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ELECTROKINETIC PROPERTIES OF (Na^+, K^+)-ATPase VESICLES AS STUDIED BY LASER DOPPLER SPECTROSCOPY

PETER SCHLIEPER [a], RAM MOHAN [b] and RAIMUND KAUFMANN [b]

[a] *Department of Pharmacology and* [b] *Department of Physiology, University of Düsseldorf, D-4000 Düsseldorf (F.R.G.)*

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The technique of laser Doppler electrophoresis was applied for the study of the surface charge properties of (Na^+, K^+)-ATPase containing microsomal vesicles derived from guinea-pig kidney. The influence of pH, the screening and binding of uni- and divalent cations and the binding of ATP show: (1) one net negative charge per protein unit with a pK = 3.9; (2) deviation from the Debye relation between surface potential and ionic strength for univalent cations, with no difference in the effect of Na^+ and K^+; (3) Mg^{2+} binds with an association constant of $K_a = 1.1 \cdot 10^2$ M^{-1} while ATP binds with an apparent $K_a = 1.1 \cdot 10^4$ M^{-1} for 1 mM NaCl, 0.2 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM Tris-HCl (pH 7.3). The binding is weaker at higher Mg^{2+} concentrations. There is no ATP binding in the absence of Mg^{2+}. In addition, the average vesicle size derived from the linewidth of the quasi-elastic light scattering spectrum is 203.7 ± 15.2 nm. In the presence of ATP a reduction in size is observed.

Introduction

The influence of the surface potential on the overall transmembrane potential in excitable tissues and its related physiological properties has been discussed by many authors [1–6]. Surface potentials play also an important role in the membrane properties of bacterial cell walls [7,8], in halobacterium [9] and in thylakoids [10]. Biochemically, surface charges may exert effects on phase transitions and long range forces in lipid bilayers [11–13]; they also modulate particle adhesion, fusion and aggregation [14–17] and may influence ligand binding [18].

Surface chemical groups of biological cells have been successfully investigated by electrophoresis [19]. In addition to the usual microelectrophoretic method, by which the particles had to be observed in an microscope, a new technique has been developed, basing on the Doppler principle (laser Doppler electrophoresis, [20,21]). This technique has been successfully applied for the characterisation of the surface groups of blood cells [22,23], chromaffin granules [24], synaptic vesicles [25] and mast cells [26].

A slightly modified technique of quasi-elastic light scattering in the absence of an electric field has been used by Selser et al. [27] to measure the size and polydispersity in size of small (less than 1 μm) spherical particles. Their measurements on sarcoplasmic reticulum vesicles are in agreement with electron microscopic pictures.

We have combined both techniques to investigate the biophysical properties of a microsomal preparation, enriched with (Na^+, K^+)-ATPase. This preparation appears to form spherical closed vesicles in which single ATPase protein units protrude from the surface. Therefore it is ideally suited to detect surface active groups and to study the binding of ions and substrates to the ATPase protein.

Materials and Methods

Vesicle preparation

The isolation of the (Na^+, K^+)-ATPase from guinea-

pig kidney was based on the method of Skou [28]. After homogenization of the kidneys in 0.25 M sucrose, 5 mM Na_2-EDTA, 30 mM histidine and 0.1% sodium desoxycholate (pH 6.8), the sample was centrifuged at 8000 $\times g$ for 10 min. The supernatant was centrifuged for 90 min at 40000 $\times g$ and the pellet was resuspended in 2 M KI, 3.3 mM $MgCl_2$, 2.5 mM Tris-EDTA, 2.5 mM cysteine and 1.3 mM Tris-ATP (pH 8.0) and gently stirred for 30 min at 4°C. Then the sample was diluted with distilled water to a KI-concentration of 0.8 M and centrifuged for 30 min at 100000 $\times g$. The pellet was resuspended in 5 mM Tris-EDTA, pH 7.4, again centrifuged and homogenized in 0.25 M sucrose, 1 mM EDTA, 3 mM Tris (pH 7.0) followed by dialysis for 18 h. The preparation was stored in small fractions at −20°C. The protein content of the preparation, determined according to the method of Lowry et al. [29] (bovine albumin as standard), was between 2 to 3 mg/ml. The activity was measured with a coupled spectrophotometric assay [30] at 37°C and expressed in μmol phosphate per mg protein per h.

Materials

ATP and ADP were obtained from Boehringer (Mannheim, F.R.G.) as disodium salts. The substances were dissolved in dust-free, double-distilled water and then titrated back to the corresponding pH, at which the experiment was performed. All other chemicals were reagent grade.

Electron microscopy

To remove the sucrose, which could damage the microsomes during electron microscopic observations, 1 ml of the enzyme preparation was dialyzed in the cold for 12 h against 10 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 1 mM Tris (pH 7.3). For negative staining a drop of the suspension was spread on the surface of a 2% phosphotungstic acid solution, neutralized by NaOH to pH 7.3. The preparation was transferred onto a carbon coated grid and immediately examined in a Siemens Elmiskop 101.

