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ORIGINAL PAPER

Monitoring of the Proton Electrochemical Gradient in Reconstituted Vesicles: Quantitative Measurements of Both Transmembrane Potential and Intravesicular pH by Ratiometric Fluorescent Probes

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Abstract Proteoliposomes carrying reconstituted yeast plasma membrane H^+-ATPase in their lipid membrane or plasma membrane vesicles are model systems convenient for studying basic electrochemical processes involved in formation of the proton electrochemical gradient ($\Delta\mu_H{}^+$) across the microbial or plant cell membrane. $\Delta\psi$- and pH-sensitive fluorescent probes were used to monitor the gradients formed between inner and outer volume of the reconstituted vesicles. The $\Delta\psi$-sensitive fluorescent ratiometric probe oxonol VI is suitable for quantitative measurements of inside-positive $\Delta\psi$ generated by the reconstituted H^+-ATPase. Its $\Delta\psi$ response can be calibrated by the K^+/valinomycin method and ratiometric mode of fluorescence measurements reduces undesirable artefacts. *In situ* pH-sensitive fluorescent probe pyranine was used for quantitative measurements of pH inside the proteoliposomes. Calibration of pH-sensitive fluorescence response of pyranine entrapped inside proteoliposomes was performed with several ionophores combined in order to deplete the gradients passively formed across the membrane. Presented model system offers a suitable tool for simultaneous monitoring of both components of the proton electrochemical gradient, $\Delta\psi$ and ΔpH. This approach should help in further understanding how their formation is interconnected on biomembranes and even how transport of other ions is combined to it.

Keywords Transmembrane potential · Intracellular pH · Oxonol dyes · Pyranine · Proteoliposomes

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Introduction

Proteoliposomes are artificially prepared lipid vesicles carrying membrane proteins incorporated into their membrane during reconstitution. Proteoliposomes with reconstituted yeast plasma membrane H^+-ATPase or plasma membrane vesicles (PMVs) represent an *in vitro* model system suitable for studying basic electrochemical phenomena involved in the formation of proton electrochemical gradient ($\Delta\mu_H{}^+$) across the yeast plasma membrane. Active $\Delta\mu_H{}^+$ build-up in yeast involves action of the H^+-ATPase, a P-type ATPase transporting protons outside the cell [1–5]. $\Delta\mu_H{}^+$ consists of two components—difference in electric potential across the membrane, i.e. the transmembrane potential $\Delta\psi$, and difference in proton concentrations, i.e. the pH gradient ΔpH. Since proton concentration defines pH on both sides of the membrane, the maintenance of ΔpH should be directly related to the mechanism of intracellular pH regulation [6, 7]. The transmembrane electrogenic transport of protons

combines with the transport of other ions, either active driven by specific energy-dependent ion transporters, or passive through specific ion channels or through membrane lipid matrix itself. Among other ions, potassium cations K^+ play an important role in yeast cells [7–12].

In the proteoliposomes carrying various types of charge-translocating systems basic electrochemical phenomena free of interfering metabolic factors can be monitored by means of fluorescence spectroscopy using fluorescent dyes [5, 13–15]. Anionic fluorescent dyes oxonol V and oxonol VI belong to the group of $\Delta\psi$-sensitive distribution fluorescent probes [14, 16–18]. These dyes have been used to monitor inside-positive transmembrane potentials generated on reconstituted vesicles [5, 13–15]. Charged molecules of the dyes redistribute according to the gradient in electric potential. Since they carry negative electric charge, they accumulate inside vesicles with inside-positive $\Delta\psi$ built up. The dye molecules bind to the membrane in dependence on their concentration in surrounding liquid phase, i.e. the higher the dye concentration inside the vesicles is the more the dye molecules bind to inner membrane surface. The binding is accompanied by changes in dye fluorescence, which can be used for $\Delta\psi$ monitoring. Although simple intensity measurements are often used with both oxonols, especially with oxonol V that exhibits pronounced fluorescence quenching, intensity-independent methods are more convenient for quantitative measurements since they increase accuracy of the measurements that is in the case of intensity-based methods limited by variability in either dye and vesicle concentrations or by photobleaching. Both oxonols bound to the membrane exhibit an about 20 nm shift of emission spectrum towards longer wavelengths. Transmembrane potential can thus be monitored when tracking the emission spectrum position. Ratiometric mode of measurements is a convenient way, how the position can be tracked. Fluorescence intensities are measured at two separate wavelengths, either of excitation or emission, corresponding to fluorescence bands of free and bound dye. Then ratio of the intensities is calculated, which is related directly to the spectrum position. Ratiometric monitoring proved to be useful in quantitative measurements of $\Delta\psi$ generated by the reconstituted H^+-ATPase [19].

To collect complete information on the proton electrochemical gradient $\Delta\mu_H{}^+$, $\Delta\psi$–sensitive fluorescent dyes should be combined with pH-sensitive ones to monitor both components of the gradient. Redistribution pH-sensitive fluorescent indicators such as ACMA (9-amino-6-chloro-2-methoxyacridine) have often been used to monitor the electrochemical gradient formed on the reconstituted vesicles [5, 20, 21]. However, e.g. ACMA reports on both parts of the electrochemical gradient of protons [22] and it cannot be thus easily quantified separately for pH. The use of *in situ* pH-sensitive fluorescent indicators entrapped inside the vesicles seems to be a better possibility. Additionally, hydrophilic fluorescent pH indicators such as pyranine are more convenient than hydrophobic ones such as carboxySNARF because of their low interaction with the lipid matrix of the membrane which often results in leakage of the dye from the vesicles and in unspecific changes of the dye fluorescence [23].

The dyes oxonol VI and pyranine were chosen for $\Delta\psi$ or ΔpH monitoring on reconstituted vesicles, since their use allows the quantification of fluorescence measurements. The quantification means that the response of the fluorescent probes is translated into units of electric voltage (mV) for $\Delta\psi$ or into units of pH for ΔpH. This requires the use of calibration procedures such as the K^+/valinomycin calibration method for $\Delta\psi$, see e.g. [14, 24, 25]. In this method, different concentration gradients of potassium ions are imposed on the proteoliposomal membrane and the ionophore valinomycin is used to enhance potassium permeability for K^+. Then $\Delta\psi$ that can be calculated from the Nernst equation [26, 27] is formed. The K^+ diffusion transmembrane potentials were used for quantifying oxonol VI fluorescence response in proteoliposomes with reconstituted Na^+/K^+-ATPase [14]. Večeř et al. [23] modified the K^+/valinomycin calibration method for measurements of inside-negative $\Delta\psi$ with diS-$C_3(3)$ in liposomes. This method was adjusted for inside-positive $\Delta\psi$ and used to quantify $\Delta\psi$ measurements with oxonol VI in proteoliposomes with H^+-ATPase reconstituted from the plasma membrane of *S. pombe* [19].

