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Inhibitory Effect of Ethanol on the Na^+-ATPase Activity of Rat Kidney Proximal Tubular Cell Plasma Membranes

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Summary

The inhibitory effect of 2 % ethanol (400 mM) in the incubation medium on several characteristics of the Na^+-ATPase of basolateral plasma membranes from rat kidney proximal tubular cells was investigated. Ethanol did not change the K_m of the enzyme for Mg^{2+}, ATP or Na^+; it did not change either the optimal pH or temperature values of the incubation medium for the enzyme to act and finally, it did not affect the apparent energy of activation of the enzyme. It was also found that 2 % ethanol produced stronger inhibition of the ATPase when it is in an activated or stimulated state, than when it is working at its lower basal level. The presented results can be explained by assuming that 2 % ethanol in the incubation medium inhibits Na^+-ATPase activity by affecting the enzyme structure as well as its activating mechanism.

Key words
Ethanol – Na^+-ATPase – Kidney – Rat

Introduction

Ethanol easily penetrates the lipid bilayer of biological membranes, producing physical-chemical and structural changes, affecting the activity of membrane bound enzymes. Working with basolateral plasma membranes of rat kidney proximal tubular cells, we have shown in a previous report (Rothman *et al.* 1992) that 2 % ethanol in the incubation medium inhibits the activity of Na^+,K^+-ATPase, as well as of Na^+-ATPase in these membranes. It is well known that the effect of this concentration of ethanol on Na^+,K^+-ATPase is associated with a reduction of the affinity of ATPase for K^+, allowing in this way Na^+ competition which consequently inhibits the activity of ATPase (Israel *et al.* 1965, Israel and Salazar 1967, Swann 1986, Rothman *et al.* 1994). This does not apply Na^+-ATPase. The activity of this enzyme is inhibited to the same extent by 2 % ethanol, independently of the presence or absence of different concentrations of K^+ (Rothman *et al.* 1994). Even more, there is not yet a clear cut explanation as to how ethanol, at this concentration, affects the activity of this ATPase.

Even when the activity of both ATPases, Na^+ and Na^+,K^+, is related to the extrusion of Na^+ from the cells, the Na^+,K^+-ATPase is particularly involved in the control of the cell Na^+ and K^+concentrations (extrusion of Na^+ in exchange for K^+), while the Na^+-ATPase is mainly involved in the active control of cell volume (extrusion of Na^+, accompanied by Cl^- and water) (Whittembury and Proverbio 1970, Proverbio *et al.* 1991). Consequently, inhibition of one enzyme or the other, could drive cells to very different conditions. In this connection, it is very important that 50 mM ethanol (0.25 %), concentration that can be attained in rat blood plasma after large ingestion of alcohol (Akera *et al.* 1973) inhibits *in vitro* the activity of Na^+-ATPase by about 20 %, without producing any effect on the activity of the Na^+,K^+-ATPase (Rothman *et al.* 1992). The aim of the present work was to study how ethanol, on a 2 % concentration, affects the activity of Na^+-ATPase.

Methods

Membrane preparation

Outermost slices of the kidney cortex (rich in proximal tubules) of 3-month-old healthy male Sprague-Dawley rats were obtained as previously described (Whittembury 1961). Each gram of tissue was homogenized at 4 °C with eight strokes at 2500 rpm in an Eberbach homogenizer with a Teflon pestle, in 3 vol. of a solution of 0.25 M sucrose/20 mM Tris-HCl (pH 7.2)/0.5 mM dithiothreitol/0.2 mM phenylmethylsulfonylfluoride (sucrose/Tris medium) at 4 °C. Plasma membrane enriched fractions were prepared according to the method described elsewhere (Marín *et al.* 1983). The final pellet was resuspended in the sucrose/Tris medium, frozen, and kept at −20 °C.