Experimental procedure

The instrumental setup for the measurement of Doppler shift of the scattered laser light in electrophoresis has been described [31]. The electrophoresis cell was made of Perspex and consisted of at least four compartments. The two outer buffer compartments contained the platinum electrodes used to apply the constant electrical field of 100 V. They were separated from the two inner sample compartments by dialysis paper (small pore size). The two sample compartments, with a total content of 5 ml, were connected by a 17-mm long glass capillary coated with methylcellulose. For measurement, the electrode compartments contained the same buffer as the sample chambers. 50 μl of the enzyme suspension was diluted into 5 ml of the corresponding buffer. Only buffers with an ionic strength below 14 mM could be used. Higher ionic strengths could lead to Joule heating phenomena and to the formation of bubbles on the electrodes. Experiments were performed at room temperature, 20–23°C. The ATPase preparation was used without sonication. A He-Ne laser (Spectraphysics) of 15 mW strength and 632.8 nm wavelength was used in all experiments.

Independent quasi elastic light scattering experiments for the measurements of diffusion coefficients in the absence of an electric field were conducted in 2 mm wide rectangular quartz cells.

Theory

The average number of vesicles in the final diluted sample was $2 \cdot 10^9$ per ml. This was determined by counting the number of protein units for an individual average size vesicle on electronmicrographs. Knowing the protein concentration of a given sample and the total number of protein molecules (a molecular weight of $3 \cdot 10^5$ was used) the number of average size vesicles could be calculated. The number density corresponds to an average interparticle distance of 8 μm, which is far enough to preclude any long range interactions between particles. Therefore, the results of diffusion and electrophoretic mobility measurements correspond to a population of non-interacting particles.

Particle size and distribution of sizes were obtained from the experimental light scattering power spectrum adjudged as more reliable than the comparative linewidth from the electrophoresis experiments. For a better procedure for size estimation, the power spectrum was subjected to Fourier transformation to yield the autocorrelation function.

Unlike the power spectrum the autocorrelation function involves fewer assumptions for analysis of polydispersity [32]. Following the technique of Selser et al. [27], the autocorrelation function was acquired at low angles of light scattering (30° angle and below) which are also the suitable angles for electrophoretic light scattering measurements.

In the frequency power spectrum, the half-width of the peak depends only on the diffusion coefficient (D) and the scattering vector (K) according to Eqn. 1 [31]

$$\Gamma = K^2 D \tag{1}$$

with

$$K = \frac{4\pi}{\lambda} \sin\frac{\vartheta}{2} \tag{2}$$

where λ is the wavelength of the He-Ne-laser and ϑ is the angle at which the experiment was performed, i.e. the angle between the incident laser light and the photomultiplier. The diffusion coefficient is calculated from

$$D = kT/6\pi\eta a \tag{3}$$

where k is the Boltzmann constant, T the absolute temperature, η the viscosity and 'a' the particle radius. Combining these three equations allows the determination of the mean particle radius. The electrophoretic mobility was calculated from the Doppler shift in frequency, according to the equation

$$\Delta\omega = \mu E K \cos\alpha \tag{4}$$

where μ is the electrophoretic mobility ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$), E is the applied electrical field ($V \cdot cm^{-1}$), K the scattering vector, and α the angle between the direction of the applied electric field and the scattering vector K. The cell was adjusted that way that E and K were parallel ($\cos\alpha = 1$). Mobilities were converted to ζ-potentials by the Henry equation:

$$\zeta = \frac{3\eta\mu}{2\epsilon_r\epsilon_0 f(\mathcal{H}a)} \tag{5}$$

$\epsilon_r\epsilon_0$ are the dielectric constant and the permittivity of free space, respectively, and $f(\mathcal{H}a)$ is the Henry's function, which varies between 1.0 and 1.5 as $\mathcal{H}a$ goes from zero to infinity. $1/\mathcal{H}$ is the thickness of the diffuse double layer. $\mathcal{H}$ is calculated by

$$\mathcal{H} = 0.328 \cdot 10^8 \sqrt{cz^2} \tag{6}$$

c is the concentration and z the valency of a symmetrical electrolyte. The charge density σ at the surface of shear is calculated from the Gouy-Chapman equation:

$$\sigma = \frac{\sqrt{c}}{13\,660} \sin h \frac{zF}{2RT} \zeta \tag{7}$$

where σ is in e^-/nm^2, R gas constant, F Faraday constant and T the absolute temperature. Binding constants were determined according to the Langmuir adsorption isotherm

$$\sigma = \frac{\sigma_{initial}}{1 + K_a C_0} \tag{8}$$

where $\sigma_{initial}$ is the charge density before administration of ATP, K_a the association constant and C_0 the concentration of drug or ion at the plane of shear. C_0 is related to the bulk concentration c by the Boltzmann expression:

