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## ETHANOL STIMULATES EXPRESSION OF FUNCTIONAL H<sup>+</sup>,K<sup>+</sup>-ATPase IN Sf9 CELLS

Corné H.W. Klaassen, Herman G.P. Swarts and Jan Joep H.H.M. De Pont\*

Department of Biochemistry, University of Nijmegen, PO Box 9101,  
6500 HB Nijmegen, The Netherlands

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The baculovirus expression system is suitable for functional expression of gastric H<sup>+</sup>,K<sup>+</sup>-ATPase. Expression of functional H<sup>+</sup>,K<sup>+</sup>-ATPase in Sf9 cells is accompanied by synthesis of large amounts of non-functional subunits. When H<sup>+</sup>,K<sup>+</sup>-ATPase is synthesised in the presence of 150-250 mM ethanol in the culture medium, two to threefold higher levels of functional H<sup>+</sup>,K<sup>+</sup>-ATPase are produced due to the formation of more functional subunits rather than to an increase of subunits *per se*. The catalytical properties of the ethanol-produced H<sup>+</sup>,K<sup>+</sup>-ATPase are indistinguishable from control preparations. The mechanism by which ethanol stimulates the formation of functional H<sup>+</sup>,K<sup>+</sup>-ATPase probably involves a direct effect on the physical properties of Sf9 membranes. In addition there also might be an indirect effect through ethanol inducible stress proteins acting as molecular chaperones. © 1995 Academic Press, Inc.

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The baculovirus expression system has been widely used for expression of foreign genes in insect cells. Both soluble cytosolic proteins and complex membrane proteins have successfully been expressed using this system (1-4). Expression of foreign genes is usually regulated by the well characterised and very strong baculovirus polyhedrin and p10 promoters (5-7). Both promoters are activated in the very late phase of infection and can mediate expression levels of up to 30-50% of total cellular protein, making them ideal promoters to regulate expression of large amounts of foreign proteins.

We have employed the baculovirus expression system for studying the characteristics of gastric H<sup>+</sup>,K<sup>+</sup>-ATPase (E.C. 3.6.1.36). This enzyme can be expressed in insect cells as a functional enzyme with similar catalytical properties as native H<sup>+</sup>,K<sup>+</sup>-ATPase purified from pig gastric mucosa (8). Although vast amounts of H<sup>+</sup>,K<sup>+</sup>-ATPase subunits can be produced in insect cells, comparison between the activities of native H<sup>+</sup>,K<sup>+</sup>-ATPase and recombinant H<sup>+</sup>,K<sup>+</sup>-ATPase leads to the conclusion that more than 90% of the subunits produced in Sf9

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\*Corresponding author. fax: +31-80-540525.

Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SCH 28080, 3-(cyanomethyl)-2-methyl-8(phenylmethoxy)imidazo[1,2a]pyridine; I<sub>0.5</sub>, half maximal inhibitory concentration.

cells are inactive, most probably due to improper folding of the peptide backbone. This phenomenon is not specific for H<sup>+</sup>,K<sup>+</sup>-ATPase since the same is true for a variety of both related as well as unrelated proteins that are expressed using the very strong baculovirus polyhedrin promoter (9-11). Therefore, attempts to improve functional expression of recombinant proteins in Sf9 cells, have focussed on the use of alternate less strong promoters, that are activated at an earlier stage in infection (12,13).

In this paper we will show that functional expression of gastric H<sup>+</sup>,K<sup>+</sup>-ATPase in Sf9 cells, using the polyhedrin and p10 promoter to drive expression of the H<sup>+</sup>,K<sup>+</sup>-ATPase subunits, can be enhanced upon addition of ethanol to the culture medium.

