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Protonation-dependent inactivation of Na,K-ATPase by hydrostatic pressure developed at high-speed centrifugation

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Abstract

Irreversible inactivation of membranous Na,K-ATPase by high-speed centrifugation in dilute aqueous solutions depends markedly on the protonation state of the protein. Pig kidney Na,K-ATPase is irreversibly inactivated at pH 5 but is fully protected at pH 7 and above. Shark rectal gland Na,K-ATPase is irreversibly inactivated at neutral or acidic pH and partially protected at an alkaline pH. The overall Na,K-ATPase activity and the K-dependent pNPPase activity were denatured in parallel. Cryoprotectants such as glycerol or sucrose at concentrations of 25–30% fully protect both enzymes against inactivation. The specific ligands NaCl and KCl protect the Na,K-ATPase activity partially and the pNPPase activity fully at concentrations of 0.2–0.3 M. Electron microscope analysis of the centrifuged Na,K-ATPase membranes revealed that the ultrastructure of the native membranes is preserved upon inactivation. It was also observed that the sarcoplasmic reticulum Ca-ATPase and hog gastric H,K-ATPase are susceptible to inactivation by high-speed centrifugation in a pH-dependent fashion. H,K-ATPase is protected at alkaline pH, whereas Ca-ATPase is protected only in the neutral pH range.

Keywords: Na,K-ATPase; H,K-ATPase; Ca-ATPase; Denaturation; Ultrastructure; Water activity

1. Introduction

It is well known that reversible as well as irreversible inactivation of enzymatic activity may occur at elevated hydrostatic pressures and pressure changes have been used as a technique to characterize the relation between enzyme structure and function (see e.g. [6,7]). Thus, reversible inhibition of Na,K-ATPase activity was reported by De Smedt et al. [2] and Chong et al. [3], and Champeil et al. [4] noted marked changes in electron spin resonance spectra of Ca-ATPase caused by large hydrostatic pressures developed during centrifugation experiments. Pressure effects on this enzyme have also been studied in detail by Varga et al. [5].

High-speed centrifugation is a routine step for the concentration and separation of membranes derived from plasma and subcellular membranes, and it is therefore essential to define the conditions where centrifugation-induced hydrostatic pressures influence the overall enzyme function as well as partial activities. Here we show that high-speed centrifugation leads to a pH-dependent irreversible inactivation of

Abbreviations: E_1, protein conformation of Na,K-ATPase predominant in Na^+-containing media; E_2, protein conformation of Na,K-ATPase predominant in K^+-containing media

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Na,K-ATPase and compare hydrostatic pressure effects on the inactivation of pig kidney and shark rectal gland enzymes. In addition we describe the protective effects of ligands such as NaCl and KCl and of the commonly used cryoprotectants glycerol and sucrose. The specific ligands, e.g. Na^+, which induces the E_1 conformation (protein conformation of Na,K-ATPase predominant in Na^+-containing media) characterized by high affinity nucleotide binding, and K^+, which induces the E_2 conformation (protein conformation of Na,K-ATPase predominant in K^+-containing media) with K^+ occluded and low affinity for nucleotides (see e.g. [6,7]), both protect against inactivation but at much higher concentrations than those required to induce the specific protein conformations. Eosin fluorescence is used to estimate the conformational state in the pH range 5.5–9.5, and there appears to be no correlation between the conformational state of the enzyme and the susceptibility to irreversible inactivation.

Electron microscopy of negatively-stained as well as thin-sectioned preparations shows that the inactivated membrane pellets contain membrane structures indiscernible from the native membranes, ruling out the formation of closed vesicles or an extensive fragmentation of the membranes as the cause of inactivation.