Materials and methods

Chemicals

Egg yolk lecithin (L-α-phosphatidylcholine from dried egg yolk, type X-E), dioleoyl L-α-phosphatidylcholine (DOPC), Sephadex G 25 fine, ATP (adenosine 5′-triphosphate disodium salt), hexokinase (from bakers yeast, type III), n-octyl glucoside (N-octyl β-D-glucopyranoside), CCCP (carbonyl cyanide m-chlorophenylhydrazone) and MES (2-(N-morpholino) ethanesulfonic acid) were purchased from Sigma, valinomycin from Fluka, oxonol V (bis-(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol), oxonol VI (bis-(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol) and pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid) from Molecular Probes.

Strains, media and growth conditions

The yeast *Schizosaccharomyces pombe* 972h$^-$ was grown in a minimal medium [28] containing 2.5 % glucose at 38°C. Cells in the early stationary phase were harvested and homogenized as previously described [29].

Isolation of plasma membranes

Membranes were isolated essentially as previously described [20, 30] without additional solubilization and purification. The protein content of the plasma membrane fraction was determined according to Bradford [31] with bovine serum albumin as a standard.

Assay of ATPase activity

ATPase activity was determined by measuring the rate of inorganic phosphate (Pi) release from ATP as previously described [20, 30]. The reaction was performed at 30°C under continuous shaking and was started by adding isolated plasma membranes or reconstituted proteoliposomes (see below) to a total of 3 ml of 50 mM MES (pH 6.0) containing 5 mM ATP and 12 mM $MgCl_2$. Aliquots of 0.5 ml of the mixture were withdrawn every minute within the first five minutes of incubation. The reaction was stopped by adding the aliquot to 1.5 ml of 1% (w/w) sodium dodecyl sulfate. The amount of inorganic phosphate in the aliquots was determined 15 min after an addition of 0.5 ml of ammonium molybdate (40 mM $(NH_4)MoO_4$ in 2.5 N H_2SO_4) and 0.5 ml of Photorex solution (10 g Photorex, 30 g $NaHSO_3$ in 1 l) by measuring the absorbance at 660 nm. The rate of phosphate release was calculated as a slope of the time dependence related to the plasma membrane protein content (Pi/mg.min).

Preparation of proteoliposomes (PMVs) with reconstituted H^+-ATPase

S. pombe plasma membrane H^+-ATPase was reconstituted into proteoliposomes as previously described [20] with slight modifications: Egg yolk lecithin (20 mg/ml) was dissolved together with 40 mg octylglucoside in 1 ml of chloroform, dried by nitrogen gas on the walls of a tube and desiccated in vacuum for 45 min. The resulting detergent-phospholipid film was hydrated by 10 mM MES (pH 6.0 by NaOH) and isolated plasma membranes were added to a total volume of 1 ml so that the final protein content was 200 μg/ml. The mixture was dialysed in the "Mini-Lipoprep" apparatus (Diachema AG, Zürich) at 4°C against 0.5 l of 10 mM MES (pH 6.0). The buffer was replaced after 1.5 h dialysis and the reconstitution was complete after 3 h.

The resulting proteoliposomes were collected, rapidly frozen in liquid nitrogen, thawed at room temperature, sonicated by a probe type sonicator (Cole-Palmer Instruments Co.) two times for 2–3 s and kept on ice. When $MgCl_2$, KCl, choline chloride or pyranine were required to supplement the composition of the reconstitution buffer, the ions were added to the suspension of thawed proteoliposomes and the freeze-thaw sonication procedure was repeated two times. The dye from the extraliposomal volume was removed by gel filtration on 6 cm columns filled with Sephadex G 25 equilibrated in the appropriate buffer. The columns were centrifuged at 600 g for 5 min, then the sample suspension was loaded on the top, the columns were centrifuged again and the eluted sample was collected.

Preparation of liposomes, SUVs and LUVs

Pure liposomes or sonicated unilamellar vesicles (SUVs) were prepared from egg yolk lecithin (20 mg/ml) similarly as the proteoliposomes, the only difference being that the plasma membranes were not added after the detergent-phospholipid film was hydrated by 1 ml of 10 mM MES. Large unilamellar vesicles (LUVs) were prepared as previously described [23]. Ten milligrams of phospholipid (DOPC) was dissolved in 0.5 ml of chloroform, dried in a stream of nitrogen gas in an Eppendorf vial to give a thin film on its walls and kept in vacuum for 10 min. Then 0.5 ml of 50 mM TRIS, pH 7.5, 0.5 mM KCl was added. The solution was mixed in a wortex mixer to form multilamellar vesicles. LUVs 400 nm in diameter were prepared by the extrusion method using the LipoFast extrusion apparatus [32].

Fluorescence measurements

Fluorescence measurements were performed on a Fluoromax 2 spectrofluorometer (photomultiplier tube photocatode sensitive to 700 nm) at an excitation wavelength of 560 nm selected from the xenon lamp spectrum. The scattered excitation light was eliminated using a red cut-off filter with full transparency from about 600 nm.

Results and discussion

Calibration of dye response to positive $\Delta\psi$ in proteoliposomes with reconstituted plasma membrane H^+-ATPase

The calibration of the dye response to $\Delta\psi$ is a necessary prerequisite for evaluation of $\Delta\psi$ to which the electrogenic proton-motive pump charges the proteoliposomal membrane. When defined K^+ gradients are imposed on the membrane, $\Delta\psi$ according to the Nernst equation, $\Delta\psi = (RT/F)\ln([K^+]_{out}/[K^+]_{in})$ [26, 27] is formed after the membrane permeability to K^+ is selectively enhanced by the ionophore valinomycin. The calculated values of transmembrane potential are then used to calibrate the dye response. In our method, potassium ions are complemented by choline ions on one side of the membrane to maintain osmotic equilibrium between proteoliposomal lumen and medium in different K^+ gradients [33]. The K^+ gradients were adjusted so that concentrated proteoliposomes prepared in 0.5 mM KCl,

149.5 mM choline chloride, 10 mM MES (pH 6.0) were diluted into MES of the same concentration where appropriate KCl concentrations were complemented by choline chloride to reach a total of 150 mM. Valinomycin (1 nM) was added to proteoliposomes stained by one of the dyes and then the fluorescence intensity ratio corresponding to adjusted $\Delta\psi$ was measured.

Choline chloride does not serve only to balance osmotically the volumes but also to keep constant ionic strength during either the calibration or the measurements of $\Delta\psi$ formation by H^+-ATPase. This is one of the prerequisites necessary for accurate quantitative measurements of the generated $\Delta\psi$ with fluorescent dyes [19] since the dye binding to the membrane surface is affected by the ionic strength in the liquid phase. Keeping the overall ionic strength constant ensures that the observed changes in the dye fluorescence report solely on $\Delta\psi$ and not on ion concentrations changing during the measurement.