ATPase activity determination

The ATPase activity was determined as already described (Proverbio *et al.* 1986). Briefly, 180 μl of the incubation medium containing (final concentrations): 50 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 2 mM Na^+-free Tris-ATP, 6 mM ouabain (Sigma, St. Louis, MO), were preincubated for 5 min at 37 °C in the presence or absence of 100 mM NaCl. The ionic strength of the incubation medium was kept constant with Tris-HCl (pH 7.2). The reaction was started by addition of 20 μl of the membrane suspensions (0.1 mg protein/ml), and continued for 10 min. At the end of the incubation, the reaction was stopped and the liberated inorganic phosphate was determined according to the method already described (Marín *et al.* 1986). Before the ATPase assays, the membranes were treated with sodium dodecyl sulfate (SDS, Bio-Rad Laboratories, Richmond, CA), using optimal ratios of SDS/protein, in order to avoid plasma membrane vesicle formation (Marín *et al.* 1986). Preliminary experiments showed that under all the tested conditions, the reaction was linear for even 30 min of incubation. The Na^+-ATPase activity was calculated as the difference between the P_i liberated in a medium containing Mg^{2+} + ouabain and that liberated in the presence of Mg^{2+} + Na^+ + ouabain. The ATPase activity was expressed as nanomoles of P_i per milligram of protein per minute. The enzyme activities were determined for six separate preparations which were assayed in quadruplicate at each point. Each preparation consisted of pooled membranes from the kidneys of six rats. The protein concentrations were determined by the Coomassie blue dye binding assay (Bio-Rad Laboratories, Richmond, CA) (Bradford 1976).

Statistical Analysis

Data are expressed as the mean ± S.E.M., *n* represents the number of experiments performed with different preparations. Differences between the results were analyzed by Student's t-test. Significance was accepted at $P<0.05$.

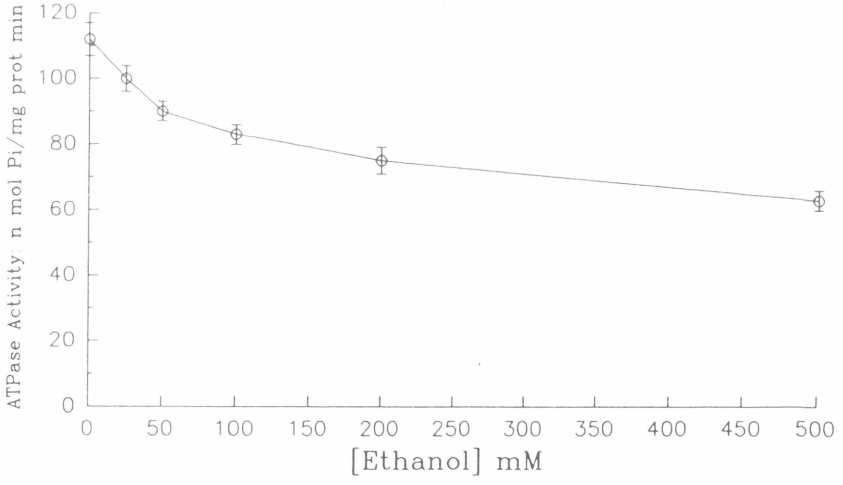


Fig. 1
Na^+-ATPase activity of basolateral plasma membranes from rat kidney proximal tubular cells as a function of the ethanol concentration in the incubation medium. Values are means ± S.E.M. (for six separate membrane preparations).

Results

Fig. 1 shows the effect of different ethanol concentrations on the activity of Na^+-ATPase. The higher was the ethanol concentration, the higher was its inhibitory effect, reaching a value of 44 % inhibition at a concentration of 500 mM (2.5 %). This inhibition is not due to a denaturalizing effect of ethanol on the ATPase, since membranes pretreated with 500 mM ethanol, washed and assayed in the absence of alcohol, had a similar Na^+-ATPase activity as control membranes (Rothman *et al.* 1994).

The inhibitory effect of ethanol on Na^+-ATPase can be ascribed to several causes: 1) changes in the affinity of the substrate or any ligand for their sites; 2) changes of the optimal conditions for the enzyme to be active; 3) lowered turnover rate of the enzyme; 4) diminution in the number of active or functional ATPases and 5) any combination of these possibilities.

