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# Phosphoinositide–protein interactions of the plasma-membrane $Ca^{2+}$-transport ATPase as revealed by fluorescence energy transfer

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**Fluorescence energy transfer has been used to study the interaction of various phospholipids with the erythrocyte ($Ca^{2+}$ + $Mg^{2+}$)-ATPase. The fluorescence energy transfer between tryptophan residues of the ($Ca^{2+}$ + $Mg^{2+}$)-ATPase purified from erythrocytes and pyrene-labelled analogues of phosphatidylcholine (Pyr-PC), phosphatidylinositol (Pyr-PI), phosphatidylinositol 4-phosphate (Pyr-PIP), phosphatidylinositol 4,5-bisphosphate (Pyr-$PIP_2$), phosphatidylglycerol (Pyr-PG) and phosphatidic acid (Pyr-PA) was measured. A positive correlation was found between the number of negative charges on the phospholipids ($PIP_2$ > PIP > PA > PI = PG > PC) and the potency of their pyrene-labelled analogues to act as quantum acceptors in fluorescence energy transfer from the tryptophan residues of the ($Ca^{2+}$ + $Mg^{2+}$)-ATPase. This is the first time that a physical interaction between PIP / $PIP_2$ and an intrinsic membrane protein has been demonstrated. The dependence of the energy transfer on the number of negative charges of the phospholipids closely resembles the previously demonstrated charge dependence of the enzymatic activity of the ($Ca^{2+}$ + $Mg^{2+}$)-ATPase (Missiaen, L., Raeymaekers, L., Wuytack, F., Vrolix, M., Desmet, H. and Casteels, R. (1989) Biochem. J. 263, 687–694). It is concluded that the stimulation of the ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity by negatively charged phospholipids is based on a binding of these lipids to the ($Ca^{2+}$ + $Mg^{2+}$)-ATPase and that the negative charges are a major modulatory factor for this interaction.**

## Introduction

The $Ca^{2+}$-transporting ATPase of the plasma membrane extrudes $Ca^{2+}$ out of the cell against its large electrochemical gradient. This ($Ca^{2+}$ + $Mg^{2+}$)-ATPase belongs to the class of P-type ATPases: it forms an aspartyl-phosphate intermediate during the reaction cycle [2]. It is a single polypeptide with a $M_r$ of about 138 000 [3] whose activity is stimulated by the binding of the $Ca^{2+}$-calmodulin complex, by negatively charged phospholipids, by partial proteolysis and by cAMP-dependent phosphorylation of a residue in the C-terminal domain of the enzyme [4].

The stimulatory effect of the negatively charged polyphosphoinositides on the plasma-membrane $Ca^{2+}$-transporting ATPase activity might be of physiological importance. First, there is the observation made by Vrolix et al. [5] that the stimulation of the $Ca^{2+}$-transporting ATPase by cyclic GMP-dependent protein kinase can be mediated by the generation of phosphatidylinositol 4-phosphate (PIP) from phosphatidylinositol (PI) by activation of a PI kinase. Secondly, it was observed that the decreased $Ca^{2+}$-transporting ATPase activity induced by $Ca^{2+}$-mobilizing agonists [6] is accompanied by a reduction in phosphatidyl-

Abbreviations: ($Ca^{2+}$ + $Mg^{2+}$)-ATPase, ($Ca^{2+}$ + $Mg^{2+}$)-dependent ATPase; Pyr-PI, *sn*-2-(pyrenyldecanoyl)phosphatidylinositol; Pyr-PIP, *sn*-2-(pyrenyldecanoyl)phosphatidylinositol 4-phosphate; Pyr-$PIP_2$, *sn*-2-(pyrenyldecanoyl)phosphatidylinositol 4,5-bisphosphate; Pyr-PC, *sn*-2-(pyrenyldecanoyl)phosphatidylcholine; Pyr-PG, *sn*-2-(pyrenyldecanoyl)phosphatidylglycerol; Pyr-PA, *sn*-2-(pyrenyldecanoyl)phosphatic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylcholine; PA, phosphatidic acid; PG, phosphatidylglycerol; DMSO, dimethylsulfoxide; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; $\lambda_{ex}$, excitation wavelength; $\lambda_{em}$, emission wavelength.

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inositol 4,5-bisphosphate ($PIP_2$) and PIP levels in the plasma membrane [7].