## MATERIALS AND METHODS

**Cells and viruses.** Sf9 cells (ATCC CRL-1711) were maintained either as monolayer cultures in tissue culture flasks (Greiner, Alphen a/d Rijn, The Netherlands) or as 100 ml suspension cultures in 250 ml spinner flasks (Bellco, Vineland, NJ, USA) in vitamin free TNM-FH medium supplemented with 5 mg/ml bovine serum albumin, 5 U/ml penicillin, 5 µg/ml streptomycin and 10% fetal calf serum (Gibco, Breda, The Netherlands). In suspension cultures, an additional 0.1% (w/v) of pluronic F-68 (Sigma, Axel, The Netherlands) was added to the culture medium. For production of H<sup>+</sup>,K<sup>+</sup>-ATPase, Sf9 cells were grown to 1.5\*10<sup>6</sup> cells per ml in spinner flasks, pelleted by centrifugation for 10 min at 100 x g at ambient temperature and infected with DLZαASβ viruses encoding both H<sup>+</sup>,K<sup>+</sup>-ATPase subunits (8) at 1\*10<sup>7</sup> cells/ml using a multiplicity of infection of 3. After 1 h at 27°C cells were transferred to 100 ml fresh culture medium with various additions as indicated, and incubated at 27°C until they were harvested at 2 days post infection. Occasionally, cells were grown and infected as monolayer cultures in 175 cm<sup>2</sup> tissue culture flasks.

**Preparation of Sf9 membranes.** Cells were centrifuged at 2,000 x g for 5 min at ambient temperature and frozen at -20°C until further processing. This was done by resuspending the cell pellets at 1\*10<sup>7</sup> cells/ml in icecold homogenisation buffer (25 mM HEPES/Tris pH 7.0, 10% (w/v) sucrose, 2 mM EDTA, 5 µg/ml leupeptin). Membranes were disrupted by sonication using 3 pulses of 15 s with a probe sonicator set at 60 W (Branson Power Company, Denbury, USA) with subsequent cooling on ice. After centrifugation for 30 min at 10,000 x g (4°C) the supernatant was centrifuged for 60 min at 100,000 x g (4°C). Pelleted membranes from this step were resuspended in 0.2 initial volume of storage buffer (25 mM HEPES/Tris pH 7.0, 10% (w/v) sucrose, 2 mM EDTA) and stored at -20°C. The supernatant containing the soluble protein fraction was also stored at -20°C. Functional H<sup>+</sup>,K<sup>+</sup>-ATPase in this crude membrane preparation could be further purified from contaminating non-specific (auto)phosphorylating enzymes and from non-functional H<sup>+</sup>,K<sup>+</sup>-ATPase subunits using a sucrose density step gradient by centrifugating the membranes for 1 h at 100,000 x g (4°C) over a cushion of 25% (w/v) sucrose in 25 mM HEPES/Tris pH 7.0, 2 mM EDTA followed by 38% (w/v) sucrose in the same buffer. The 25%-38% (w/v) sucrose interfase was collected, diluted to 10% (w/v) sucrose in the same buffer, pelleted by centrifugation for 1 h at 100,000 x g (4°C), resuspended in 25 mM HEPES/Tris pH 7.0, 10% (w/v) sucrose, 2 mM EDTA and stored at -20°C.

**Enzyme activity assays and protein analysis.** The functional (SCH 28080 sensitive) H<sup>+</sup>,K<sup>+</sup>-ATPase content of the membrane preparations was determined by phosphorylation with [γ-<sup>32</sup>P]ATP (Amersham, Buckinghamshire, UK) as follows: 5 µl of membrane fraction (10 to 50 µg protein) was incubated in 50 µl of 25 mM Tris/acetate pH 6.0, 1 mM MgCl<sub>2</sub> either

with or without 100  $\mu\text{M}$  SCH 28080 as a specific  $\text{H}^+, \text{K}^+$ -ATPase inhibitor (14) for 1 h on ice. Next, 10  $\mu\text{l}$  of 0.6  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP was added and the reaction was allowed to proceed 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 over 0.8  $\mu\text{m}$  filters (type ME-27, Schleicher and Schull, Dassel, Germany) and repeated washing with stopping solution. Filters were analysed by liquid scintillation analysis.

The ATPase activity of the  $\text{H}^+, \text{K}^+$ -ATPase produced in the membrane preparations was measured as the SCH 28080 sensitive liberation of inorganic phosphate from [ $\gamma$ - $^{32}\text{P}$ ]ATP as follows: 2-10  $\mu\text{g}$  of membrane protein was incubated in 100  $\mu\text{l}$  of 100 mM Tris/acetate pH 7.0, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{NaN}_3$ , 1.5 mM KCl, 0.1 mM EGTA, 10  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP either with or without 100  $\mu\text{M}$  SCH 28080 at 37°C for 20 min. Then, the reaction mixture was placed on ice and mixed with 900  $\mu\text{l}$  of 10% (w/v) charcoal, 5.5% (w/v) trichloroacetic acid. After centrifugation for 30 s in a microfuge, 200  $\mu\text{l}$  of the clear supernatant containing the liberated inorganic phosphate was analysed. In the absence of added KCl, no SCH 28080 sensitive ATPase activity could be detected.