Interaction of the dye with agents added to vesicles during experiments is another possible source of effects interfering with reliable quantitative measurements. For example, interaction of anionic dyes with the K^+-valinomycin complex was reported [34]. We took care to exclude such effects by testing in suspensions of pure liposomes how the chemicals used in our experiments affect fluorescence of the dyes. No significant interaction hampering quantitative measurements was observed. Another prerequisite of successful calibration is that the calibration should be performed on the system identical to the system used in actual experiments. Special attention should be paid to the ion composition of the measurement buffer since ions in liquid phase can affect the dye interaction with membranes or proteins.

Calibration measurements with pure liposomes showed that the K^+ concentration in the inner volume does not necessarily equal the K^+ concentration in the buffer used for liposome preparation. The liposomes were prepared in the buffer of defined K^+ concentration and were then diluted into the same buffer. Addition of valinomycin to the liposome suspension stained by oxonol V or oxonol VI led to a shift of the dye emission spectrum (Fig. 1), which is supposed to report on the presence of $\Delta\psi$. This effect, which appeared independently of the procedure used for the preparation (extrusion, dialysis or sonication), indicates a gradient of K^+ concentrations across the membrane, which after the addition of valinomycin causes build-up of $\Delta\psi$. The spectrum shift could be alternatively explained by the binding of anionic dye molecules to the K^+-valinomycin complex [34] as mentioned already above. This valinomycin-induced fluorescence shift is eliminated when choline chloride is used to complement the concentration of K^+ ions, e.g. 75 mM (inset of Fig. 1) to the total of 150 mM. The absence of the effect in such combination of ions shows that the spectrum shift under investigation can hardly be explained by the formation of the putative dye-K^+-valinomycin complex. It is more probable that the ion composition inside the vesicles need not be identical with that in the buffer used for vesicle preparation. This holds also for inner pH as indicated by changes in fluorescence of pyranine entrapped in the vesicles that were observed shortly after dilution to measurement buffer before the proteoliposomes were energized by ATP, see Fig. 8A.

The presence of choline ions helps to reach the same K^+ concentration on both sides of the proteoliposomal membrane. The origin of this effect is not clear. Competition of choline ions with K^+ in binding to the membrane surface can play an important role. Anyway, the absence of the emission shift when K^+ is complemented by choline confirms the possibility to set zero $\Delta\psi$, which is necessary for successful calibration. The fact that the inner K^+ concentration can be clearly defined is also important for the $\Delta\psi$ calculation using the Nernst equation.

Calibrations carried out with pure liposomes and proteoliposomes at the same lipid concentration revealed that the pure liposomes show a more pronounced fluorescence response to $\Delta\psi$ although the intensity ratio measured for zero transmembrane potential was the same for both systems. This observation can be explained since a large portion of the reconstituted vesicles need not be tightly sealed in the presence of plasma membrane fragments. Then, $\Delta\psi$ is not formed on the unsealed vesicles, the dye does not accumulate inside these vesicles and their contribution is missing in the overall fluorescence response as compared to the pure liposomes. This again demonstrates that the calibration has to be performed on the system identical to that where the active $\Delta\psi$ generation is studied. Whether $\Delta\psi$ described by the Nernst equation is formed on membrane of the sealed proteoliposomes and whether the K^+/valinomycin calibration method can be used was tested by experiments with different salts used to prepare the calibration buffers. When choline was replaced by sodium and chloride by sulphate we obtained spectral shifts almost identical to those obtained with choline chloride itself in the sample buffer (Fig. 2). These ions should differ in their membrane permeabilities due to their size. This means that in the presence of valinomycin the diffusion $\Delta\psi$ is set preferentially by K^+ conductivity and not by its combination with conductivities of the remaining relevant ions in the system, which would be the case when the formed $\Delta\psi$ depends on how tightly sealed the proteoliposomes are and when it does not thus correspond to the Nernst equation.

Fluorescence monitoring of H^+-ATPase-generated $\Delta\psi$

The potential-sensitive fluorescent probes oxonol V and oxonol VI were used to monitor the H^+-ATPase-generated $\Delta\psi$.

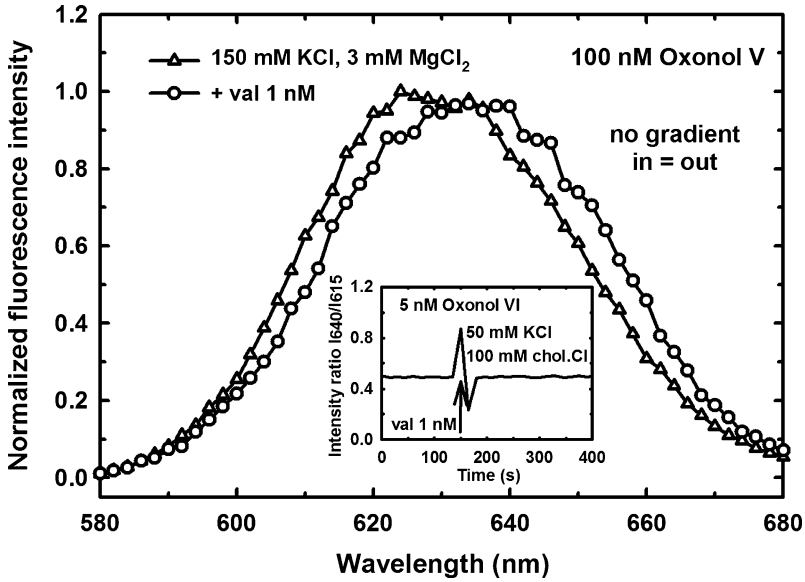

Fig. 1 Adjustment of zero transmembrane potential; pure liposomes (SUVs) prepared in 10 mM MES, pH 6.0 by dialysis and supplied with 150 mM KCl, 3 mM $MgCl_2$ during two freeze-thaw cycles each followed by sonication were diluted into the same buffer for the measurement. Fluorescence spectra of 100 nM oxonol V in the liposome suspension were acquired before and after addition of 1 nM valinomycin. Lipid concentration was 2 μg/ml. In **Inset,** 1 nM valinomycin was added to proteoliposomes prepared and diluted in 10 mM MES, pH 6.0, 3 mM $MgCl_2$ containing 50 mM KCl suplemented by 100 mM choline chloride. The intensity ratio I_{640}/I_{615} was monitored for 5 nM oxonol VI in the proteoliposome solution before and after the valinomycin addition. Lipid concentration in the sample was 70 μg/ml

Potential-dependent accumulation of dye molecules inside the vesicles is accompanied by changes of fluorescence characteristics due to the binding of the dye to the vesicular membrane. The emission spectrum of both dyes shifts upon binding about 20 nm towards longer wavelengths (a red shift). Moreover, especially oxonol V exhibits pronounced quenching of its fluorescence probably due to aggregation of bound dye molecules. Hence, $\Delta\psi$ generation can be monitored by tracking the position of emission spectrum or by measuring fluorescence intensity at a defined wavelength. Intensity ratio measurements at two wavelengths (640 nm/620 nm for oxonol V and 640 nm/615 nm for oxonol VI) were found to be sufficient for tracking the spectrum position.