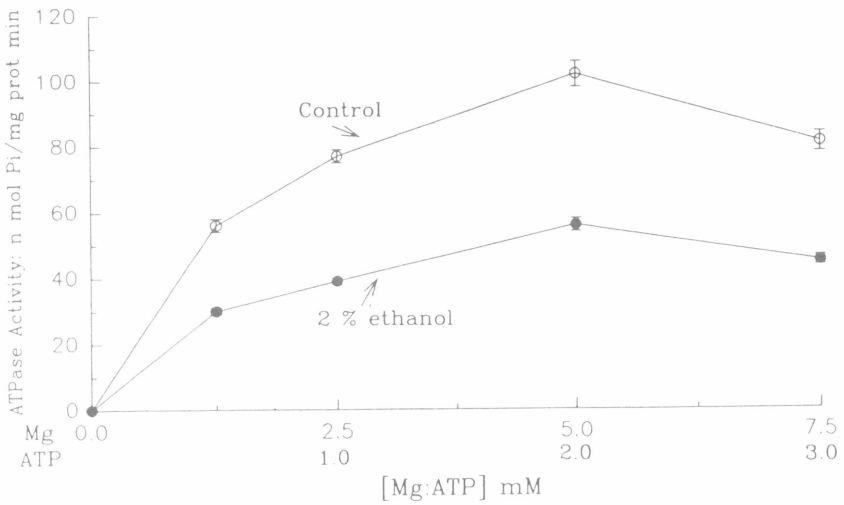


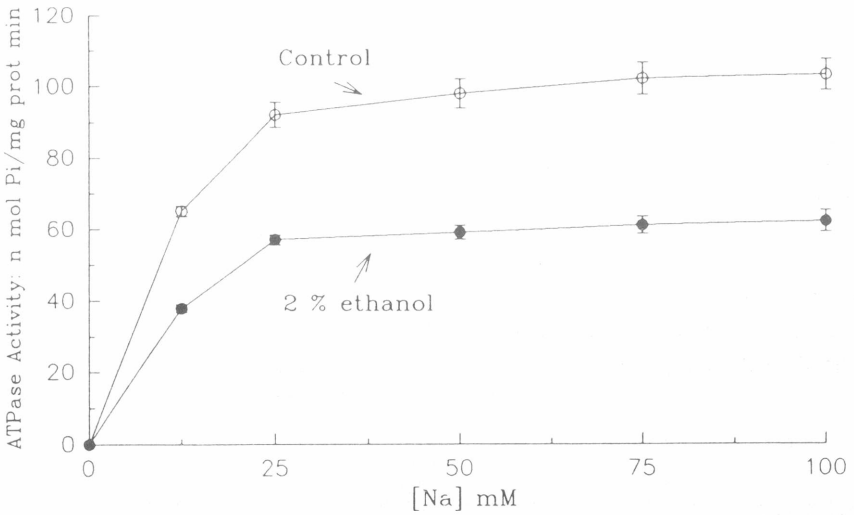
Fig. 2
Effect of increasing the Mg^{2+}: ATP concentration (at a fixed ratio of 2.5:1) in the incubation medium on the Na^+-ATPase activity of basolateral plasma membranes from rat kidney proximal tubular cells. The experiments were carried out in the presence or in the absence of 2 % ethanol. Values are means ± S.E.M. (for six separate membrane preparations).

1) Changes in the affinity of the substrate or any ligand for their sites

Fig. 2 shows the Na^+-ATPase activity of purified kidney plasma membranes as a function of the Mg^{2+}: ATP concentration in the incubation medium (in a previously determined optimal ratio of 2.5:1), in the presence or absence of 2 % ethanol. The presence of alcohol in the incubation medium lowered the ATPase activity for all the tested Mg^{2+}: ATP concentrations in a proportional way. The K_m for Mg^{2+}: ATP is around 0.50:0.20 (mM:mM), either in the presence or absence of ethanol. Similarly, 2 % ethanol in the incubation medium proportionally lowered the Na^+-ATPase activity when assayed as a function of the Na^+ concentration (Fig. 3). The K_m for Na^+ in both cases, in the presence or absence of ethanol, is around 8 mM.

On the other hand, furosemide, a known inhibitor of the Na^+-ATPase activity (Proverbio *et al.* 1991), showed a very similar effect on the enzyme, both in the presence or absence of 2 % ethanol. The K_i of inhibition, in both cases, was around 1 mM (data not shown).

Fig. 3
Effect of increasing the Na^+ concentration (as NaCl) in the incubation medium, on the Na^+-ATPase activity of basolateral plasma membranes from rat kidney proximal tubular cells. The experiments were carried out in the presence or in the absence of 2 % ethanol. Values are means ± S.E.M. (for six separate membrane preparations).