Negatively charged phospholipids strongly stimulate the purified plasma-membrane $Ca^{2+}$ pump from erythrocytes and from smooth muscle by increasing the $V_{max}$, the affinity and the co-operativity for $Ca^{2+}$ [1]. The order of potency in stimulating the enzyme ($PIP_2$ > PIP > PA > PI = PS) is proportional to the number of negative charges on these lipids [1]. This stimulatory effect could be reduced by complexing the negative charges with polyamines, neomycin [8], Ruthenium red or compound 48/80 [9]. These findings suggest that the negative charges are important for the modulation of the activity of the enzyme by phospholipids.

However, the mode of activation by these negatively charged phospholipids has not yet been elucidated. First, the binding of the phospholipids to the $(Ca^{2+} + Mg^{2+})$-ATPase has not been independently demonstrated in a direct way. Secondly, the nature of such an interaction remains unspecified. It could be controlled mainly by the negative charges on the hydrophilic head group of these phospholipids or by factors other than simple electrostatics, e.g., steric interaction and hydration. Under the latter condition mainly hydrophobic associations may determine the affinity of the lipid molecules for the $(Ca^{2+} + Mg^{2+})$-ATPase while the hydrophilic head group could be responsible for a more specific functional modification of the enzyme.

Recently a method has been developed for the synthesis of pyrenylacyl-labelled PIP and $PIP_2$ [10]. In this study, we have used a spectroscopical method for measuring the interaction of these probe lipids with the $(Ca^{2+} + Mg^{2+})$-ATPase. This method is independent of enzyme activity measurements, and should allow us to test the hypothesis of binding of the phospholipids with the $(Ca^{2+} + Mg^{2+})$-ATPase and to establish the nature of this interaction.

## Materials and Methods

*Synthesis of pyrene-labelled phospholipids*

*sn*-2-(Pyrenyldecanoyl)-PC (Pyr-PC) was synthesized from egg yolk PC (Sigma) as described by Somerharju et al. [11]. *sn*-2-(Pyrenyldecanoyl)-PG (Pyr-PG) and *sn*-2-(pyrenyldecanoyl)-PA (Pyr-PA) was synthesized from Pyr-PC using phospholipase D as described [11]. *sn*-2-(Pyrenyldecanoyl)-PI (Pyr-PI) was synthesized from yeast PI as described by Somerharju and Wirtz [12] and Somerharju et al. [11]. *sn*-2-(Pyrenyldecanoyl)-PIP (Pyr-PIP) and *sn*-2-(pyrenyldecanoyl)-$PIP_2$ (Pyr-$PIP_2$) were synthesized enzymatically from *sn*-2-(pyrenyldecanoyl)-PI (Pyr-PI) using partially purified PI and PIP kinase preparations from bovine brain as described by Gadella et al. [10]. The concentration of pyrene-labelled phospholipids was estimated by measuring the absorption at 342 nm in ethanol/DMSO (75:25, v/v) ($\varepsilon = 39700$ $M^{-1} cm^{-1}$) and by phosphorus determination [13]. As a control for the intactness of the pyrene moiety after synthesis, absorption spectra were recorded on a Hitachi U-3200 spectrophotometer. Pyrene-labelled lipids were stored in chloroform/methanol (7:1, v/v) under argon at −20 °C.

*Preparation of erythrocyte ghost membranes*

Erythrocyte ghosts were prepared from fresh porcine blood according to Steck and Kant [14].