The redistribution probes respond to $\Delta\psi$ by shifting their emission spectrum, only when both bound and free dye forms contribute comparably to the overall fluorescence spectrum. When one fluorescence component predominates, the response of the spectrum to changes in $\Delta\psi$ is suppressed. The concentration of vesicles for the measurement therefore has to be high enough to ensure sufficient manifestation of

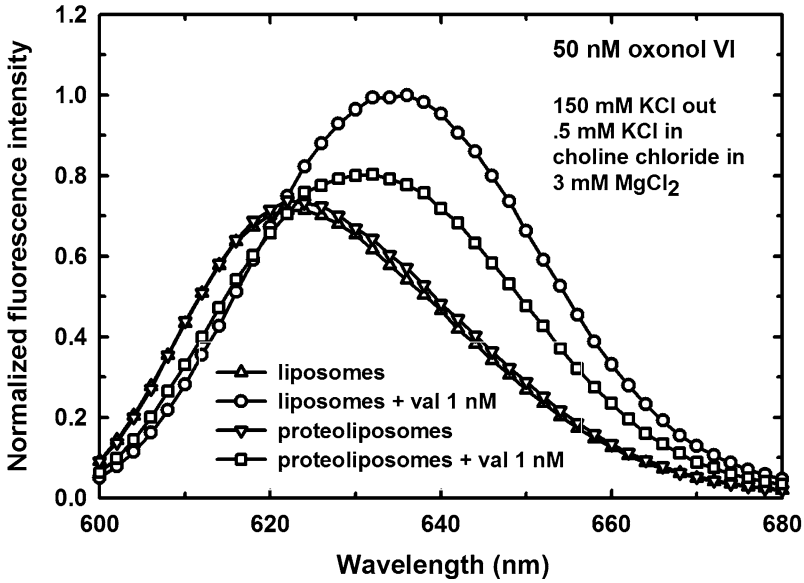

Fig. 2 Response of oxonol VI to K^+ diffusion $\Delta\psi$ in comparison of pure liposomes (SUVs) and proteoliposomes; SUVs and proteoliposomes prepared in 10 mM MES, pH 6.0 by dialysis and supplied with 0.5 mM KCl, 149.5 mM ChCl, 3 mM $MgCl_2$ during two freeze-thaw cycles each followed by sonication were diluted to 10 mM MES, pH 6.0, 150 mM KCl, 3 mM $MgCl_2$ for the measurement. Fluorescence spectra of 50 nM oxonol VI in the pure liposome and proteoliposome suspension were acquired before and after addition of 1 nM valinomycin. Lipid concentration was 50 μg/ml

the bound dye in the overall fluorescence. Since oxonol V binds much more extensively to the membrane than oxonol VI, much (about 35-times) lower concentration of the proteoliposomes is needed for proper $\Delta\psi$ − sensing. The use of oxonol V thus improves the efficiency of testing for proteoliposome tightness and quality of reconstitution. Moreover, it allows also to reduce the residual detergent level in a sample by manifold dilution of the proteoliposomes prior to the fluorescence measurements.

The upper limit for vesicle concentration arises from the demands on sample transparency. Higher dye concentrations are generally inconvenient due to the effect of the dye itself on the generated $\Delta\psi$ [19]. The lower the concentration of the dye can be used, the less the system is affected by the dye presence and the more reliable the results can be obtained. The lower concentration limit of the dye is given by the sensitivity of the spectrofluorometer Fluoromax 2 used. With oxonols, the limits of the spectrofluorometer sensitivity were reached on going to the dye concentration of 5 nM. This concentration was thus used for quantitative measurements.

When monitoring $\Delta\psi$ by tracking emission spectrum shifts, an attention should be paid to ensure that the emission spectrum is $\Delta\psi$-sensitive in the range of measured transmembrane potentials [24]. The emission spectrum appears between two limit positions given by the spectra of bound and free dye. The actual position depends on the ratio between the free and bound dye fluorescence, i.e. on relative probe-to-lipid concentration [24]. The zero $\Delta\psi$ position can be manipulated by changing the concentration of vesicles in suspension at defined dye concentration (Figs. 3A, 3B). The relative probe-to-lipid concentration should be chosen so that the overall spectrum is shifting in the whole range of measured $\Delta\psi$s (Fig. 4A). In concentrated vesicle suspensions, where the relative probe-to-lipid concentration is low, the fluorescence of bound dye prevails over that of free dye and the emission spectrum already close to the limit position need not shift appropriately in response to increasing $\Delta\psi$, Fig. 3B compared to Fig. 3C. Conversely, in dilute vesicle suspensions where the fluorescence of free dye already prevails the emission spectrum might not shift in response to decreasing $\Delta\psi$ (Figs. 3A, 4C).

Another situation can occur when the dye concentration is high relative to the concentration of available binding sites. On approaching the saturation plateau the fluorescence from bound dye is then unable to follow an increasing $\Delta\psi$ and the probe sensitivity to $\Delta\psi$ is reduced (Fig. 4C). Complete saturation of the sites could even lead to insensitivity of the probe. Increasing the dye concentration when the binding sites are occupied results in increasing portion of the free dye fluorescence which can even mean that the overall spectrum travels back to the position of the free dye (Fig. 4). This effect of probe response reduction should be considered in both spectrum position-based and intensity-based $\Delta\psi$ measurements, but it is unlikely when the dyes are used in sufficiently low concentrations required for proper $\Delta\psi$-sensing [19].

With oxonol V, pronounced fluorescence quenching takes place at higher relative dye-to-lipid concentrations due to aggregation of bound dye molecules at the membrane surface (Figs. 3A, 3C). At lower relative dye-to-lipid concentrations, oxonol V behaves similarly in pure liposomes as oxonol VI. $\Delta\psi$ can then be monitored with tracking the emission spectrum position (Fig. 4A). The case of oxonol V also shows that the fluorescent probe can respond to $\Delta\psi$ passively created by valinomycin-mediated K^+ diffusion in a different way than it does to $\Delta\psi$ actively generated by an electrogenic pump, e.g. plasma membrane H^+-ATPase. The oxonol V binding to the H^+-ATPase-containing proteoliposomes energized by ATP is accompanied by pronounced quenching of the dye fluorescence, while the shift of emission spectrum can be only small (Fig. 5A) [19]. The fluorescence intensity quenching is used in this case to monitor the transmembrane potential [5, 20]. The anomalous behavior of oxonol V in the presence of $\Delta\psi$ generated by the proton-translocating pump is likely to be caused by the aggregation of oxonol V molecules around the charged part of the energized pump [35]. The K^+ diffusion-based transmembrane potentials cannot thus be used for calibration of oxonol V response to actively generated $\Delta\psi$ and oxonol V is not suitable for quantitative measurements of H^+-ATPase-generated $\Delta\psi$ because of its difficult calibration [19].