$$C_0 = c \exp(-\zeta F/RT) \tag{9}$$

Results

Biochemistry

Specific activity of (Na^+, K^+)-ATPase preparations from guinea-pig kidney in various media have been measured with the coupled spectrophotometric assay. The activity is high in a medium of high ionic concentrations and at a temperature of 37°C (309 μmol phosphate/mg protein per h at 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 50 mM Tris). This is reduced to only 13 μmol phosphate/mg protein per h when the preparation is incubated at room temperature. At low ionic concentrations (10 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM Tris) ATPase activity is 87 μmol phosphate/mg protein per h at 37°C and correspondingly 3 at room-temperature. The temperature (room temperature) and the ionic concentration (low) used in our experiments are such that ATP is bound but the enzyme is not dephosphorylated.

Electron microscopy

Fig. 1 shows (Na^+, K^+)-ATPase containing vesicles,

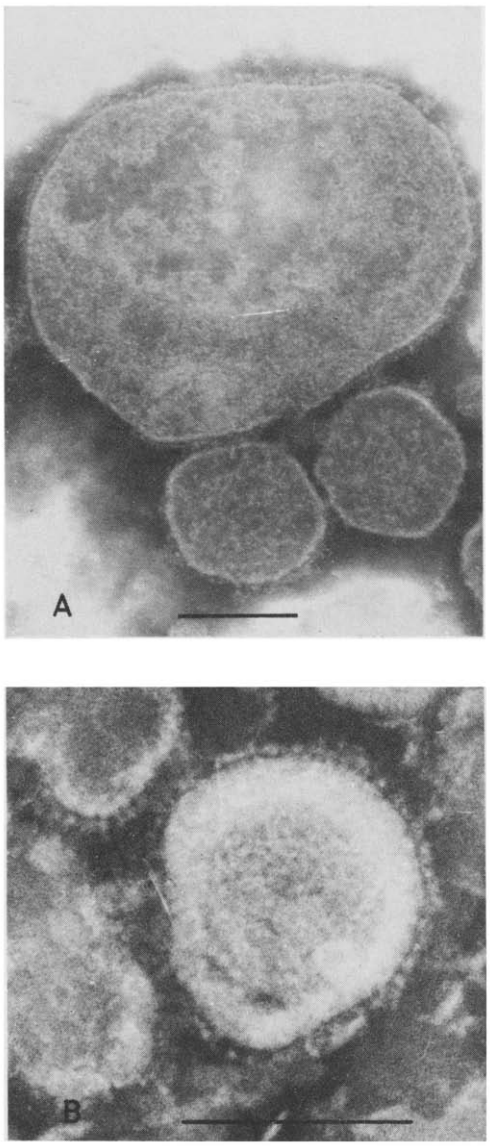

Fig. 1. Electron microscopic pictures (A and B with a higher magnification) of the (Na^+, K^+)-ATPase containing vesicles, incubated in 10 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM Tris (pH 7.3) at room temperature and negatively stained with phosphotungstic acid (pH 7.3). The bars represent 100 nm.

negatively stained with sodium phosphotungstate. It is seen that our particle preparations consist mainly of inside-out spherical vesicles. Proteins are protruding outside from the vesicle surface in a manner similar to that found in mitochondria. Most of the

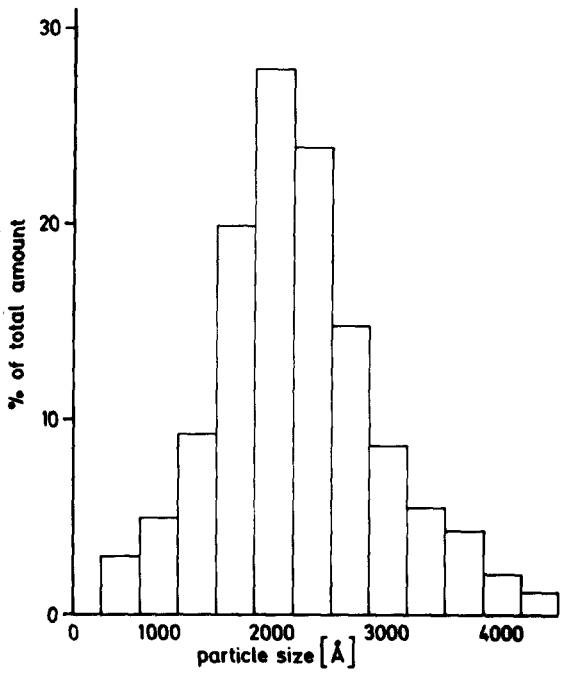

Fig. 2. Particle size distribution of (Na^+, K^+)-ATPase containing vesicles under the same conditions as described in Fig. 1, derived from electron micrographs.

proteins seem to be arranged in a quasi-crystalline lattice. An average diameter and an average extension of the protein from the vesicle surface is 6.4 × 6.4 nm.