*Purification of the $(Ca^{2+} + Mg^{2+})$-ATPase*

The plasma-membrane $Ca^{2+}$-transporting ATPase from erythrocytes was purified using a modification of the calmodulin-affinity chromatography as described by Kosk-Kosicka et al. [15]. This procedure allows purification of the enzyme in the absence of phospholipids. Briefly, erythrocyte ghosts were centrifuged in a Beckman Ti60 rotor at 50000 rev./min ($254400 \times g_{max}$) for 30 min and resuspended in 130 mM KCl, 20 mM Hepes (pH 7.4), 0.5 mM $MgCl_2$, 0.05 mM $CaCl_2$, 2 mM dithiothreitol and 20% glycerol at 8 mg membrane protein/ml. The membranes were solubilized by adding 4 mg Triton X-100 (Merck) per ml of buffer. After 10 min of incubation at 4 °C (under continuous stirring), the nonsolubilized material was removed by centrifugation in a Ti60 rotor at 50000 rev./min for 30 min. The Triton X-100-solubilized material was added to a calmodulin-Sepharose 4B affinity gel that had been equilibrated with buffer A (130 mM KCl, 20 mM Hepes (pH 7.4), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2 mM dithiothreitol, 20% glycerol and 0.4% Triton X-100). After incubation of this mixture for 1 h at 4 °C in an end over end mixer the gel was transferred into a chromatography column. The unbound proteins were removed by washing the column with 10 volumes of buffer A. Thereupon the column was washed with 10 volumes of buffer A′ (the same as A but containing 0.05% Triton X-100 instead of 0.4%). Triton X-100 was replaced by the nonfluorescent detergent Thesit (Boehringer) by passing through the column 10 volumes of buffer A″ (A″ is the same as A′ but containing 0.05% Thesit instead of Triton X-100). Finally, the $(Ca^{2+} + Mg^{2+})$-ATPase bound to the column was collected by washing the gel with the same buffer as above except that 0.1 mM $CaCl_2$ was replaced by 2 mM EDTA. The $(Ca^{2+} + Mg^{2+})$-ATPase preparation was finally concentrated in Centriprep 10-Concentrator tubes (Amicon). Different preparations were pooled and stored at −80 °C (final concentration was 280 μg/ml). A molecular mass of $M_r = 138000$ for the $(Ca^{2+} + Mg^{2+})$-ATPase as determined by SDS-gel electrophoresis was used to calculate molar amounts of the protein.

*Reconstitution of the $(Ca^{2+} + Mg^{2+})$-ATPase*

The $(Ca^{2+} + Mg^{2+})$-ATPase purified as described above did not present any detectable enzyme activity,

but the $(Ca^{2+} + Mg^{2+})$-ATPase could be reactivated with phospholipids at a ratio of total lipid to ATPase of 100 mol/mol. This ratio was obtained by adding 50 μl of the ATPase preparation to 25 μl of a lipid mixture and vortex-mixing. Lipid mixtures were made from stock solutions of phospholipids in chloroform/