2. Materials and methods

2.1. Enzyme preparation and characterization

Na,K-ATPase was purified from two sources: rectal glands of *Squalus acanthias* [8] and pig kidney [9]. Enzymatic activity and protein measurements were done as previously described [10]. Na,K-ATPase activity refers to the rate of hydrolysis of ATP at 37°C in the presence of about 5 μg protein/ml, 130 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 3 mM ATP and 30 mM histidine (pH 7.4). Shark enzyme had a specific Na,K-ATPase activity of about 1700 μmol/mg protein/h and that of kidney enzyme was about 1500 μmol/mg protein/h. The K-activated phosphatase activity (pNPPase) was determined as the rate of hydrolysis of *p*-nitrophenylphosphate (10 mM) in the presence of 150 mM KCl, 20 mM $MgCl_2$ and 30 mM histidine (pH 7.4). For shark enzyme this was about 260 μmol/mg protein/h and for kidney enzyme about 230 μmol/mg protein/h.

H,K-ATPase was purified from hog stomach and freeze-dried as described by Rabon et al. [11]. H,K-ATPase activity was measured as the rate of ATP hydrolysis at 37°C in the presence of 20 mM NH_4Cl, 5 mM $MgCl_2$, 5 mM ATP, 0.1 mM EDTA and 30 mM histidine (pH 7.4). The values were corrected for the non-specific ATPase activity measured under the same conditions but with 20 mM *N*-methyl glucamine instead of NH_4Cl.

Ca-ATPase was purified from rabbit skeletal muscles [12]. Ca-ATPase activity was about 300 μmol/mg protein/h and refers to the rate of hydrolysis of ATP at 23°C in the presence of 0.1 mM $CaCl_2$, 100 mM KCl, 6 mM $MgCl_2$, 5 mM ATP and 30 mM histidine (pH 7.0).

Data in figures are presented as the average of three or more determinations. S.E.M. is given when exceeding 10%.

2.2. Eosin fluorescence measurements

The conformational state of Na,K-ATPase was evaluated from the fluorescence of eosin bound to the protein. Shark or kidney enzyme at a final concentration of 30 μg/ml was incubated in 5 mM histidine+5 mM Tris (varying pH) with 0.4 μM eosin for 5 min at room temperature. The eosin fluorescence was followed in a Spex fluorometer at 23°C (excitation wavelength 530 nm (bandpass 0.1 nm), 550 nm cut-off filter on the emission side) [13]. Maximal eosin binding was induced by 30 mM NaCl and complete dissociation was caused by the addition of 200 μM ADP [13].

2.3. High-speed centrifugation

Stock solutions of the enzymes were diluted at least 100-fold (to a final protein concentration of about 25 μg/ml) with 5 mM histidine+5 mM Tris buffer at the desired pH and concentration of glycerol, sucrose, NaCl or KCl. Membranes were pelleted in a Beckman L70 ultracentrifuge at 10°C in 27-ml tubes with a fixed-angle rotor (Ti70). The hydrostatic pressure at the position of the pellet was calculated using a radius of 8 cm and an aqueous column height of 4 cm. Pellets were resuspended by

homogenization (3 strokes at 2000 rpm in a 2 ml Braun homogenizer) in 1 ml of 20 mM histidine, 25% glycerol (pH 7.0) and the specific activities were determined. The protein concentration in the homogenate was 0.3–0.6 mg/ml.

2.4. Electron microscopy

Membrane samples were negatively-stained with 1% uranyl acetate or fixed with 2% glutaraldehyde, post-fixed in osmium tetroxide, stained en bloc with uranyl acetate and imbedded in Epon. Thin sections were stained with uranyl acetate and lead citrate. The specimens were examined in a Philips 208 electron microscope. Details of the procedures for microscopy were as described before [14].

3. Results and discussion

3.1. Centrifugation and stability of Na,K-ATPase

Centrifugation of shark or kidney Na,K-ATPase (1 h at 340 000 × g at 10°C) leads to the formation of a membrane pellet, the enzymatic activities of which is strongly affected by the pH of the centrifugation medium (Fig. 1). Both shark and kidney enzymes were almost completely and irreversibly inhibited by centrifugation at low pH. At neutral pH most of the enzymatic activity of kidney Na,K-ATPase was preserved, whereas the shark enzyme was inactive. At pH 9 shark enzyme possessed about 60% of the initial activity, and the kidney enzyme was fully active. The pNPPase activity, which under most conditions appears to be more robust [10], was reduced in parallel with the Na,K-ATPase activity (Fig. 1).