In comparison to oxonol V, oxonol VI makes the calibration by K^+ diffusion transmembrane potentials possible since it responds similarly to both types of $\Delta\psi$ and the response consists mainly in the shift of emission spectrum (Figs. 2, 5B). The shift can be directly tracked by using the ratiometric mode of measurement, which proved to be very useful for measurements of $\Delta\psi$ generated by the reconstituted H^+-ATPase. After application of the K^+/valinomycin-based calibration, $\Delta\psi$ around 165 mV was estimated to be generated by the H^+-ATPase on the membrane of proteoliposomes [19].

Studying the formation of $\Delta\psi$ or ΔpH in reconstituted vesicles by fluorescent probes has inherent limits. The interaction of the dye with chemicals being added to the samples during experiments often results in changes of the dye fluorescence that can be hardly distinguished from the $\Delta\psi$-dependent fluorescence response. If these effects are considered and tested either on pure liposomes or unenergized proteoliposomes, the method presented above provides reliable results, i.e. results reporting on genuine properties of the transporters that are engaged in the electrochemical gradient generation. Fig. 6 shows how the momentary $\Delta\psi$ generated by the reconstituted H^+-ATPase can be monitored continuously. Hexokinase transfers the phosphate group from ATP to glucose. The energy source of the gradient generation is

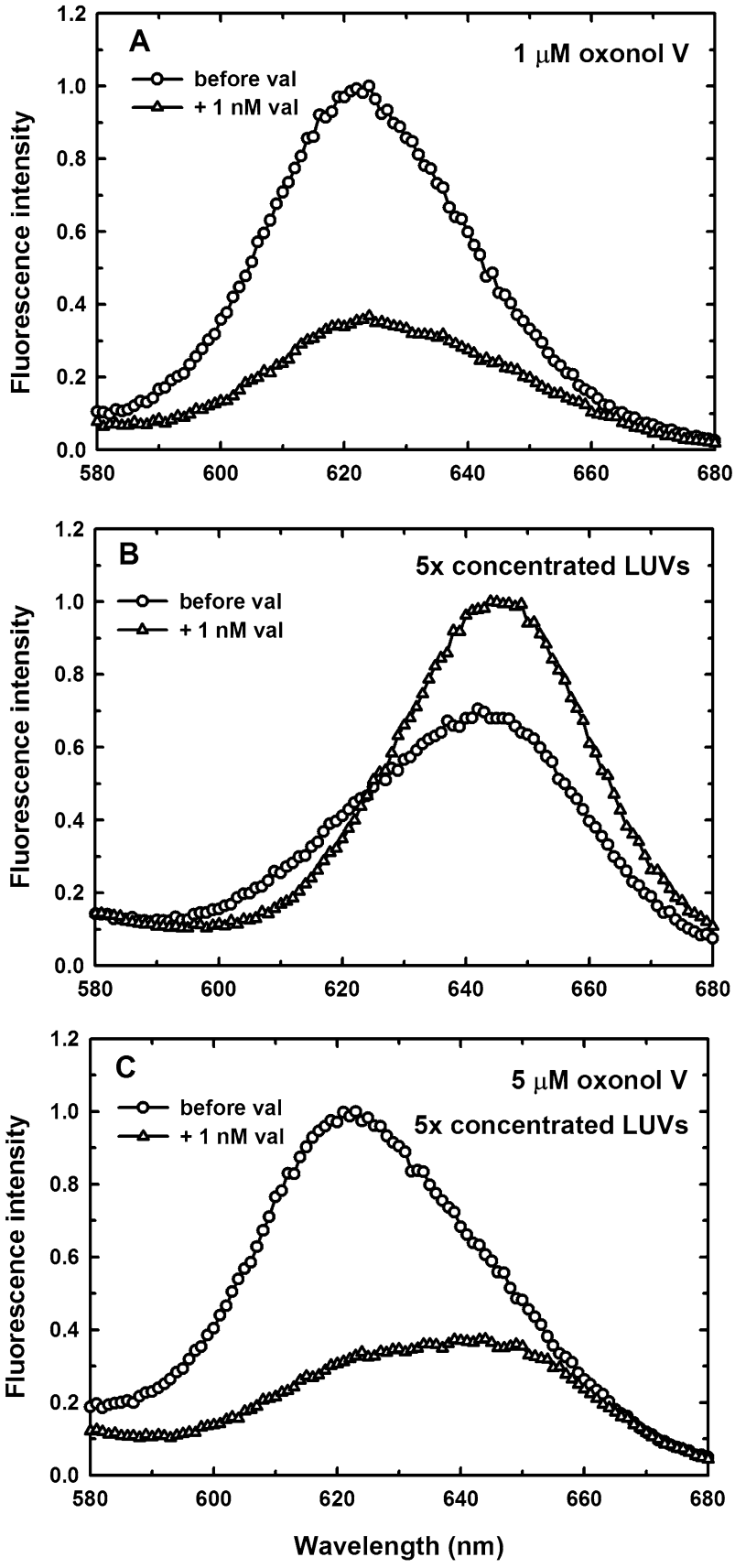

Fig. 3 Response of oxonol V to K^+ diffusion $\Delta\psi$ in LUVs measured at different relative probe-to-lipid concentrations and at two different lipid concentrations; K^+ gradient (150 mM KCl out, 0.5 mM KCl in) was imposed and $\Delta\psi$ build-up was accomplished by the addition of 1 nM valinomycin. Fluorescence spectra were measured before and after the valinomycin addition. LUVs were prepared from DOPC in 50 mM TRIS, pH 7.5, 0.5 mM KCl by extrusion through 400 nm polycarbonate filter. Concentration of oxonol V was 1 μM in **A**, **B** and 5 μM in **C**. Lipid concentration in **A** was 7 μg/ml and in **B**, **C** was 35 μg/ml