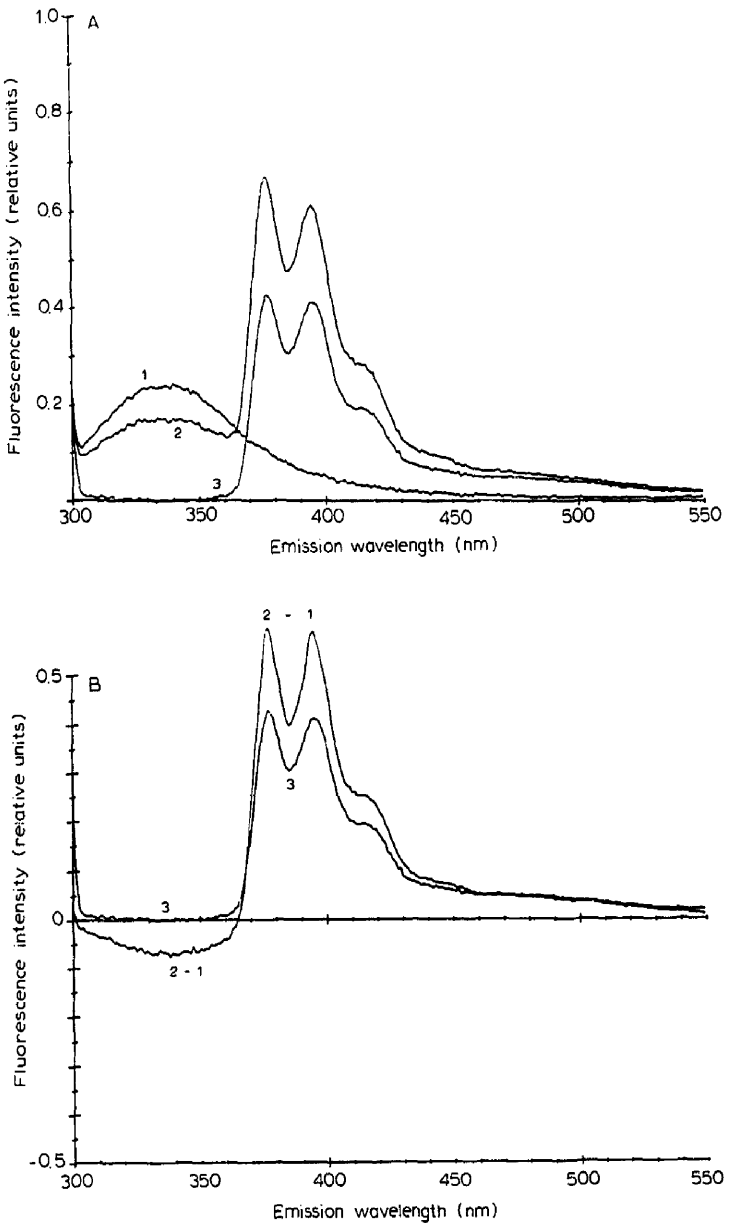


Fig. 1. (A) Fluorescence emission spectra of the purified $(Ca^{2+} + Mg^{2+})$-ATPase reconstituted in PC in the absence (1) and presence (2) of 1% Pyr-PIP, and of 1% Pyr-PIP in PC without $(Ca^{2+} + Mg^{2+})$-ATPase (3). $\lambda_{ex}$ = 290 nm. (B) Fluorescence emission spectrum of 1% Pyr-PIP in PC (3) and difference spectrum calculated by subtraction of spectrum (1) from spectrum (2).

methanol (7 : 1, v/v). The solvent was evaporated under a stream of nitrogen, and the lipids were redissolved in the $(Ca^{2+} + Mg^{2+})$-ATPase buffer (130 mM KCl, 20 mM Hepes (pH 7.4), 1 mM $MgCl_2$, 2 mM EGTA, 0.05% Thesit and 20% glycerol (v/v)) and sonicated for 6 × 5 s. The final lipid concentration was 5 mg/ml.

*Fluorescence spectroscopy*

Fluorescence emission spectra were recorded on a SLM-Aminco SPF-500 C spectrofluorimeter between 300 and 550 nm (band width 10 nm). The excitation wavelength was set at 290 nm with a slit width of 5 nm. All experiments were carried out at 25°C under continuous stirring of the sample. Spectra were corrected for background fluorescence (Raman peak). The reconstituted $(Ca^{2+} + Mg^{2+})$-ATPase (40 μl) was pipetted into a cuvette containing 0.1 M KCl, 6 mM $MgCl_2$, 5 mM $NaN_3$, 0.5 mM EGTA, 40 mM Hepes (final volume of 2 ml). The buffers used for the fluorescence measurement were routinely filtered through a Millipore filter (45 μm). The efficiency of energy transfer was calculated from the fluorescence intensities (at 340 nm) of the donor (tryptophan) in the presence ($F_{da}$) and absence ($F_d$) of the acceptor (pyrene), e.g., the equation: $E = 1 - (F_{da}/F_d)$. The fluorescence energy transfer rate was calculated according to the relationship $k = E/(1 - E)$ [16,17]. Control experiments were carried out by measuring the energy transfer between 7.5 μg bovine serum albumin (BSA, Calbiochem) and Pyr-PC or Pyr-PI containing egg-PC vesicles (16 nmol total phospholipid) in the same buffer as described above (final volume of 2 ml).

## Results and Discussion

Fig. 1A shows the fluorescence emission spectra of the purified $(Ca^{2+} + Mg^{2+})$-ATPase reconstituted in PC in the absence (1) or presence (2) of 1% pyrene-labelled PIP. Also the spectrum of 1% Pyr-PIP in PC without $(Ca^{2+} + Mg^{2+})$-ATPase is shown (3). The fluorescence spectrum of the purified $(Ca^{2+} + Mg^{2+})$-ATPase shows a maximum at 340 nm, due to the fluorescence of the 12 tryptophan residues of the enzyme. This spectrum shows a good overlap with the pyrene-absorption spectrum ensuring an efficient energy transfer from the donor tryptophan residues of the $(Ca^{2+} + Mg^{2+})$-ATPase towards the pyrene-labelled acceptor probes if a close association takes place. At 1 mol% concentration the pyrene probes appear predominantly as monomers with emission maxima at 377 and 396 nm. Only a small shoulder in the fluorescence signal corresponding to excimer formation could be detected around 470 nm, the emission maximum for excimer fluorescence. These data indicate that the probes are uniformly distributed as monomeric species both in the absence and in the presence of the $(Ca^{2+} + Mg^{2+})$-ATPase.