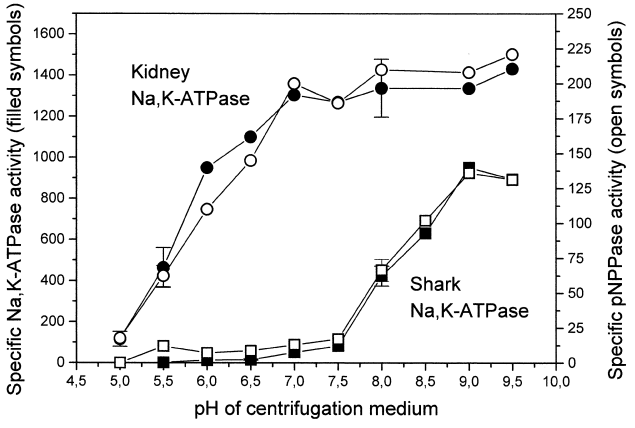

Fig. 1. pH-dependence of inactivation of Na,K-ATPase due to centrifugation. Na,K-ATPase from shark (squares) or kidney (circles) was diluted with 5 mM histidine+5 mM Tris buffer at the indicated pH to a protein concentration of 0.025 mg/ml and subjected to centrifugation for 60 min at 60 000 rpm. The pelleted enzyme was homogenized in 25% glycerol, 20 mM histidine (pH 7.0) and the specific Na,K-ATPase (filled symbols) and pNPPase (open symbols) were determined.

Loss of enzyme activity is due to the centrifugation step alone, not to the dilution or protonation of the enzyme. Fig. 2A shows that for kidney enzyme the Na,K-ATPase activity is fully retained after incubation for 3 h in the pH range of 5.5 to 9.5, both at 0 and 20°C, which is sufficient time for centrifugation and for fluorescence measurements. Shark enzyme is more labile below pH 6, but is stable in the range 6.5–9.5. Homogenization of the pellets was shown in control experiments not to cause inactivation, and sodium dodecyl sulfate gels revealed no changes in the content of subunits relative to that of native membranes.

Centrifugation for shorter periods or at lower speeds showed that the degree of inactivation is a function of time of centrifugation as well as the magnitude of the hydrostatic pressure (Table 1). At 20 000 rpm, corresponding to a hydrostatic pressure of 0.15 kbar, kidney Na,K-ATPase was stable during centrifugation up to 1 h at pH 5, suggesting that the threshold for irreversible inactivation is about 0.2–0.3 kbar (cf. Table 1). Centrifugation with a smaller aqueous column height in the centrifuge tube (and thus reduced hydrostatic pressure) also reduced inactivation (not shown).

3.2. Conformational state of centrifuged Na,K-ATPase

The $E_1 \Leftrightarrow E_2$ equilibrium between forms of Na,K-ATPase is well known to be strongly influenced by pH in the absence of added cations: enzyme is predominantly in the E_1 form at low pH and completely in the E_2 form at pH 9.4 due to the protonation of the amine-containing buffers used [15]. The conformational state of the Na,K-ATPase in the pH range of the centrifugations was studied using eosin as an E_1 marker (previous investigations of hydrostatic