Three different vesicle preparations were studied in the electron microscope and a size distribution was evaluated (Fig. 2). It shows that most of the particles have an average diameter of about 2000 Å (200 nm).

Size and polydispersity

For the determination of the vesicle size and size distribution the heterodyne autocorrelation function at 15° to 30° was utilized in a manner similar to Selser et al. [27]. The results show a particle size of 203.7 ± 15.2 nm. The size and size distribution is in agreement with the calculations from the electron-micrographs (Fig. 2). The effect on the particle size after addition of ATP is shown in Fig. 3, where the intensity fluctuation power spectrum is plotted against the frequency. Calculations based on diffusional line broadening for the linewidth showed a 20% reduction in the mean radius, if sphericity is assumed, meaning a change in the average volume

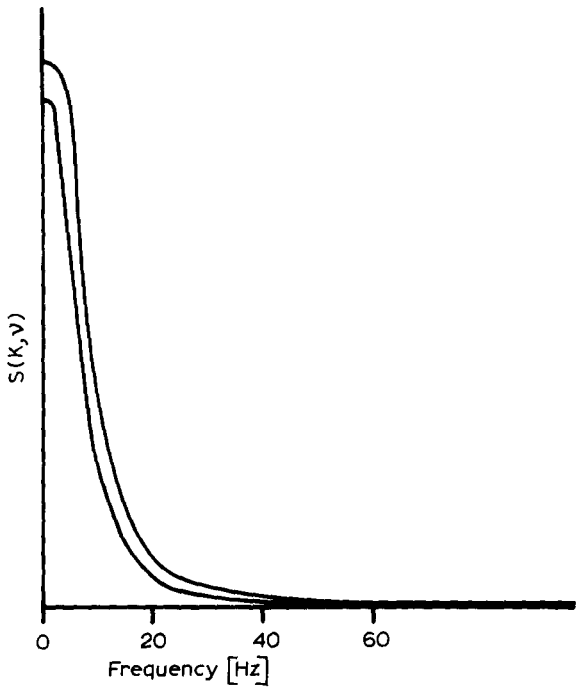

Fig. 3. Experimental curves of light intensity as a function of frequency under pure heterodyne scattering conditions. (Na^+, K^+)-ATPase particles were incubated in 10 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM Tris (pH 7.4) at room temperature. The inner curve was obtained under control conditions and the outer one was recorded after addition of 2 mM ATP.

of 50%. Such a large difference is unlikely to result from conformational changes in the protein or structural changes within the membrane. If there is no size or shape change, the alternative explanation could be refractive index changes following structural transformation. Such a phenomenon was noted by Uhl et al., [33] in their light scattering intensity measurements of Mg^{2+}-ATPase. But such refractive index changes do not contribute to the linewidth of the power spectrum. Although volume changes in activated mitochondria and in light-sensitive organelles are well known, we rule out the possibility of osmotic pressure changes in our ATPase system as it is not actively pumping ions. The change in volume observed is independent of incubation times. We verified the ATP-induced size reduction of the vesicles by the measurements of the light scattering intensities. These experiments were performed in the same experimental arrangement but with a 1-cm

TABLE I

LIGHT INTENSITY MEASUREMENTS OF (Na^+, K^+)-ATPase SUSPENDED IN DIFFERENT BUFFER SOLUTIONS, WITH AND WITHOUT ATP

The angle of detection was 15°.

Buffer	Intensity (kHz)	
	Control	0.8 mM ATP
1 mM Tris (pH 7.4)	2.94 ± 0.07	3.11 ± 0.09
0.1 mM $MgCl_2$, 1 mM Tris (pH 7.4)	3.86 ± 0.07	3.59 ± 0.08
1 mM NaCl, 0.2 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM Tris (pH 7.4)	4.18 ± 0.09	3.68 ± 0.08

diameter circular cell substituted for the 2-mm rectangular cell and with the pin-holes in the photomultiplier widened. The results are shown in Table I where the photon-counts in arbitrary units are given. As can be seen from the table, in the absence of Mg^{2+} there is little change in the light scattering intensity on addition of ATP. But in the presence of Mg^{2+} light scattering intensities decrease on the addition of ATP. This could be a consequence of decrease in size within the Rayleigh light scattering theory.

Electrophoretic mobility as a function of angle and voltage

A typical laser Doppler spectrum due to electrophoretic mobility of the vesicles taken at different angles of observation is shown in Fig. 4. The presence of a single, symmetrical peak demonstrates an electrophoretically homogeneous preparation. The Doppler shift also depends linearly on the voltage in a range of 80 to 200 V, showing the absence of any interaction between the particles.

