures. This effect was also visible in immunoprecipitates from tunicamycin-treated cultures (Figure 5; compare lane 2 with lane 1). In control precipitates from cells expressing only the H\+,K\+-ATPase \( \beta \)-subunit, essentially no \( \beta \)-subunits were precipitated with the anti-\( \alpha \)-subunit antibody (Figure 5, lane 3).

DISCUSSION

The present study shows the effects of inhibitors of glycoprotein biosynthesis and processing (tunicamycin and dMAN respectively) on the activity of recombinant H\+,K\+-ATPase expressed in insect cells. There might be some limitations on the use of tunicamycin, however, because in several reports it has been noted that the inhibition of N-glycosylation by tunicamycin in expression studies in vitro resulted in the concomitant inhibition of protein synthesis [5,18,19]. This of course complicates the interpretation of the results of such experiments. Several lines of evidence, however, show that this is not so in the present study. First, Western blot analysis reveals no apparent decrease in the total amount of either H\+,K\+-ATPase \( \alpha \)- or \( \beta \)-subunits (Figure 1). Secondly, the level of H\+,K\+-ATPase \( \alpha \)-subunits in the membrane preparations has been quantified with ELISA techniques and no decrease in the amount of H\+,K\+-ATPase \( \beta \)-subunits was found (results not shown). In addition, the levels of endogenous (auto)phosphorylating enzymes and ATPases are hardly affected by tunicamycin in concentrations up to 10 \( \mu \)g/ml. The same is true for the \( \beta \)-galactosidase activity that is used as a reporter enzyme (results not shown). It therefore seems that the inhibitory effect of tunicamycin on protein accumulation is cell-type-specific and might also be protein-specific. Apparently, adding tunicamycin to the growth medium does not affect the viability of the insect cells. Because the level of H\+,K\+-ATPase \( \beta \)-subunit synthesis was unaffected by tunicamycin, the results of our experiments can be interpreted as they are. Hence our results clearly show that the acquisition of N-linked oligosaccharide moieties is essential for obtaining a catalytically active H\+,K\+-ATPase in insect cells.

The question of which mechanism of N-glycosylation is essential for catalytic activity of H\+,K\+-ATPase remains to be answered. An obvious explanation is that the sugar moieties are essential for subunit stabilization. Such a role has also been proposed for a variety of other (membrane) glycoproteins [20–22]. Another possibility is that an essential conformational transition of the H\+,K\+-ATPase \( \beta \)-subunits is obtained only after subunit glycosylation. Such a glycosylation-dependent folding event has also been reported for other proteins [23]. This option is supported by the fact that transport of the \( \beta \)-subunit to the plasma membrane depends on N-glycosylation events as well. However, this latter criterion does not discriminate for the functional properties of the recombinant H\+,K\+-ATPase in Sf9 cells because the functional enzyme is retrieved from intracellular membranous structures. In addition, another structurally related property of the \( \beta \)-subunit, assembly with the \( \alpha \)-subunit into a heterodimeric complex, does not depend on the presence of N-linked oligosaccharides on the \( \beta \)-subunit. This could mean that subunit assembly takes place before the subunits are to be glycosylated, or that the sugar moieties are otherwise engaged in the formation of a catalytically active H\+,K\+-ATPase. Therefore a much more interesting speculation might be that (some of) the sugar residues present in all precursor, intermediate and fully complex glycosylated forms (Man\(_4\)GlcNAc\(_2\) N-linked to an Asn residue) on the H\+,K\+-ATPase \( \beta \)-subunits are themselves involved in the catalytic cycle of H\+,K\+-ATPase, but neither of the above-mentioned possibilities can be excluded by our results.

It would be interesting to examine whether the difference in dependence on N-glycosylation events between H\+,K\+-ATPase and Na\+,K\+-ATPase could be related to some other differences between the two. For instance, for reasons as yet unknown, H\+,K\+-ATPase is much more susceptible to inactivation by detergent than Na\+,K\+-ATPase. The latter enzyme is usually purified by detergent extraction [24], but the use of detergents with H\+,K\+-ATPase almost always leads to loss of catalytic activity of most of the preparation [25].

The role of N-glycosylation for H\+,K\+-ATPase activity has not been studied before. Therefore no results about the role of N-glycosylation for H\+,K\+-ATPase activity are available. In this light, it is interesting to note that in highly purified native H\+,K\+-ATPase samples from gastric parietal cells, only glycosylated \( \beta \)-subunits are present. In fact, extensive glycosylation was one of the main reasons for the late discovery of the H\+,K\+-ATPase \( \beta \)-subunit [26]. This supports our conclusion that N-glycosylation is essential for gastric H\+,K\+-ATPase activity. In turn, because expression of H\+,K\+-ATPase \( \beta \)-subunits with the baculovirus expression system results in the synthesis of glycosylated as well as non-glycosylated \( \beta \)-subunits, our findings might also account for the presence of substantial amounts of non-functional H\+,K\+-ATPase \( \beta \)-subunits when using this system.

It should be noted that the use of tunicamycin to eliminate the formation of N-linked oligosaccharide structures does not allow us to discriminate between its effect on N-linked glycosylation events on either the \( \alpha \)- or \( \beta \)-subunit of H\+,K\+-ATPase. Although we have used glycosylation patterns of the H\+,K\+-ATPase \( \beta \)-subunit to demonstrate the action of the inhibitors used in this study, the inhibitory action of tunicamycin on the catalytic properties of gastric H\+,K\+-ATPase cannot be ascribed automatically and exclusively to its effects on this H\+,K\+-ATPase.
subunit. In fact, the presence of a cytosolically located N-linked sugar moiety, has been reported for the H⁺,K⁺-ATPase α-subunit [26,27] and also for the Na⁺,K⁺-ATPase α-subunit [28]. Although we have not yet established whether or not the recombinant-baculovirus-expressed H⁺,K⁺-ATPase α-subunit is N-glycosylated, the possibility that a cytosolically located N-linked sugar moiety on the α-subunit could be involved in the catalytic cycle of H⁺,K⁺-ATPase has also to be considered seriously. This latter option challenges our current understanding of how this proton pump functions.

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