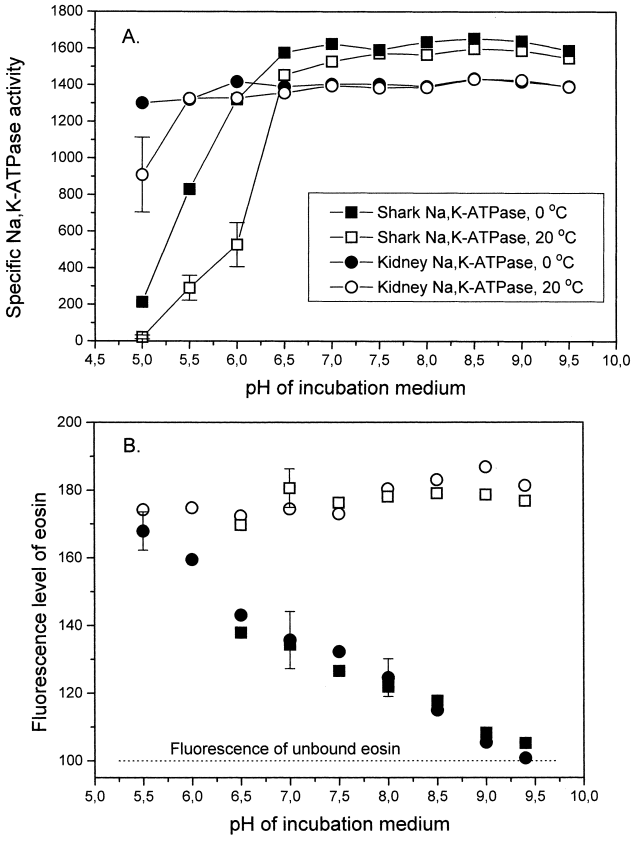

Fig. 2. pH-dependence of stability and conformational state of Na,K-ATPase. (A) Na,K-ATPase from shark (squares) or kidney (circles) was diluted with 5 mM histidine+5 mM Tris buffer at the indicated pH to a protein concentration of 0.03 mg/ml, and the enzyme activity was measured after 3 h at 0°C (filled symbols) or 20°C (open symbols). (B) Enzymes were diluted into the same buffers in the presence of 0.4 μM eosin at room temperature and the initial fluorescence level was determined (filled symbols) as well as the levels after addition of 30 mM NaCl (open symbols) and 200 μM ADP, giving the fluorescence level of free eosin (= 100%).

pressure effects on enzyme activity have emphasized the importance of the conformational state of the protein, see e.g. [3,16]). The following measurements were done at each pH value (Fig. 2B): the initial level of eosin fluorescence in the buffer in the presence of enzyme (filled symbols), the maximal level of fluorescence induced by addition of 30 mM NaCl (open symbols) and the minimum fluorescence level obtained after addition of 200 μM ADP which displaces eosin from its binding site (this level is taken as 100%). The total eosin fluorescence response (about 80%) reflects the number of functional molecules in the sample and was constant under experimental conditions used. It suggests that the dissociation constant (K_D) for eosin and the fluorescence yield are not affected by protonation in this pH range.

The titration curves were similar for kidney and shark enzyme (experiments with shark enzyme below pH 6.5 were omitted due to the spontaneous inactivation observed at room temperature, Fig. 2A). Combination of the data on initial fluorescence level (i.e. the proportion of E_1 conformation, Fig. 2B) and on inactivation by centrifugation (Fig. 1) suggests that the E_2 form of kidney Na,K-ATPase predominant at high pH is less prone to inactivation than the E_1 form predominant at low pH. Yet, half-maximal protection against inactivation occurs at pH 6, whereas a pH of about 7 is required to turn half of the kidney enzyme molecules into the E_2-form. The difference in the position of the titration curves is more pronounced for shark Na,K-ATPase, where half of the enzyme is in the E_2 form at pH 6.5, and half-maximal protection against inactivation occurs at pH 8–9. Thus, there seems to be no simple correlation between the conformational state and the susceptibility to pressure-induced inactivation.

Table 1
Time- and pressure-dependence of inactivation of Na,K-ATPase by centrifugation

Time (min)	30 000 rpm (0.34 kbar)	40 000 rpm (0.60 kbar)	50 000 rpm (0.94 kbar)	60 000 rpm (1.36 kbar)
10	76 ± 9%	72 ± 11%	46 ± 20%	15 ± 5%
30	70 ± 11%	58 ± 7%	24 ± 4%	3 ± 2%
60	58 ± 13%	46 ± 6	14 ± 1%	1 ± 1%

Kidney Na,K-ATPase (0.03 mg/ml) in 5 mM histidine+5 mM Tris buffer at pH 5.0 was centrifuged at 10°C for the indicated times at angular velocities of 30 000–60 000 rpm, and the specific Na,K-ATPase activity of the pellet was measured. The values are given in percent (± S.D.) of the activity of the native enzyme (100% = 1460 μmol/mg/h).