The energy transfer between the tryptophan residues of the $(Ca^{2+} + Mg^{2+})$-ATPase and the Pyr-PIP probe is demonstrated in Fig. 1A. This figure depicts the decrease in intensity of the tryptophan emission and the concomitant increase in the pyrene signal compared to the pyrene signal in the absence of the $(Ca^{2+} + Mg^{2+})$-ATPase. In order to demonstrate more clearly the increase of the sensitized emission of the pyrene probe we show in Fig. 1B the difference spectrum calculated by subtracting spectrum (1) from spectrum (2). This difference spectrum is compared with the spectrum of the pyrene probe in the absence of the $(Ca^{2+} + Mg^{2+})$-ATPase (3). The decrease in tryptophanyl fluorescence is due to energy transfer by close associations between the $(Ca^{2+} + Mg^{2+})$-ATPase and the acceptor-labelled lipid molecules. It can be excluded that the decrease of the tryptophanyl fluorescence would be an artifact due to direct pyrene absorption at either the 290 nm excitation or the 340 nm emission wavelengths of the donor since at these wavelengths the optical density of all samples was below 0.04. The decrease in donor emission at 340 nm was used for calculation of the fluorescence energy transfer efficiency because at this wavelength there is no acceptor emission. At other wavelengths, e.g., at 377 nm (maximum emission for pyrene), an overlap of donor and acceptor spectra obscured the calculations. We did not attempt in this study to derive absolute values for the distances between donors and acceptors from the percentages of energy transfer but we compared the energy transfer efficiencies for different pyrene-labelled phospholipids.

In the experiment illustrated in Fig. 2, we compared the efficiencies of fluorescence energy transfer between the tryptophan residues of the $(Ca^{2+} + Mg^{2+})$-ATPase and two different acceptors: the neutral Pyr-PC and the

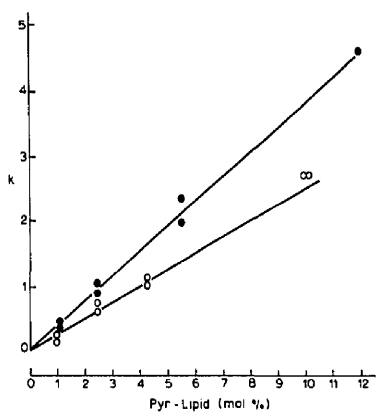


Fig. 2. The experimental rate of fluorescence energy transfer ($k = E/(1-E)$) from the tryptophan residues of the $(Ca^{2+} + Mg^{2+})$-ATPase to Pyr-PC (○) and Pyr-PI (●) acceptors as a function of acceptor concentration.

negatively charged Pyr-PI. We measured the fluorescence energy transfer efficiency ($E$) as a function of the acceptor concentration. The fluorescence energy transfer rate ($k$) for Pyr-PC and Pyr-PI at different acceptor concentrations was derived from the measured fluorescence energy transfer efficiency ($E$) according to the relationship $k = E/(1\text{-}E)$ [16]. The expected linear dependence of $k$ on the acceptor concentration was obtained, by plotting this parameter as a function of acceptor concentration and is illustrated for Pyr-PC and Pyr-PI in Fig. 2. It is obvious that the slope of the curve is greater for Pyr-PI than for Pyr-PC. It can therefore be concluded that the negatively charged phospholipid Pyr-PI was more efficient in quenching the tryptophan fluorescence of the $(Ca^{2+} + Mg^{2+})$-ATPase than the corresponding PC derivative. This is probably due to a higher affinity of the $(Ca^{2+} + Mg^{2+})$-ATPase for Pyr-PI as compared to Pyr-PC. This can be expected as the negatively charged inositol headgroup of PI would be directly involved in providing a lipid binding site for the $(Ca^{2+} + Mg^{2+})$-ATPase.