Electrophoretic mobility as a function of ionic concentration

The influence of ionic concentration and the effects of Na^+ and K^+ in the range of 1 to 10 mM on the electrophoretic mobility were studied. In calculating the ζ-potential Henry's equation was used (Eqn. 5), which takes into account the size of the particle relative to the thickness of the double layer in the

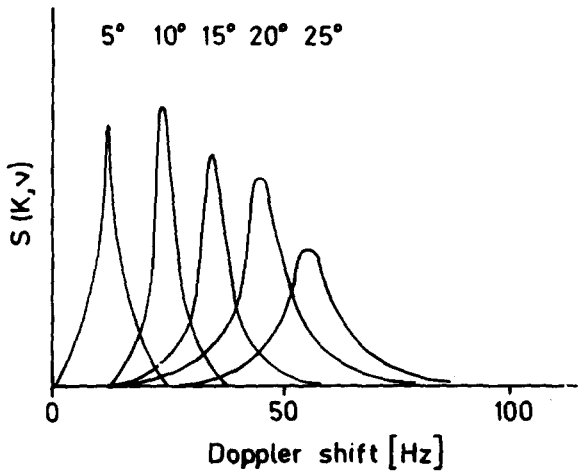

Fig. 4. Experimental laser Doppler spectra under electrophoretic conditions, taken at different angles of observation. The (Na^+, K^+)-ATPase vesicles were incubated in 10 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM Tris (pH 7.4) at room temperature.

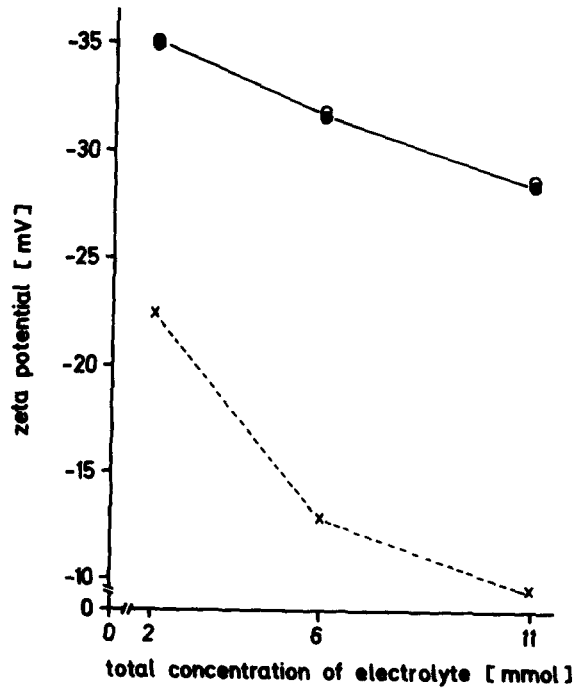

Fig. 5. ζ-potentials, calculated from Eqn. 5 as a function of concentration of NaCl (o———o) and KCl (•———•). The buffer concentration was kept constant at 1 mM Tris (pH 7.4). x----x, represents calculated ζ-potential data from a ζ/c (c = electrolyte concentration) dependence, according to the Debye-Hückel theory.

low ionic concentrations (1 mM) where $\mathcal{H}a < 300$. The potentials thus calculated from the electrophoretic mobilities are plotted against ionic concentration in Fig. 5. The errors in using concentrations instead of activities are small as all concentrations were dilute. It is seen that there is no difference between Na^+ and K^+. The ionic strength dependence of mobility does not follow the simple screening theory (dashed curve in Fig. 5). The reasons could be that the Gouy-Chapman theory (or the Debye relation) do not necessarily hold for realistic biological cells. Models, treating the membrane surface as composed of discrete charges (instead of a uniform distribution in Gouy-Chapman theory) or treating the cell as a porous polyanion are new alternatives [34,35]. Ion binding, if any, seems to show anionic binding if Gouy-Chapman theory is valid. It is well known that Na^+ and K^+ selectively influence the ATPase activity. Such a selective behaviour is not contradicted by these studies since the binding sites for Na^+ and K^+ could be located differently from the charged surface layer (the Stern layer). Furthermore it is possible that the selective binding occurs only during active pumping when the translocation of the ions could be descerned by changes in the electrophoretic mobilities [36]. The difference between screening, site-binding or territorial binding have been elucidated in recent times but mainly devoted to divalent cations while univalent cations are discussed only in the context of polyelectrolyte solutions [37–39]. The inter-charge distances between the enzyme units are probably too far for condensation of the counter-ions to occur.

Determination of the association constant for Mg^{2+} from changes in mobility

The binding of Mg^{2+} to the ATPase containing vesicles is shown in Fig. 6. Under the influence of Mg^{2+}, the mobility is decreased. With increasing Mg^{2+} concentrations, the linewidth of the Doppler shifted peak did not change, indicating that there was no change in particle size. Association constants for binding were derived by fitting Eqns. 5–9 directly to the experimental points. The binding constants were corrected for the influence of the screening effect by application of the Debye-Hückel relation. An approximate average association constant $K_a = 1.1 \cdot 10^2$ (M^{-1}) was derived.