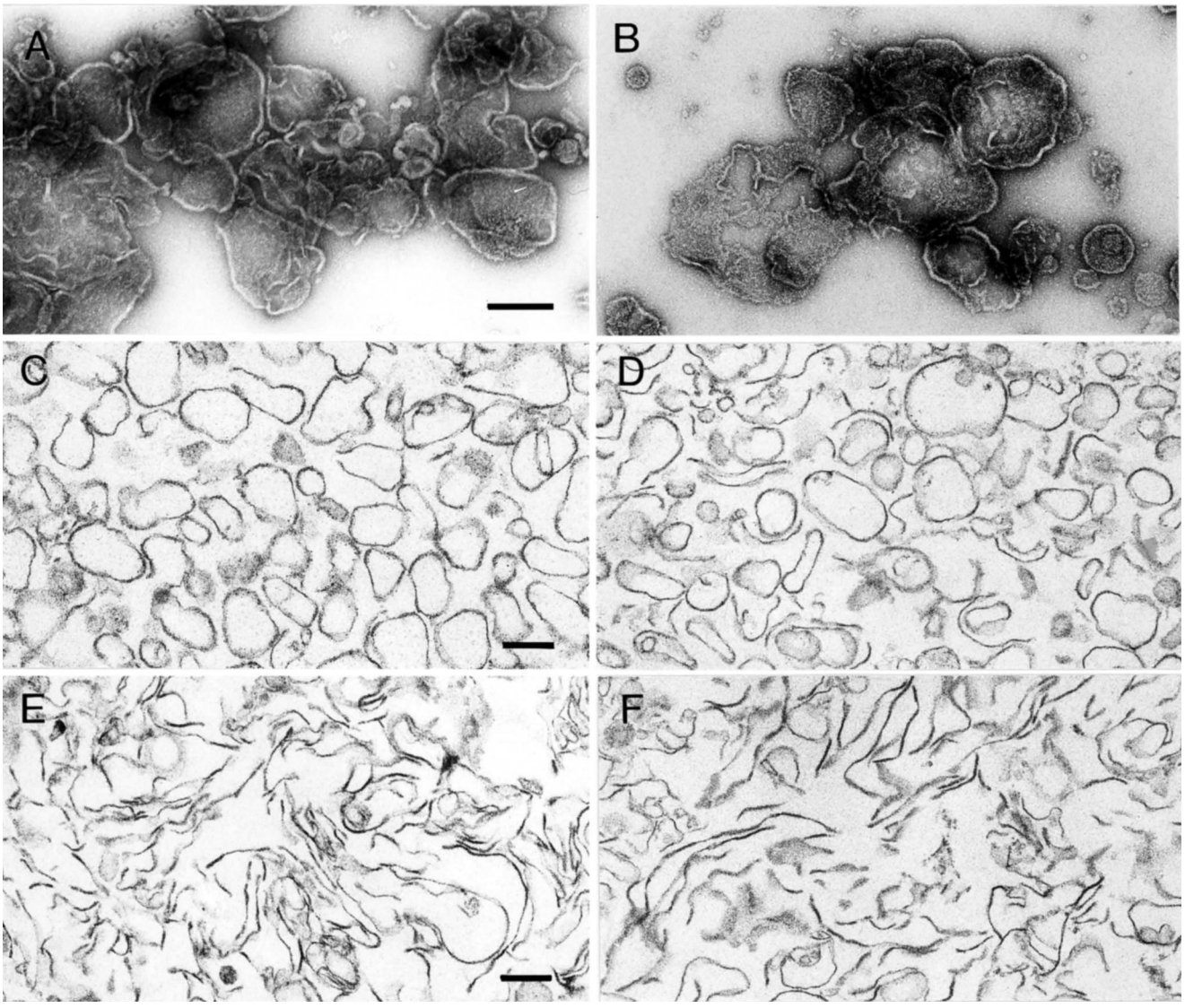

Fig. 3. Electron microscopy of centrifuged Na,K-ATPase membranes. Shark membranes were centrifuged at pH 5.0 (A) and pH 9.5 (B) and negatively stained with 1% uranyl acetate and recorded in the electron microscope. The membranes vary in diameter and have a tendency to stick together but there is no systematic difference in structure between membranes centrifuged at low and high pH. (C) and (D) show the corresponding shark preparations following thin sectioning. There is no obvious difference between membranes centrifuged at pH 5.0 (C) and pH 9.5 (D). Membranes appear either as open cups or rounded profiles depending upon the plane of sectioning. (E) and (F) show kidney membranes centrifuged at pH 5.0 (E) and pH 9.5 (F). Kidney membranes characteristically appear as flattened or curved discs but there is no difference between membranes centrifuged at low and high pH. Bar: 0.2 μm.

3.3. *Ultrastructure of centrifuged Na,K-ATPase membranes*

Electron microscopy of resuspended membrane pellets demonstrated that preparations from both shark and kidney contained membrane fragments of diameters ranging from 0.05 to 0.2 μm in diameter. Following negative staining, the shark membranes were either extended flat on the support film or appeared as open but flattened sacs (Fig. 3A,B). Importantly there was no structural difference between membranes centrifuged at pH 5.0 and pH 9.5 (Fig. 3). Also the kidney membranes were similar at low and high pH and exhibited the characteristic

surface particles corresponding to the enzyme protein (not shown, see [17]). In thin sections the shark membranes appeared as ring-like or opened profiles corresponding to a cup-shaped structure cut at different angles (Fig. 3C,D), while kidney membranes were more open and flattened (Fig. 3E,F). Also in thin sections there were no structural differences between the appearance of the membranes prepared at pH 5.0 (Fig. 3C,E) and pH 9.5 (Fig. 3D,F).