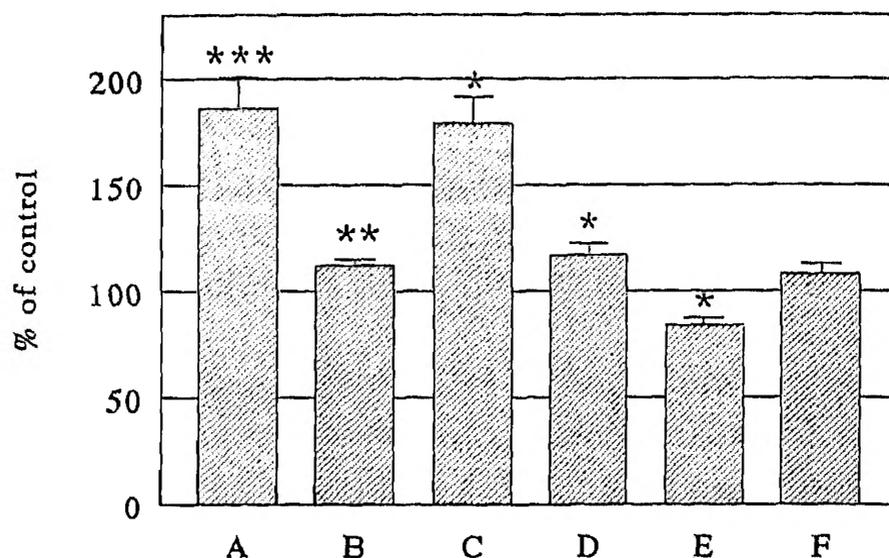
The  $\beta$ -galactosidase content of infected cells was determined by its activity in 0.6 ml reaction medium containing 82 mM  $\text{Na}_2\text{HPO}_4$ , 18 mM  $\text{NaH}_2\text{PO}_4$ , 1 mM  $\text{MgCl}_2$ , 5 mM dithioerythritol, 2.9 mM *o*-nitrophenol- $\beta$ -D-galactopyranoside and 0.2-1.0  $\mu\text{g}$  soluble proteins. After incubation at 37°C for 30 min, the reaction was terminated upon addition of 2.4 ml of 1 M  $\text{Na}_2\text{CO}_3$ . The extinction of *o*-nitrophenol at 420 nm was used for quantification of the  $\beta$ -galactosidase activity.

Protein was determined using the modified Lowry method described by Peterson (15) using bovine serum albumin as a standard. The amount of  $\text{H}^+, \text{K}^+$ -ATPase (in  $\mu\text{g}/\text{mg}$  protein) of the membrane fractions was determined with a monoclonal antibody to the  $\alpha$ -subunit (16) according to a previously described quantitative enzyme linked immunosorbent assay (17).

## RESULTS AND DISCUSSION

A recombinant baculovirus (DLZ $\alpha$ AS $\beta$ ) has been constructed in which expression of the  $\text{H}^+, \text{K}^+$ -ATPase  $\alpha$ -subunit and  $\beta$ -subunit is regulated by the polyhedrin and p10 promotor, respectively, and in which the *E. coli* LacZ coding sequences are expressed by the drosophila hsp70 promotor. Infection of Sf9 cells with DLZ $\alpha$ AS $\beta$  virus leads to synthesis of a catalytically active membrane embedded  $\text{H}^+, \text{K}^+$ -ATPase as well as functional soluble  $\beta$ -galactosidase. The catalytic properties of recombinant  $\text{H}^+, \text{K}^+$ -ATPase can be measured as a SCH 28080 sensitive phosphorylation capacity that is also sensitive towards potassium ions, as well as by a  $\text{Mg}^{2+}$  dependent and  $\text{K}^+$  stimulated ATPase activity that is inhibited by SCH 28080. Activity of the recombinant expressed  $\beta$ -galactosidase can be measured by a colorimetric assay using *o*-nitrophenol- $\beta$ -D-galactopyranoside as an artificial substrate. Although Sf9 cells are capable of producing functional  $\text{H}^+, \text{K}^+$ -ATPase, less than 10% of the produced  $\text{H}^+, \text{K}^+$ -ATPase subunits are involved in the formation of a catalytically active enzyme (8).