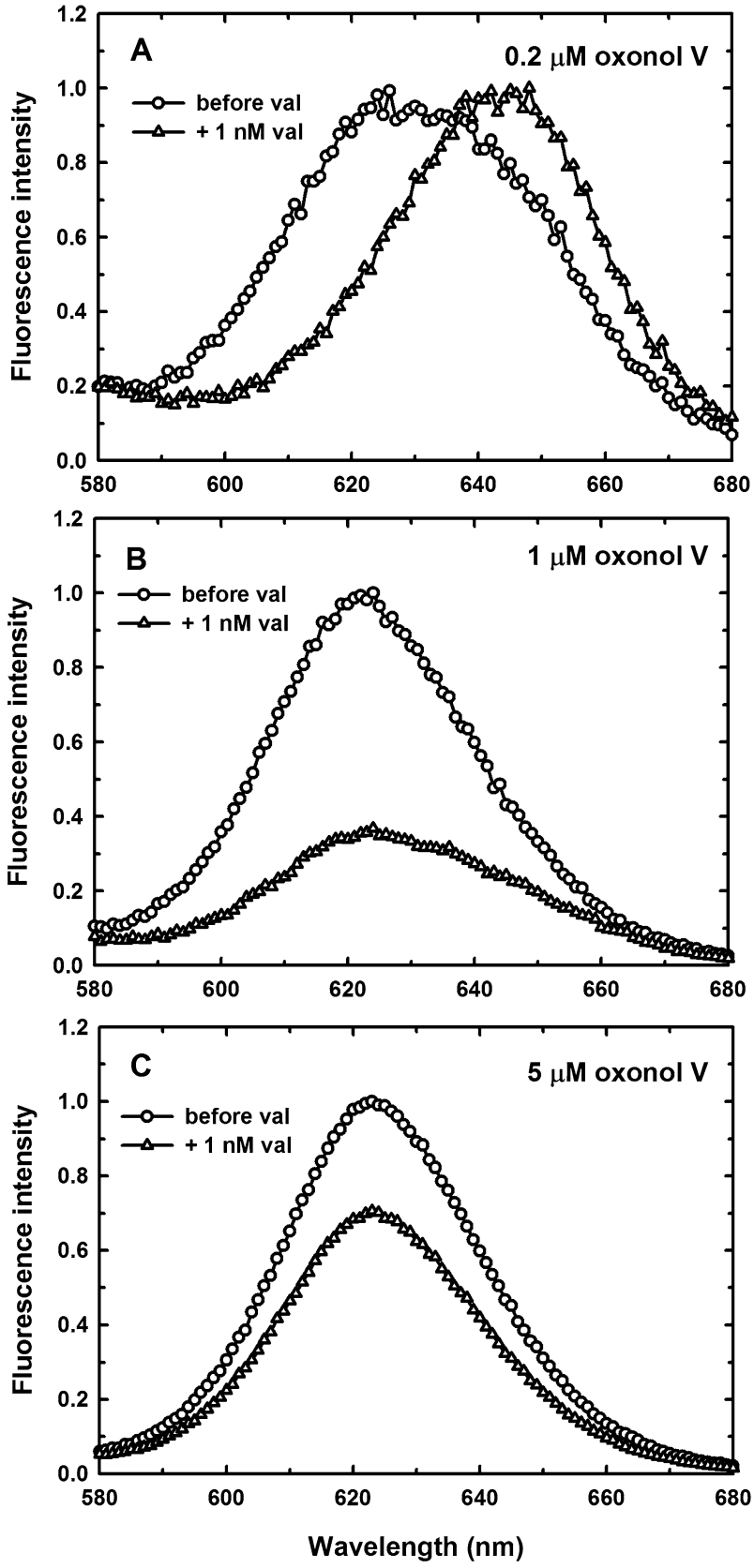

Fig. 4 Response of oxonol V to K^+ diffusion $\Delta\psi$ in LUVs measured at different oxonol V concentrations; K^+ gradient (150 mM KCl out, 0.5 mM KCl in) was imposed and $\Delta\psi$ build-up was accomplished by the addition of 1 nM valinomycin. Fluorescence spectra were measured before and after valinomycin addition. LUVs were prepared in 50 mM TRIS, pH 7.5, 0.5 mM KCl from DOPC by extrusion through 400 nm polycarbonate filter. Concentration of oxonol V was 0.2 μM in **A**, 1 μM in **B** and 5 μM in **C**. Lipid concentration in the samples was 7 μg/ml

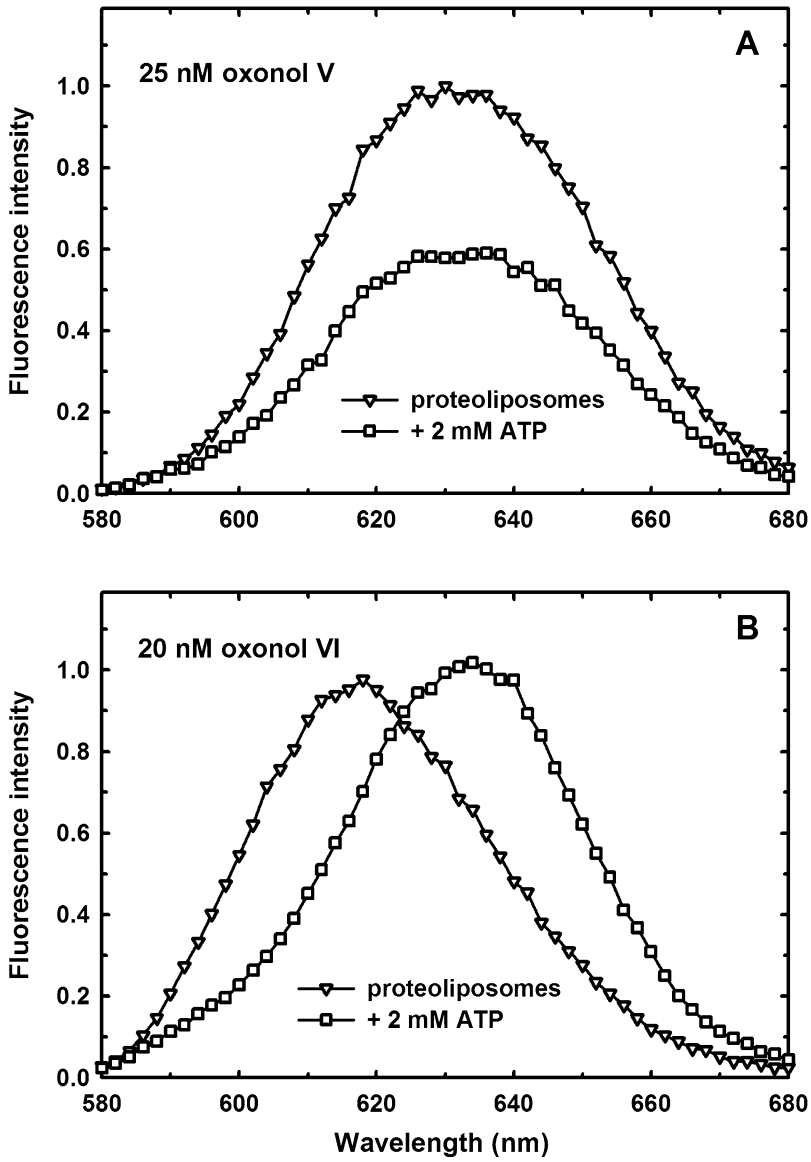

Fig. 5 Response of oxonol V and oxonol VI fluorescence spectrum to $\Delta\psi$ generated by the reconstituted plasma membrane H^+-ATPase; Plasma membrane H^+-ATPase-containing reconstituted proteoliposomes suspended in 10 mM MES, pH 6.0, 3 mM $MgCl_2$ were energized with 2 mM ATP. **A** Fluorescence spectrum of 25 nM oxonol V was measured before and after ATP addition. Lipid concentration was 2 μg/ml, content of plasma membrane proteins was 20 ng/ml. **B** Fluorescence spectrum of 20 nM oxonol VI was measured before and after ATP addition. Lipid concentration was 70 μg/ml, content of plasma membrane proteins was 0.7 μg/ml

thus depleted, which is reflected subsequently in a gradual decay of $\Delta\psi$.