These observations demonstrate that the difference in activities measured after centrifugation at low and high pH is not related to, for example, opening/closure of membrane vesicles, or to fragmentation or packing of the membranes. Solubilization of the pelleted membranes in the nonionic detergent octaethyleneglycoldodecylmonoether [18] did not lead to the recovery of enzymatic activity (not shown), which also suggests that the formation of closed vesicles is not the reason for loss of activity.

3.4. Protection of Na,K-ATPase by ligands

One of the explanations of pressure-induced inactivation of enzymes is that water molecules are pressed in the protein interior thus changing the environment and organization of the catalytic center [1]. Chong et al. [3] suggested that the ion-bound or ion-occluded forms of Na,K-ATPase occupy a smaller volume. As a compact molecule can be expected to have higher stability towards inactivation by hydrostatic pressure we studied the ability of the specific cations to protect enzyme during centrifugation. Fig. 4A shows the activity of shark enzyme when NaCl or KCl were present in the centrifugation medium at pH 6.5. There is an almost complete protection of the pNPPase activity (open symbols) but only partial protection of the Na,K-ATPase activity at 300 mM NaCl. KCl appears to protect slightly better than NaCl. Similar experiments with kidney enzyme at pH 5 are shown in Fig. 4B. Again, the enzyme possesses almost full pNPPase activity and partial Na,K-ATPase activity, and at low concentrations KCl protects better than NaCl. The extent of cation protection depends on the pH in the centrifugation medium. Centrifugation of shark enzyme at pH 6.0 instead of pH 6.5 requires about five times more NaCl to protect against inactivation, i.e. protonation of the enzyme seems to be antagonistic to-