2) Changes of the optimal conditions for the enzyme work

To study the possibility that ethanol could be changing the optimal pH value for Na^+-ATPase, we studied the effect of different pH values of the incubation medium on the activity of this enzyme, in the presence and absence of 2 % ethanol. The results of this experiment are shown in Fig. 4. Under both conditions, in the presence or absence of ethanol, the enzyme responded similarly to the different tested pH values, and attained optimal activity at a pH value around 7. Similar results were obtained when we tested Na^+-ATPase activity as a function of the temperature of the incubation medium (Fig. 5). The optimal temperature value was found to be around 47 °C, both in the presence as well as in the absence of 2 %

ethanol. Utilizing the data of Fig. 5, we calculated the apparent energy of activation of Na^+-ATPase (assayed in the presence or absence of ethanol), by means of the Arrhenius plot. The results are shown in Table 1. In both cases it was observed, as had already been demonstrated for other ATPases (Charnock *et al.* 1971), that two slopes are present with a transition point at around 20 °C. The activation energy for each range of temperatures (10–20 °C or 20–47 °C) was the same, regardless of the presence or absence of 2 % ethanol.

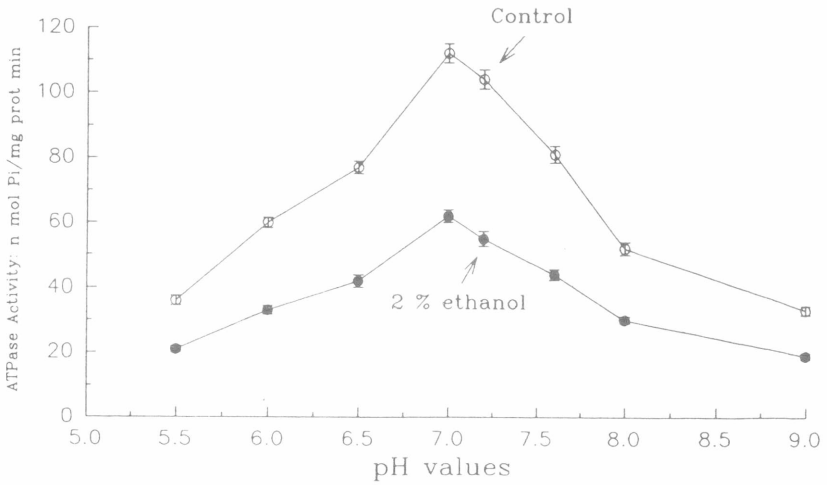


Fig. 4
Effect of increasing pH of the incubation medium on the Na^+-ATPase activity of basolateral plasma membranes from rat kidney proximal tubular cells. The experiments were carried out in the presence or in the absence of 2 % ethanol. Values are means ± S.E.M. (for six separate membrane preparations).

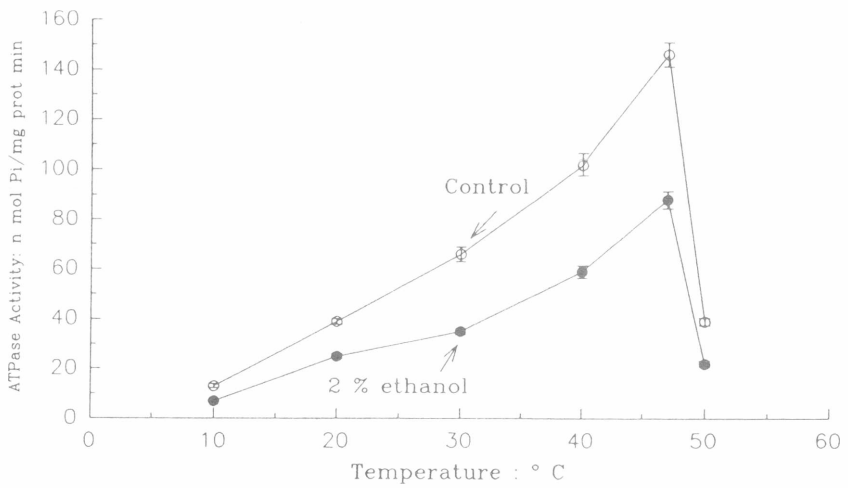


Fig. 5
Effect of increasing temperature of the incubation medium on the Na^+-ATPase activity of basolateral plasma membranes from rat kidney proximal tubular cells. The experiments were carried out in the presence or in the absence of 2 % ethanol. Values are means ± S.E.M. (for six separate membrane preparations).