Measurements of pH inside proteoliposomes

The function of the reconstituted H^+-ATPase can be studied in a more complex way, when the pH inside proteoliposomes is monitored with the aid of pH-sensitive fluorescent indicators. The redistribution pH indicator ACMA reporting on pH gradient is widely used for this purpose [5, 20, 21]. Unfortunately, the fluorescence signal of ACMA cannot be easily calibrated in units of pH. The calibration method useful for *in situ* pH-sensitive fluorescence indicators entrapped inside the vesicles, described later, cannot be applied to redistribution ones since they sense the pH gradient, and the method consists in collapsing the gradient with ionophores. Moreover, the pH manipulation inside and outside vesicles is easier to perform on unenergized proteoliposomes (data not shown); this is in contrast with the fact that ACMA requires energized proteoliposomes to respond to ΔpH [22]. Since the use of ACMA for quantitative measurements involves serious problems, we tested two *in situ* fluorescent pH indicators, carboxy SNARF-1 and pyranine, for possible use in pH measurements inside the reconstituted vesicles. While the hydrophobic dye carboxy SNARF-1 was found to be insensitive to the inner pH apparently due to its strong interaction with the lipid phase [23], the hydrophilic dye pyranine is better suited for the measurements of pH inside the vesicles [36–39]. Its usefulness for proteoliposomes with active transport of protons was proven in [40–43]. Fluorescence response of pyranine to ΔpH can be calibrated by using a mixture of ionophores that equilibrate the inner pH

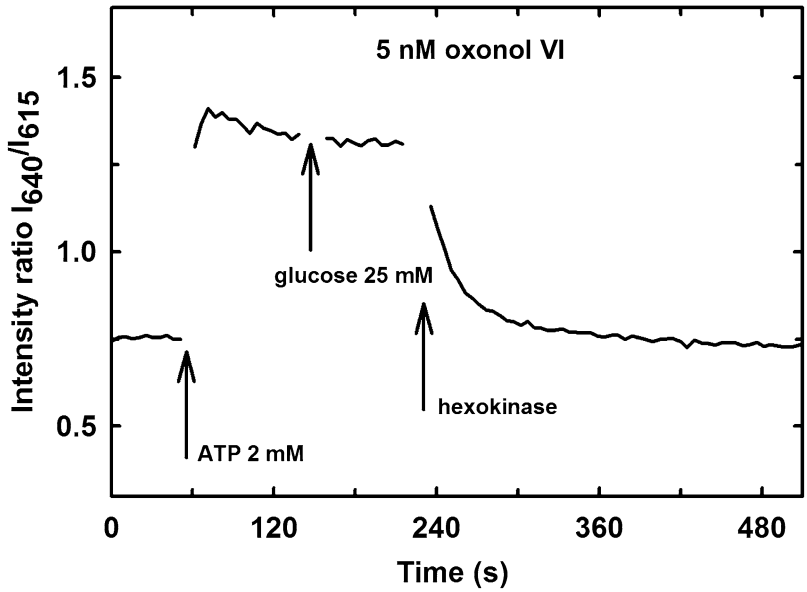

Fig. 6 Continuous monitoring of $\Delta\psi$ generated by H^+-ATPase on the reconstituted proteoliposomes; Time dependence of intensity ratio I_{640}/I_{615} of 5 nM oxonol VI was monitored in a suspension of proteoliposomes prepared and diluted in 10 mM MES (pH 6.0), 10 mM KCl and 140 mM choline chloride. Proteoliposomes were energized by an addition of 2 mM ATP. Afterwards, the proteoliposome suspension was supplemented with 25 mM glucose. Finally, 22 units of hexokinase were added to 3 ml of the sample in order to deplete the residual ATP, which caused dissipation of the generated $\Delta\psi$. Lipid concentration in the samples was 70 μg/ml, content of plasma membrane proteins 0.7 μg/ml

with the outer one in the presence of the same K^+ concentration on both sides of the membrane. The inner pH can be then manipulated by changing outer pH with successive additions of defined amounts of concentrated base or acid to the sample, see inset of Fig. 7. The calibration curve can be then measured (Fig. 7). Fig. 8A illustrates the use of pyranine for monitoring inner pH of energized proteoliposomes. Excitation intensity ratio I_{404}/I_{460} of 2 mM pyranine entrapped

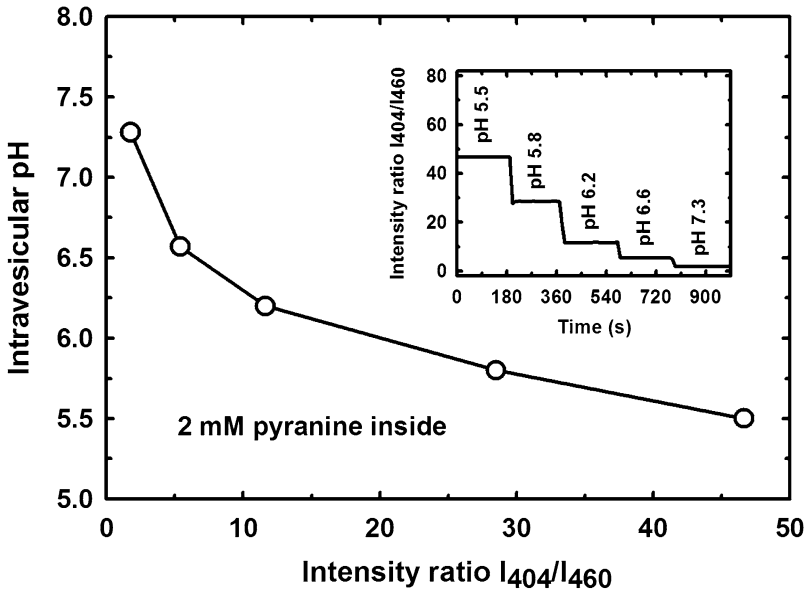

Fig. 7 Calibration curve measured for pH-sensitive fluorescent probe pyranine (2 mM) entrapped inside the proteoliposomes; The calibration of entrapped pyranine fluorescence response to pH was performed by adjusting the pH outside the proteoliposomes by small amounts of NaOH or HCl to obtain defined calibration values for pH and equilibrating inner pH with the outer by a mixture of three ionopohores: 0.3 μM valinomycin, 3.3 μM CCCP and 2.2 μM nigericin, which should totally collapse the pH gradient. The samples were incubated with the ionophore mixture 1 h prior to the measurement of fluorescence excitation intensity ratio I_{404}/I_{460}. The intensity ratio measurement is presented in **Inset**. Lipid concentration in the samples was 100 μg/ml, content of plasma membrane proteins 1 μg/ml