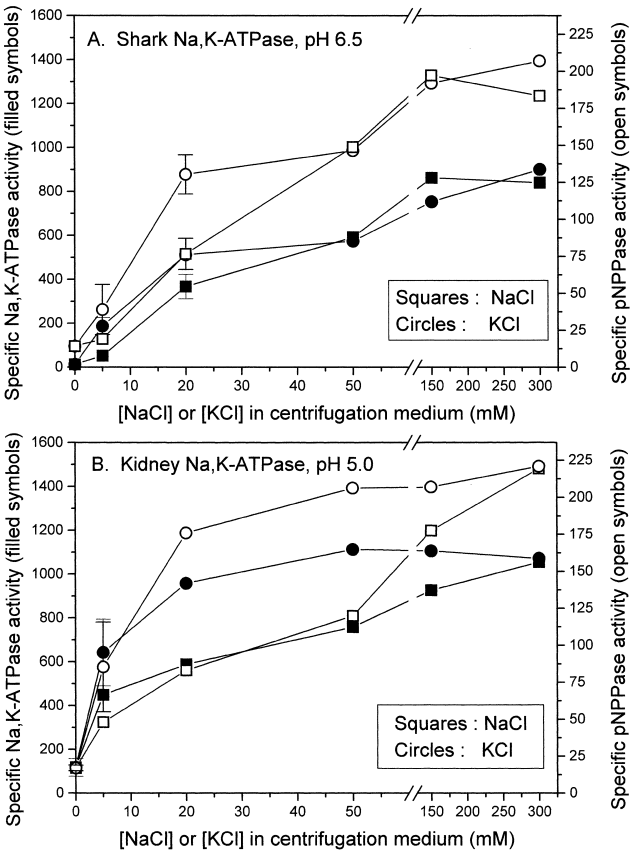

Fig. 4. NaCl and KCl protect against pressure-induced inactivation of Na,K-ATPase. (A) Shark enzyme was centrifuged at pH 6.5 (see legend to Fig. 1) in the presence of NaCl (squares) or KCl (circles) and specific Na,K-ATPase (filled symbols) and pNPPase (open symbols) activities of the pellets were determined. (B) Results obtained with kidney enzyme treated similarly, but at pH 5.0.

wards the protective effect of ions (not shown). The addition of 100 μM ADP to protect the nucleotide binding site had no effect on inactivation (not shown).

The difference between shark and kidney Na,K-ATPase in susceptibility to inactivation could stem from the different lipid environments. Lipids extracted from shark membranes exhibit a much larger fluidity than those from kidney membranes (Esmann and Marsh, unpublished observations), and the stiffening effect of the high pressure might thus be more deleterious to the shark enzyme than to the kidney enzyme. Other factors such as changes in oligomerization might play a role in the inactivation (see Champeil et al. [4] for a detailed discussion of Ca-

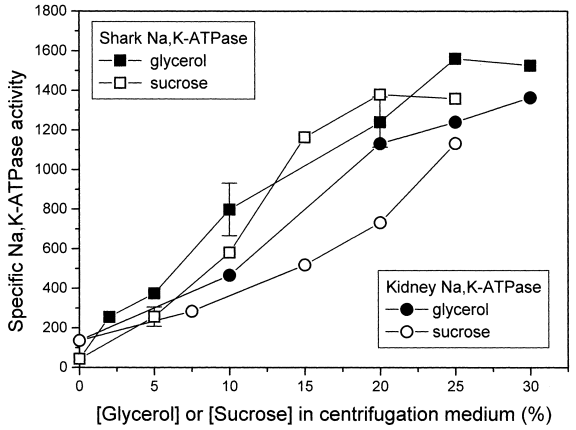

Fig. 5. Glycerol and sucrose protect against pressure-induced inactivation of Na,K-ATPase. Shark enzyme (squares) was centrifuged at pH 7.0 (see legend to Fig. 1) in the presence of glycerol (filled symbols) or sucrose (open symbols). Kidney enzyme (circles) was treated similarly, but at pH 5.0.

ATPase). It is known that the shark enzyme in the solubilized state favors a diprotomeric structure whereas kidney is more protomeric (see e.g. [18,19]). The disruption of protomer interactions by high pressure would thus affect the shark enzyme relatively more than the kidney enzyme.

3.5. Protection by glycerol or sucrose

The pressure-induced inactivation of Na,K-ATPase was reduced by the presence of glycerol or sucrose, in agreement with previous reports of Champeil et al. [4] and Varga et al. [5] on Ca-ATPase. Fig. 5 shows that the presence of glycerol (or sucrose) at high concentrations (20–30%) in the centrifugation buffer at pH 7.0 protects shark enzyme against inactivation. Protection of kidney enzyme is shown for centrifugation at pH 5.0 (Fig. 5). Note, that kidney enzyme usually is purified at neutral pH, where inactivation is negligible even in the absence of sucrose. For both enzymes it was found that the pNPPase activity was recovered parallel with the Na,K-ATPase activity (not shown). The protective effect of glycerol and sucrose is non-specific and probably due to their influence on the degree of water organization (see [5]).