When infected cells are incubated in culture medium supplemented with ethanol, considerably higher  $\text{H}^+, \text{K}^+$ -ATPase specific activity can be measured in crude Sf9 membrane preparations as shown in figure 1. This effect is demonstrated both for the  $\text{H}^+, \text{K}^+$ -ATPase



**Figure 1.** Effect of addition of ethanol (172 mM) to the culture medium on the expression and activity of various enzymes in crude Sf9 membrane preparations. A: Phosphorylation capacity of H<sup>+</sup>,K<sup>+</sup>-ATPase (SCH 28080 sensitive); B: Non-specific phosphorylation capacity (SCH 28080 insensitive); C: ATPase activity of H<sup>+</sup>,K<sup>+</sup>-ATPase (SCH 28080 sensitive); D: ATPase activity of non-specific enzymes (SCH 28080 insensitive); E: H<sup>+</sup>,K<sup>+</sup>-ATPase content; F:  $\beta$ -Galactosidase activity in the soluble protein fraction. Values are mean  $\pm$  SEM relative to control preparations. Paired t-test statistics: \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

specific phosphorylation capacity and for its K<sup>+</sup> stimulated ATPase activity and is maximal around 150-250 mM ethanol. This stimulation seems to be specific for the abundantly expressed H<sup>+</sup>,K<sup>+</sup>-ATPase since both the level of endogenous (auto)phosphorylating proteins and the activity of endogenous ATP hydrolysing enzymes in the Sf9 membrane fractions as well as of the co-expressed  $\beta$ -galactosidase in the soluble protein fraction remain virtually unaffected. Theoretically, addition of ethanol to the cell culture medium could stimulate cellular metabolism by supplying the cells with an additional energy and carbon source. In that case, infected cells are likely to produce more recombinant protein(s). The stimulatory effect of ethanol on the production of functional H<sup>+</sup>,K<sup>+</sup>-ATPase could then result in a stimulation of H<sup>+</sup>,K<sup>+</sup>-ATPase subunit production *per se*. This proves not to be the case for expression of H<sup>+</sup>,K<sup>+</sup>-ATPase since the H<sup>+</sup>,K<sup>+</sup>-ATPase subunit content of the membrane preparations was actually decreased to 86% of control levels. Therefore, the phosphorylation capacity per mg H<sup>+</sup>,K<sup>+</sup>-ATPase is even more than doubled.

Another explanation for the differences in activity between the routinely expressed H<sup>+</sup>,K<sup>+</sup>-ATPase and H<sup>+</sup>,K<sup>+</sup>-ATPase expressed in ethanol containing medium could reside in altered biochemical properties of the latter one. The most likely biochemical parameter to explain the doubled phosphorylation capacity is an increased affinity for ATP in the phosphorylation assay. This could have an effect since we routinely measure H<sup>+</sup>,K<sup>+</sup>-ATPase activities at suboptimal ATP concentrations (8). It turns out that both H<sup>+</sup>,K<sup>+</sup>-ATPase preparations virtually have the same affinity for ATP (table 1). Also, the I<sub>0.5</sub> for K<sup>+</sup> on the