The fact that 2 % ethanol in the incubation medium does not produce any change in the affinity of the substrate or any ligand for their sites, or changes in the optimal conditions for the enzyme to work, can be taken as an indication that the inhibitory effect of ethanol on Na^+-ATPase is due to a lowered turnover rate of the enzyme or/and to a reduced number of active or working ATPases. We do not yet have any experimental results to clearly differentiate between these two possibilities. However, we have demonstrated that the Na^+-ATPase of kidney tissue increases its activity upon cell swelling. This increased activity can be explained by an increased number of active or functional ATPases (Proverbio *et al.* 1988, 1991). If the effect of 2 % ethanol is to diminish the number of active enzymes, then we could expect the activity of Na^+-ATPase of membranes from swollen cells to exhibit a similar percentage of inhibition by 2 % ethanol as the activity of enzymes from cells with normal volume. To test this possibility, we studied the effect of 2% ethanol on the Na^+-ATPase activity of homogenates from normal or previously swollen cells. The results of this experiment are shown in Table 2. Note that the Na^+-ATPase activity of total homogenates from swollen cells increases around 4 times, as compared with the activity of homogenates from control cells. It is also evident that 2 % ethanol produces stronger inhibition of ATPase activity when it is in its "activated" state, i.e., working at a higher rate (72 % inhibition), than when the ATPase is working at a lower rate, i.e., at its "basal" level (39 % inhibition).

Table 1
Apparent energies of Na^+-ATPase activation in basolateral plasma membranes of rat kidney proximal tubular cells assayed in the presence or absence of 2 % ethanol in the incubation medium

Incubation condition	Apparent energy of activation $(kcal.mol)^{-1}$ Temperature range 10–20 °C	20–47 °C
Control	20.96±1.5	9.15±0.6
+ 2 % ethanol	20.04±0.1	8.93±0.7
Variation	−0.92±1.50#	−0.22±0.92#

The apparent energies of activation were calculated from the Arrhenius plot of the data shown in Fig. 5, by means of the following expression: $E_a = -m\ 2.303\ .\ R$, where m is the slope of the Arrhenius plot and R is the gas constant (1.987 $cal.mol^{-1}.°K^{-1}$). Values are expressed as means ± S.E.M. of six determinations.
 # not significant

Table 2
Effect of 2 % ethanol in the incubation medium on the Na^+-ATPase activity of homogenates from rat cortex kidney slices, freshly prepared (normal volume) or preincubated to increase their cell volume (activated)

Incubation medium	Na^+-ATPase activity $[nmol\ P_i.(mg\ protein)^{-1}.min^{-1}]$ Normal cell volume	Swollen cells
Control	18±1	53±2
+ 2 % ethanol	11±1	15±1
Ethanol effect	−7±1.4*	−38±2.2*
% inhibition	39	72

Incubations were carried-out as indicated under Materials and Methods. The cells were swollen by incubating the kidney cortex slices for 15 min at 0 °C in a medium containing 17 mM Tris-HCl (pH 7.2). Values are means ± S.E.M. (for six separate preparations)
** $P<0.001$.*

Since the activity of Na^+-ATPase of membranes from swollen cells is more sensitive to 2 % ethanol than the activity of the enzymes from normal volume cells, we may assume an extra effect of ethanol on the enzyme, when it is in its activated state. This extra effect could be exerted on the ATPase activating mechanism. To test this possibility, rat kidney cortex slices were preincubated for 15 min at 0 °C in a homogenization medium (isotonic) or in a hypotonic (10 mM Tris-HCl) medium, in the presence or absence of 2 % ethanol. After washing-out the alcohol, the tissue was homogenized and tested for Na^+-ATPase activity. The results of this experiment are shown in Table 3. It is clear that the preincubation of slices in the isotonic solution, in the presence of 2 % ethanol, does not affect the Na^+-ATPase activity of the homogenates; it is also clear that if preincubation is carried-out in the hypotonic solution, the presence of 2 % ethanol inhibits, almost totally, the activating effect of cell swelling on Na^+-ATPase activity. The presence of alcohol in the isotonic preincubation medium, did not have any effect on cell volume: 1.87±0.08 g cell water/g solid in the absence of ethanol and 1.92±0.06 g cell water/g solid in its presence. On the other hand, cellular swelling was about the same, in the presence or absence of ethanol, when the preincubation was carried out in the hypotonic medium (2.91±0.10 g cell water/g solids in the absence and 2.76±0.11 g cell water/g solids in the presence of ethanol).