In the next series of experiments, we measured the efficiency of fluorescence energy transfer between tryptophan residues of the $(Ca^{2+} + Mg^{2+})$-ATPase and Pyr-PI, Pyr-PIP and Pyr-$PIP_2$ at a concentration of 1% in PC vesicles (Fig. 3). Control spectra were measured in the presence of 1% of unlabelled PI, PIP and $PIP_2$ in order to exclude charge effects on the tryptophanyl fluorescence. At this low concentration of labelled phospholipids the order of potency in decreasing the tryptophan fluorescence of the $(Ca^{2+} + Mg^{2+})$-ATPase due

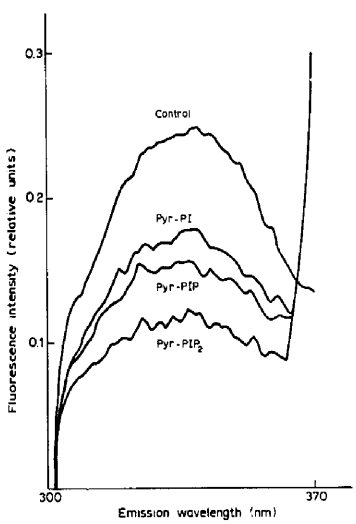


Fig. 3. Fluorescence emission spectra of the purified $(Ca^{2+} + Mg^{2+})$-ATPase reconstituted in PC in the absence of pyrene-labeled lipids (control) and in the presence of 1% Pyr-PI, 1% Pyr-PIP and 1% Pyr-$PIP_2$.

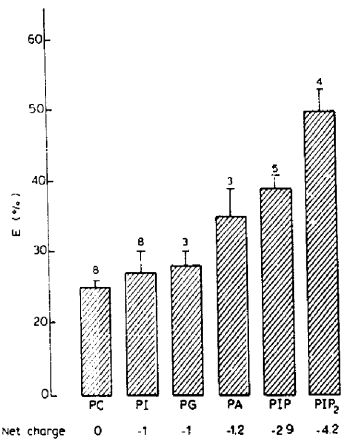


Fig. 4. Fluorescence energy transfer efficiency ($E$) between donor tryptophan residues of the $(Ca^{2+} + Mg^{2+})$-ATPase and different fluorescent acceptor probes. The results are expressed as means ± S.E. for the number of observations given above each bar. The net charge of the lipids at pH 7.4 [18,19] is given below.

to energy transfer was Pyr-$PIP_2$ > Pyr-PIP > Pyr-PI in correlation with the number of negative charges on the inositol headgroup.

In order to find out whether the inositol headgroup contributes to the specificity of interaction between the $(Ca^{2+} + Mg^{2+})$-ATPase and the polyphosphoinositides we also measured the energy transfer between the $(Ca^{2+} + Mg^{2+})$-ATPase and pyrene-labelled negatively charged phospholipids with different headgroups. At a concentration of 1%, Pyr-PG and Pyr-PA decreased the tryptophanyl fluorescence by 28% and 35%, respectively. Taken together our data indicate that for all the phospholipid species tested there exists a positive correlation between the relative potency of the lipid to act as a fluorescence acceptor and its number of negative charges at physiological pH ($PIP_2$ > PIP > PA > PI = PG > PC) as illustrated in Fig. 4.

Missiaen et al. [1] reported a similar correlation between the number of negative charges on the phospholipids and the magnitude of their effect on the $V_{max}$ and the $K_{0.5}$ for $Ca^{2+}$. Because in their experiments the effect of the negatively charged phospholipids on the $(Ca^{2+} + Mg^{2+})$-ATPase activity reached a maximum only at high concentrations of these lipids (20 to 50% of the total lipid) a specific lipid–protein interaction could not be discriminated from an overall charge effect of the lipids surrounding the $(Ca^{2+} + Mg^{2+})$-ATPase. However, some evidence was provided for a functionally important electrostatic interaction between the acidic lipids and arginine residues on the protein [20].

In this study we have investigated the lipid–protein interactions of the erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase

by using a direct physical method which did not depend on enzyme activity measurements. Using this method of fluorescence energy transfer we were able to confirm that the $(Ca^{2+} + Mg^{2+})$-ATPase has a preferential selectivity for interaction with negatively charged lipids and that the degree of interaction is correlated with the number of negative charges on the lipids thus mainly depending on electrostatic interactions. This means that the $(Ca^{2+} + Mg^{2+})$-ATPase protein would be preferentially surrounded by the highly charged polyphosphoinositides PIP and $PIP_2$ which argues in favor of a functional role of these lipids and their metabolism in the modulation of the $(Ca^{2+} + Mg^{2+})$-ATPase activity. This study is to our knowledge the first report in which such interactions were directly measured by means of fluorescence energy transfer. Furthermore it is demonstrated that the pyrene-labelled polyphosphoinositides are useful fluorescent probes to investigate the lipid–protein interactions of the erythrocyte $Ca^{2+}$-transport ATPase [10].

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