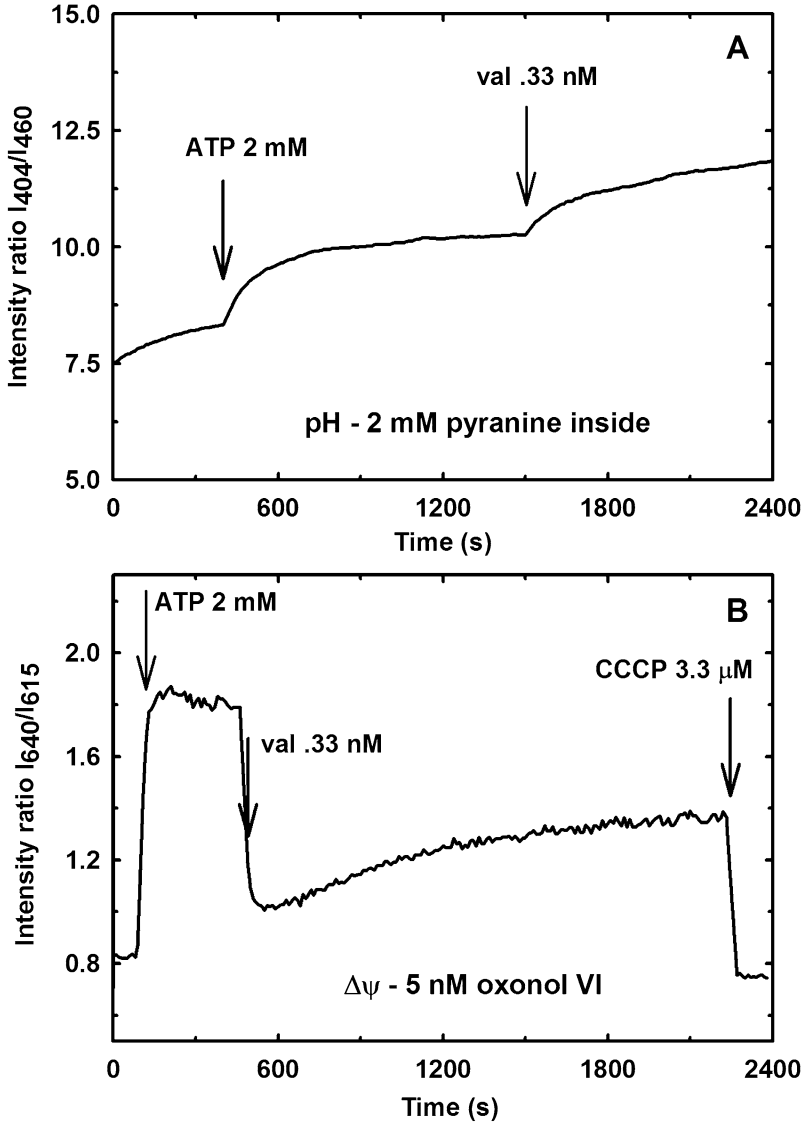

Fig. 8 Monitoring of intravesicular pH and $\Delta\psi$ in the H^+-ATPase-containing reconstituted proteoliposomes; Proteoliposomes were prepared and diluted in 10 mM MES, 100 mM KCl, 3 mM $MgCl_2$, pH 6.0 and 2 mM pyranine was entrapped inside. **A** Fluorescence excitation intensity ratio I_{404}/I_{460} of 2 mM pyranine entrapped inside was monitored before and after their energization by 2 mM ATP. Gradual acidification was observed even prior to ATP addition. Rate of the acidification increased after the addition of ATP and it was further magnified by the addition of 0.33 nM valinomycin. Lipid concentration in the samples was 100 μg/ml, content of plasma membrane proteins 1 μg/ml. In **B**, $\Delta\psi$ generated by the reconstituted H^+-ATPase was monitored by 5 nM oxonol VI for the proteoliposomes with 2 mM pyranine entrapped inside. Lipid concentration in the samples was 70 μg/ml, content of plasma membrane proteins 0.7 μg/ml

inside the reconstituted vesicles was tracked before and after addition of 2 mM ATP. Gradual acidification was observed even prior to ATP addition. These pH transients observed in unenergized proteoliposomes with pyranine shortly after dilution to the sample buffer, even when identical to that used in the preparation, indicate that the pH inside is not equilibrated with the pH of the buffer. It takes tens of minutes for the system to reach equilibrium. ATP addition boosts the intensity ratio growth. This increase indicates acidification of inner volume of the vesicles associated with proton pumping by the reconstituted H^+-ATPase. This agrees with the fact that the H^+-ATPase pumps protons inside the vesicles. Considering the calibration curve, the inner pH changes in the range of tenths of pH during the pumping. The extent of the acidification was further increased by the addition of 0.33 nM valinomycin, which is in accordance with the assumption that valinomycin-transported K^+ ions act as counter-ions to protons transported inside by the pump; this changes the concentration of either protons or K^+ ions inside vesicles. Since pyranine fluorescence is dependent also on the concentration of other cations, such as K^+ or Na^+, in the medium (unpublished data), the concentration of these ions inside the vesicles should be controlled in addition to ensure proper quantitative measurements of inner pH transients when both active and passive transport mechanisms are combined on the membrane of reconstituted vesicles. With the proteoliposomes containing pyranine inside, the H^+-ATPase-driven $\Delta\psi$ build-up was measured with oxonol VI (Fig. 8B). This measurement shows that the entrapment of the dye inside does not affect substantially the $\Delta\psi$ formation, which proves that this system can be used to simultaneous monitoring of both components of the proton electrochemical gradient. Subsequent addition of 0.33 nM valinomycin brought about a fast $\Delta\psi$ drop. Valinomycin apparently

increased the contribution of K^+ passive diffusion to ion fluxes across the membrane. Since the initial K^+ concentrations on both sides of the proteoliposome membrane are supposed to be identical, the K^+ passive diffusion alone would be setting a zero $\Delta\psi$. The $\Delta\psi$ drop can be thus explained that after the K^+ diffusion has been facilitated by valinomycin the K^+ diffusion $\Delta\psi$ prevails on the membrane, which brings $\Delta\psi$ down. The subsequent $\Delta\psi$ build-up can then be explained by redistribution of K^+ ions between the two compartments, which is driven by H^+-ATPase-mediated proton pumping.

Although the results presented in this chapter are still preliminary, the model system of reconstituted proteoliposomes with entrapped pyranine offers a promising tool for further studies of interconnections between electrogenic proton pumping, regulation of bulk pH and transport of other ions across the membrane.

Conclusions

In this paper we describe the application of ratiometric fluorescent probes oxonol VI and pyranine for studying the basic mechanisms of transmembrane transport in model systems of reconstituted vesicles. We used the ratiometric fluorescent probes for simultaneous monitoring of both components of the proton electrochemical gradient in real time, and present here the basic principles of the proper selection of fluorescent dyes for monitoring electrochemical gradients in reconstituted systems. An optimisation procedure for both the dye concentration and the concentration of reconstituted vesicles is also discussed together with the limits of the calibration methods used in this work. Special attention was paid to minimization of artefacts stemming from the interactions of the dyes with different chemical compounds used in the experiments. We expect that the calibration of fluorescence responses of both the $\Delta\psi$-sensitive dye oxonol VI and the pH-sensitive dye pyranine will allow quantification of the fluorescence measurements which can lead in the future to interpretation of the data by mathematical models using basic electrochemistry.

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