Table 3
Na^+-ATPase activity of homogenates from rat kidney cortex slices preincubated in an isotonic or in a hypotonic medium, in the presence or absence of 2 % ethanol

Preincubations	Na^+-ATPase activity $[nmol\ Pi.(mg\ protein)^{-1}.min^{-1}]$
Isotonic medium	17±1.1
Isotonic medium + 2 % ethanol	16±0.8
Hypotonic medium	50±3.0
Hypotonic medium + 2 % ethanol	22±1.5

Incubations were carried out as indicated under Materials and Methods. Before homogenization, the kidney cortex slices were preincubated for 15 min at 0 °C in the homogenization medium (isotonic) or in a hypotonic medium containing 17 mM Tris-HCl (pH 7.2). In both cases the preincubations were done in the presence or absence of 2 % ethanol, which was washed out before homogenization. Values are means ± S.E.M. (for six separate preparations).

Discussion

Ethanol inhibits Na^+-ATPase in a concentration-dependent way. In 2 % ethanol, the ATPase is inhibited by about 40 % (Fig. 1), which was the reason why we used this alcohol concentration in the present study. Several possibilities were tested in order to explain this inhibitory effect of ethanol on this enzyme.

The possibility of K_m changes for any of the substrates or ligands of the enzyme were involved, was eliminated by considering the results shown in Figs 2 and 3. K_m of the enzyme for Mg^{2+}: ATP as well as for Na^+ is the same, regardless of the presence or absence of 2 % ethanol in the incubation medium. Furthermore, the behaviour of the enzyme in the presence of different concentrations of its inhibitor furosemide is exactly the same, whether assayed in the presence or absence of 2 % ethanol.

The results shown in Figs 4 and 5 indicate that 2 % ethanol in the incubation medium does not alter the optimal conditions for the activity of the enzyme, since in both, the absence or presence of ethanol, the maximal activity of the ATPase was reached at pH 7 and at 47 °C.

The fact that the optimal temperature and the apparent energy of activation of the enzyme are the same both in the presence or absence of 2 % ethanol (Fig. 5 and Table 1), argues against, even though it does not exclude, the possibility that they play an important role in affecting the turnover rate of the ATPase. On the other hand, ethanol inhibition is stronger when the ATPase is in its "activated", than in its "lower" basal state (Table 2). Finally, the activating effect of cell swelling on Na^+-ATPase is lost when the cell volume is increased in the presence of ethanol (Table 3). These results might be explained if it is assumed that 2 % ethanol in the incubation medium affects Na^+-ATPase in two different ways: 1) by inhibiting the activity of the enzyme, either by diminishing the number of active ATPases and/or by diminishing their turnover rate, and 2) by interfering with the activating mechanism of the ATPase, inhibiting its expression.

Activity of membrane-bound enzymes can be affected by membrane lipid fluidity (Farias *et al.* 1975). A decrease in the lipid microviscosity may displace membrane proteins towards the aqueous face and *vice versa* (Shinitzky 1979), exposing or hiding alternately active sites, and thus regulating the protein functions. Ethanol, which has been shown to increase membrane lipid fluidization (Goldstein and Chin 1981, Taraschi and Rubin 1985), may interfere with this passive regulation of membrane function, affecting in this way the activity of membrane-bound enzymes. In this respect, the activity of Na^+-ATPase has been shown to be affected by various factors: a) the pH of the membrane preparation medium (Proverbio and del Castillo 1981); b) the number of Ca^{2+} ions within the membrane (Proverbio *et al.* 1982); c) hydrostatic pressure (Proverbio *et al.* 1988, 1991), and d) the presence of ethanol (Rothman *et al.* 1992). Similarly, acidity, calcium, pressure and ethanol, are known to affect membrane lipid fluidity (Shinitzky 1984, Goldstein and Chin 1981, Taraschi and Rubin 1985).

Finally, we have shown that NaI, a chaotropic agent, is an inhibitor of Na^+-ATPase (Proverbio *et al.* 1986). NaI is known to produce its effects on biological membranes, mainly by interfering with lipid-protein and protein-protein interactions (Hatefi and Hanstein 1969). Ethanol, by perturbing hydrogen bonds, might affect lipid-protein or protein-protein interactions (Zahler and Niggli 1977), inhibiting in this way the activity of Na^+-ATPase in a similar way as NaI.

In summary, the presented results constitute a clear indication that ethanol inhibits the Na^+-ATPase of rat kidney proximal tubular cells, by disturbing the physico-chemical properties of the cellular membranes. This effect seems to be exerted both on the Na^+-ATPase structure, as well as on its activating mechanism (triggered by the cell volume).

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