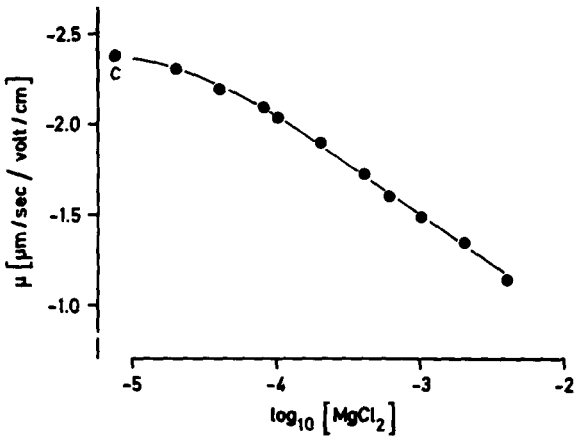

Fig. 6. Effect of increasing concentrations of $MgCl_2$ on the mobility of (Na^+, K^+)-ATPase vesicles, suspended in 1 mM NaCl, 1 mM Tris (pH 7.3).

Electrophoretic mobility as a function of pH

The pH dependence of electrophoretic mobility of the (Na^+, K^+)-ATPase containing vesicles at different ionic concentrations is shown in Fig. 7. From the figure an isoelectric point of pK = 3.95 for the lowest ionic strength (1 mM NaCl, 1 mM Tris) was determined. This pK value may be specifically associated with aspartic acid (pK = 3.65) alone and/or glutamic acid (pK = 4.25). Aspartic acid is known

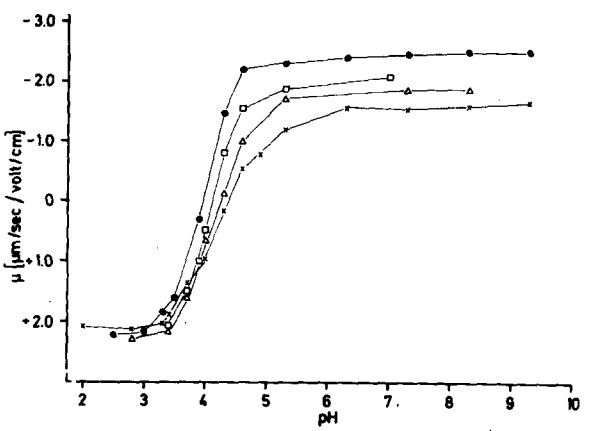

Fig. 7. Electrophoretic mobility as a function of pH. The (Na^+, K^+)-ATPase vesicles were suspended in different electrolyte media: ●———●, 1 mM NaCl, 1 mM Tris; □———□, 1 mM NaCl, 0.2 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM Tris; △———△, 5 mM NaCl, 1 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM Tris; ×———×, 10 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM Tris.

to be present and to be responsible for the ATP interaction [40]. It could be suggested therefore that the measured pK of 3.95 corresponds to that of aspartic acid, especially because the value would decrease further when measured in very dilute electrolytes. The surface charge density of the vesicle calculated at pH 7.3 in the same electrolyte is σ = 1 e^-/41.49 nm^2. Using the electron-microscopic data of 6.4 × 6.4 nm per protein unit, this corresponds to one net negative charge per ATPase molecule. It should be pointed out that this charge density corresponds to the density at the plane of shear, which can be expected to be located near the ends of the protruding protein units. This means that charged lipids deeper in the membrane core do not contribute to electrophoretic mobility, i.e. to surface charge density.

The pK is shifted towards a higher pH by increasing Mg^{2+} concentrations. This shift with a reduction in net negative charge and with no further change in the shape of the pH-mobility curve suggests a direct binding of Mg^{2+} to aspartic acid. Besides specific binding, also nonspecific binding of Mg^{2+} to the inner lipid surface is also possible.

Determination of association constant for ATP from changes in mobility

Only in the presence of Mg^{2+} there is a change in the electrophoretic mobility when ATP is added to the vesicle suspension. Calcium can substitute for Mg^{2+}. In the absence of Mg^{2+}, ATP binding could be observed only at a pH below the isoelectric point. While Mg^{2+} reduces the net negative charge of the vesicles, ATP tries to bring it back.

Fig. 8 shows the ζ-potential as a function of ATP and ADP concentrations at different ionic strengths at a fixed pH of 7.3. The binding was calculated from EC_{50} concentrations ignoring the screening contributions from the added ATP as ATP is a complex charged molecule [41]. Since the uni- and divalent cations are present in higher concentrations in comparison to ATP, the error in the calculation for the ATP binding is not large. The association constants for ATP determined for the three different electrolyte concentrations were: K_a = $1.11 \cdot 10^4$ at 1 mM NaCl, 0.2 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM Tris; $K_a = 2.39 \cdot 10^3$ at 5 mM NaCl, 1 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM Tris; $K_a = 1.06 \cdot$