We have previously noted a 20–30% decrease in specific Na,K-ATPase activity when solubilized Na,K-ATPase was precipitated with Mn^{2+}, whereby membranous structures are formed [20]. The procedure involved a centrifugation step in 13% glycerol. From the experiments shown in Fig. 5 it seemed possible that the decrease in specific activity was due to the non-optimal glycerol concentration, and we are now able to precipitate solubilized Na,K-ATPase with Mn^{2+} and carefully centrifuge the membranes under conditions where the loss of specific activity is less than 10% (Esmann and Fedosova, unpublished observations).

3.6. Protonation-dependence of inactivation of H,K-ATPase and Ca-ATPase

Two other P-type ATPases [6] used in this study exhibited similar protonation-dependent behavior (Fig. 6). H,K-ATPase was found to be stable in the pH range of the experiment (not shown) and the inactivation by centrifugation (squares) followed a pattern similar to that of kidney Na,K-ATPase with almost full inactivation at pH 5 and complete protection of activity at pH 8–9.

Ca-ATPase, on the other hand, appeared to be more sensitive and was inactivated by centrifugation both at low and high pH. At pH 6.0 the specific

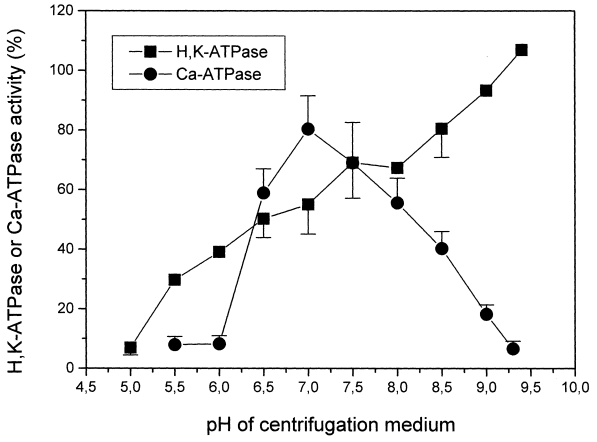

Fig. 6. pH-effect on inactivation of H,K-ATPase and Ca-ATPase due to centrifugation. H,K-ATPase (squares) was diluted with 5 mM histidine+5 mM Tris buffer (about 0.03 mg protein/ml) and Ca-ATPase (circles) was diluted with 80 mM KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 mM histidine+5 mM Tris buffer (about 0.03 mg protein/ml) at the indicated pH-values and subjected to centrifugation for 60 min at 60 000 rpm. The pelleted enzymes were homogenized in 20 mM histidine +25% glycerol (pH 7.0) and the specific activity of the pellets was determined. The activities are given in percent of the activity prior to centrifugation.

activity is only about 5% of maximal, whereas the specific activity is almost fully recovered in the pH range 7–8. Above pH 8 considerable inactivation is observed (cf. Fig. 6). Champeil et al. [4] also observed that inactivation of Ca-ATPase at 0.8 kbar was more marked at pH 6 than at pH 7.5 (cf. discussion in [4]).

3.7. Conclusion

Membranous preparations of three cation transport ATPases have a striking pH-dependence of the susceptibility to irreversible inactivation due to large hydrostatic pressures developed during centrifugation at high speeds. For Na,K-ATPase there is a marked difference between the mammalian source (pig kidney) and the shark rectal gland enzyme, the latter being more sensitive towards inactivation at neutral pH. The related cation transport ATPases, H,K-ATPase and Ca-ATPase, are also inactivated in a protonation-dependent fashion, and Ca-ATPase displays a bell-shaped activity curve with inactivation at high and low pH, but protection in the neutral range. The centrifugation method can be used to study the influence of hydrostatic pressure on partial reactions such as cation occlusion, ouabain and nucleotide binding and stress the importance of monitoring the effects of centrifugation in handling of membranes containing P-type ATPases.

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