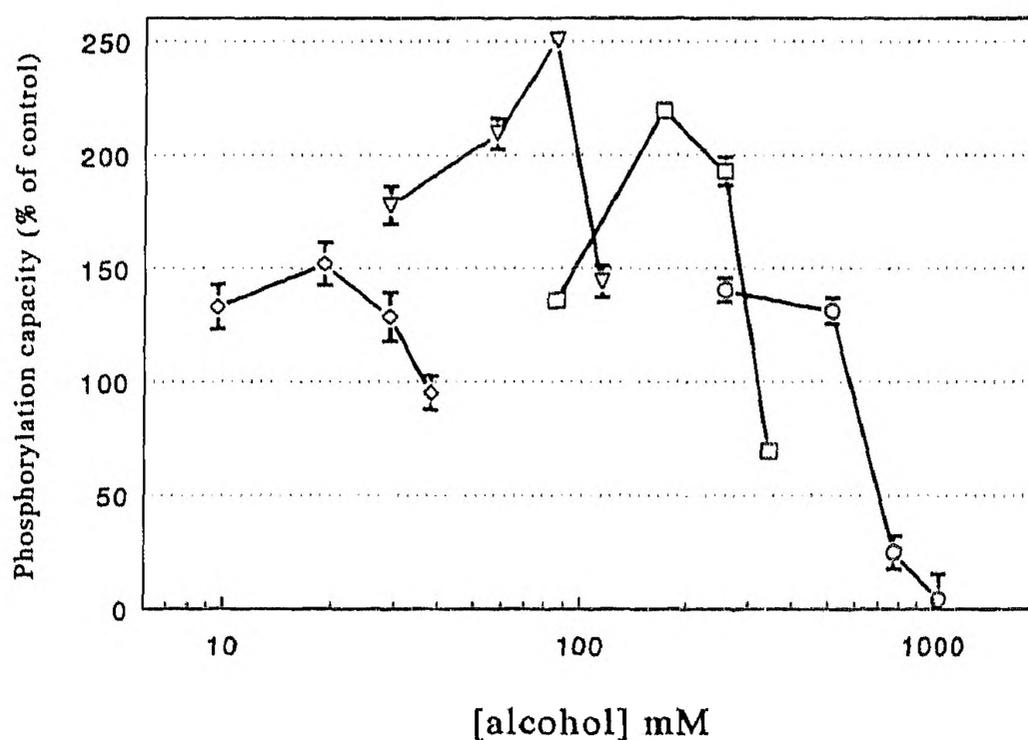
**Table 1.** Apparent  $K_m$  for ATP and  $I_{0.5}$  for KCl on the steady state phosphorylation capacity of  $H^+,K^+$ -ATPase in a purified membrane preparation from control or ethanol treated cells

	Control	Ethanol treated
$K_m$ (ATP)	12 nM (7-17)	9 nM (4-14)
$I_{0.5}$ (KCl)	520 $\mu$ M (430-630)	430 $\mu$ M (360-500)

Values between parentheses indicate the 95% confidence levels.

steady state phosphorylation capacity is also unaltered in both preparations (table 1). This demonstrates that both  $H^+,K^+$ -ATPase preparations have similar biochemical properties.

The effect of other small alcohols was also investigated (figure 2). The maximal stimulating effect was different for each of the alcohols tested, but was obtained at alcohol concentrations which decrease by a factor three per additional C-atom. This phenomenon therefore obeys to Traube's rule. This suggests that at least part of the stimulation is due to the lipophilic nature of these compounds. A likely explanation for the stimulation could be that the alcohols affect the fluidity of the Sf9 membranes in a way that facilitates a proper folding of the peptide backbone and/or proper assembly of recombinant expressed  $H^+,K^+$ -ATPase subunits and thus leads to higher levels of functional  $H^+,K^+$ -ATPase. In that



**Figure 2.** Effect of addition of various small alcohols to the culture medium on the synthesis of functional  $H^+,K^+$ -ATPase, measured as the SCH 28080 sensitive phosphorylation capacity in crude membrane preparations. Values are mean  $\pm$  standard deviation, relative to control preparations. Alcohols included in this test are methanol (circles), ethanol (squares), 1-propanol (triangles) and 1-butanol (diamonds).

case, this stimulatory effect should be specific for high-level expressed recombinant transmembrane proteins. This option is supported by the finding that the phosphorylation capacity and the ATPase activity of endogenous membrane embedded enzymes and the activity of the recombinant expressed soluble  $\beta$ -galactosidase are virtually unaffected by the presence of ethanol. Since little is known about the structure and composition of Sf9 cell membranes, this option needs further investigation.

Addition of ethanol to the culture medium inflicts a general form of stress upon the Sf9 cells and could result in the synthesis of stress proteins (18). Since stress proteins are believed to act as molecular chaperones involved in a proper folding of proteins, this also could explain the formation of increased levels of functional  $H^+,K^+$ -ATPase in ethanol treated baculovirus infected Sf9 cultures.

In conclusion, our results clearly demonstrate that addition of ethanol to the culture medium stimulates the formation of functional  $H^+,K^+$ -ATPase in baculovirus infected Sf9 cells. All results indicate that this stimulation is due an increase in the number of functional subunits. This stimulatory effect of ethanol on expression of functional  $H^+,K^+$ -ATPase in insect cells may have important implications for expression of other foreign proteins using the baculovirus expression system.

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