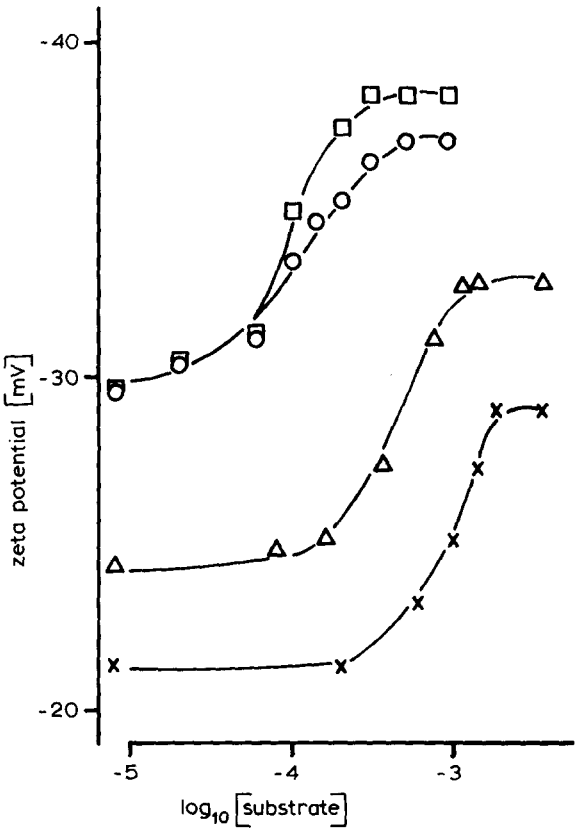

Fig. 8. The vesicle zeta potential as a function of substrate concentration. The particles were suspended in different media: □———□ and ○———○, 1 mM NaCl, 0.2 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM Tris, effect of ATP and ADP, respectively; △———△, 5 mM NaCl, 1 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM Tris, effect of ATP; X———X, 10 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM Tris, effect of ATP. All experiments were performed at pH 7.3 and room temperature.

10^3 at 10 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM Tris, all at pH 7.3. A Hill plot for the binding gives a value of $n = 2$, indicating cooperative binding. This probably means that the binding of one ATP molecule to one protein unit facilitates the binding of a second ATP molecule to a neighbouring enzyme complex probably via the Mg^{2+}.

It is seen from an analysis of the curves:

(1) ζ decreases with increasing concentrations of uni- and divalent cations partly due to ionic screening but mainly due to divalent ion binding. The parallel shifts of the curves at the three different electrolyte concentrations is an indication for the absence of heterogenity in ATP binding. It also shows only a weak screening due to ATP.

(2) ATP binds specifically to the enzyme as $\Delta\zeta$, the difference in ζ between the control and the ATP saturation value is the same for all the three curves. $\Delta\zeta$ is a measure of the number of available binding sites and should be the same for all Mg^{2+} concentrations.

(3) The Mg^{2+} concentration affects the binding of ATP. Higher ATP concentrations are needed to produce the same effect when the vesicles are suspended in buffers of higher Mg^{2+} concentrations. In this sense Mg^{2+} seems to be antagonistic, although it is required for ATP binding. These results show furthermore that Mg^{2+} is binding to and not shielding surface sites. Similar studies were done with ADP, which showed that this molecule binds to the enzyme to almost the same extent as ATP: $K_a = 1.02 \cdot 10^4$ at 1 mM NaCl, 0.2 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM Tris (pH 7.3).

Discussion

Various investigators have measured the size, size distribution and fusion of vesicles by the method of quasi elastic light scattering [15,16,27]. In this paper we showed the feasibility of simultaneously measuring the surface charge and the size of vesicle preparations by applying the technique of laser Doppler electrophoresis. A vesicle size in the range of 200 nm is not easily amenable to electrophoretic measurements by conventional methods. As a technique, however, the laser Doppler electrophoresis method needs improvement: to be able to work at the higher ionic strengths which correspond to physiological conditions. Also the instrumentation needs to be modified so as to make the linewidth measurements as precise as in normal quasi-elastic light scattering without the electric field. Although the shifted peak is an advantage to measure the linewidth properly, our measurements show that there is a small instrumental linebroadening in electrophoresis compared to pure elastic light scattering measurements. The interpretation of the experimental results is complicated by the vesicle size being intermediate ($\mathcal{K}a < 300$) so that corrections using Henry's equation are necessary for the calculation of the ζ-potentials. Our calculations here are based on the simple

Debye-Hückel theory which is a mean-field approximation to the Poisson-Boltzmann equation. Although it is known that these approximations are not valid for biological cells, there have been various improvements on the basis of models [34,35]. We further assume that the double layer is in equilibrium with fixed surface charges as the value of the ζ-potential did not change with the voltage employed in the region of 80 to 200 V/cm.

The errors in the calculation of the surface charge density from the experimental mobility values by using the Gouy-Chapman approximation are not high because the surface potential of our vesicles is below −40 mV and the vesicle surface appears to be smooth, spherical and mostly of one chemical group. The Debye-Hückel theory probably yields a better fit to our data under equilibrium conditions than under non-equilibrium conditions where electric conductance changes as a function of ionic binding [43]. The (Na^+, K^+)-ATPase vesicle suspension is electrophoretically homogenous (a single peak) with particles of an average radius of 203.7 nm and a single size distribution of ±15.2 nm. The electron micrographs show these are spherical and closed particles. From the activity measurements it is deduced that they are inside-out vesicles. If it had been a mixture of inside-out and right side out particles one would have expected two mobility peaks because of the asymmetric nature of the membrane surfaces. This allowed us to study the ATP binding to the catalytic site. In the final suspension for the electrophoretic measurements there were about $2 \cdot 10^9$ particles which gives an average interparticle distance of about 8 μm, sufficiently long to prevent the occurrence of any kind of interparticle interaction. From the mobility studies we have deduced that there is one net negative charge per enzyme molecule complex and from the surface pK value (Fig. 7) that the negative chemical group is most probably aspartic acid.

It is known that sodium and potassium ions are needed for the phosphorylation and dephosphorylation of the ATPase molecule [44]. Attempts have been made to show Na^+ and K^+ binding selectivity to macromolecules but they have never been successful [45]. As is shown in Fig. 5 the effect of Na^+ and K^+ concentration on the mobility is the same. Furthermore, it is seen that the Debye relation between ζ-potential calculated by the Henry equation, and ionic strength ($\zeta \sim C^{-0.5}$) is not followed: the exponents are −0.57 for 2 mM, −0.67 for 6 mM and −0.74 for 11 mM concentration of the electrolyte. This effect could be due to anionic binding with increasing ionic strength or to changes in the distance between the cell surface and the ζ plane. Alternatively, this result could be due to nearest-neighbour discrete charge-charge interactions in a porous model which depend on the ionic strength. In this case at higher salt concentrations the charge density is increased compensating the inverse relation between potential and salt concentration according to Debye. A different kind of a deviation from the Debye relation was found by Uzgiris and Kaplan [23] in lymphocytes. At low salt concentrations the mobility reached a plateau, which they explained to be due to flexible molecules in the double layer.

The binding constant for Mg^{2+} obtained by us for the ATPase vesicles $K_a = 1.1 \cdot 10^2\ M^{-1}$ is in general higher than that found by other investigators by other methods [43]. In the few experiments done we find no difference between Ca^{2+} and Mg^{2+}, although such a difference has been observed in phospholipid bilayers [37]. Since we find that ATP alone in the absence of Mg^{2+} does not bind to (Na^+, K^+)-ATPase, the first step in binding could be the Mg-ATP^{2-} ligand. The scheme involving the carboxylic acid group of aspartic acid and the Mg-ATP^{2-} in the phosphorylation step is given by Repke [46]. A value of 2 for the Hill coefficient for ATP binding could reflect the role of Mg^{2+} in the cooperative binding. In a study similar to ours but using a fluorescent probe and microelectrophoresis Aiuchi et al. [47] investigated the changes in the ζ-potential of mitochondria induced by ATP. They found that the negative ζ-potential was decreased on the addition of ATP and this was explained by postulating a conformational change. Our studies show that Mg^{2+} binds to the aspartic acid site of the ATPase molecule. Binding of ATP is triggered by Mg^{2+} and could be related with conformational changes of the protein, by which surface charges are internalized. Such conformational changes have been observed in (Na^+, K^+)-ATPase preparations with the aid of fluorescent probes [48–50].

The reduction in the size of the spherical vesicles upon binding of ATP (or ADP) observed in the linewidths of electrophoresis and light scattering experiments and from light scattering intensity measurements amount to a reduction in the surface area of the vesicle of 35%. In the case of uniform distribution of fixed surface charges this should lead to a corresponding increase in σ which is not observed. If instead of a uniform distribution of surface charges there are patches of protein molecules as sometimes seen in electron micrographs, then the size reduction on the vesicles need not lead to an increase in σ. Furthermore, ATP does not bind to nonspecifically bound Mg^{2+}. This taken together with volume changes would mean long-range structural transformations on and in the membrane. It is not known if the lipid base is involved in these structural transformations nor is it known whether a kind of 'desensitization' of available binding sites occurs. It is likely either that the 'desensitized' ATPase conformer is not available to Mg^{2+} or that the Mg^{2+}-ATPase conformer is not available for ATP binding. There could be a correlation between the amount of size reduction and the number of enzyme sites which are not available for binding. For example at 35% surface contraction nearly 30% of the enzyme sites are not reactive to ATP.

To examine in detail the actual cause for the linewidth broadening on addition of ATP to (Na^+, K^+)-ATPase vesicles further experiments in quasi-elastic light scattering and in light scattering intensity are needed. However, the electrophoretic light scattering method has proved to be a promising instrument to characterize the surface and size properties of a vesicle preparation as well as to study the interaction of ligands to receptors on membranes.

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