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Monoclonal antibody to phosphatidylserine inhibits Na^+/K^+-ATPase activity

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Abstract

A monoclonal IgG, directed to phosphatidylserine (PS1G3), partially (40–50%) inhibited Na^+/K^+-ATPase activity (forward running reaction cycle) without affecting the $K_{0.5}$ values for Na^+, K^+ and MgATP. The Hill or interaction coefficients (n_H) for Na^+ and K^+ for this reaction were reduced from 3.0 to 1.6 and from 1.6 to 0.8, respectively. The K^+-stimulated *p*-nitrophenylphosphatase activity (*p*-NPPase), which is a partial reaction sequence of the Na^+/K^+-ATPase system (but in the backward running mode), was inhibited more strongly (about 70%) due to an increase in K^+/substrate antagonism. In this system $K_{0.5}$ and n_H values for both *p*-nitrophenyl phosphate (*p*-NPP) and K^+ were increased by the mAb. At the maximally inhibitory concentration of PS1G3 the V_{max} of the *p*-NPPase was also reduced. Partial reactions, which were inhibited by PS1G3, are: (1) the Na^+-activated phosphorylation (non-competitive vs. Na^+), (2) the Rb^+ occlusion (competitive vs. Rb^+). Partial reactions not harmed by PS1G3 are: (3) the K^+-dependent dephosphorylation, (4) the K^+-dependent $E_1 + K^+ \rightleftharpoons E_2K$ transition. We conclude that PtdSer is involved in cation occlusion, possibly by forming part of the access gate.

Key words: ATPase, Na^+/K^+-; Phosphatidylserine; Enzyme inhibition

1. Introduction

In a foregoing study on the site of inhibition by the activating cation antagonist ethylenediamine (Eda) [1] we detected that a prominent site of interaction between the inhibitor and the enzyme-lipid complex is phosphatidylserine (PtdSer) [2]. This finding, in agreement with previous interpretations [3], hinted at the involvement of phosphatidylserine in cation activation.

Involvement is, of course, a multivalent term, which may imply that the phospholipid via its negative charges: (1) gathers alkali cations from the surrounding fluid for transfer to the protein, (2) facilitates conformational changes in the protein, thereby modulating affinity for binding of the cations, or (3) forms the actual activating phospholipid-protein complex. Recent developments [4] indicate that the impact is on the conformational change to E_2 which has a high affinity to K^+ (low to Na^+).

Meanwhile [5], monoclonal antibodies to PtdSer have been raised by injection of PtdSer-coated *Salmonella* directly into mouse spleen. Hence, a specific tool was available to test the role of PtdSer in the reaction mechanism of Na^+/K^+-ATPase and to compare the results with those obtained using bis-phosphatidylseryl substituted ethylenediamine (PS_2Eda, [2]). Inhibitions of partial and overall reactions of Na^+/K^+-ATPase in the forward and reverse reaction mode [6] by the antibody support the conception of a membrane (more in particular PtdSer) stimulated pump mechanism.

Abbreviations: PtdSer, phosphatidylserine; Eda, ethylenediamine; PS_2Eda, *N,N'*-bis(*O*-phosphatidylseryl)ethylenediamine; EDTA, ethylenediaminetetraacetic acid; PS1G3, monoclonal IgG to PtdSer; PS4A7, monoclonal IgM to PtdSer; mAb, monoclonal antibody; PBS, phosphate-buffered saline; *p*-NPP, *p*-nitrophenyl phosphate; *p*-NPPase, *p*-nitrophenylphosphatase. FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay.

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2. Materials and methods

2.1. Enzyme preparation and overall hydrolytic activity

Na^+/K^+-ATPase was prepared and purified from rabbit kidney outer medulla according to the isopycnic

zonal centrifugation procedure of Jørgensen [7], following solubilisation of contaminating protein by dodecylsulfate treatment. The preparation, after the final spin, was resuspended and stored at −20°C in 50 mM imidazole-HCl (pH 7.0), containing 10% (w/v) sucrose. Protein of the enzyme preparation was determined by the method of Lowry et al. [8], following trichloroacetic acid precipitation and using bovine serum albumin as a standard [7]. Specific Na^+/K^+-ATPase activity under optimal conditions at pH 7.4 and 37°C ranged between 1500 and 2200 μmol/mg protein per h.

2.2. Induction and purification of phosphatidylserine antibodies

Antibodies to PtdSer were raised by repeated intrasplenic injection (covering a 1-week interval) of PtdSer-coated *Salmonella* into BALB/c mice, followed by five injections via the lateral tail veins (each injection every 3 weeks: long-term immunisation). 3 days after the last injection the spleen cells were fused with P3-X63-Ag.653 cancer cells, the hybridomas were cultured and selected on PtdSer-antigenicity via ELISA of the hybridoma supernatant on phospholipid coated microtiter plates [5]. PS4A7, an IgM [5], was isolated by $(NH_4)_2SO_4$ precipitation and purified by HPLC; PS1G3, an IgG [5], was also isolated by $(NH_4)_2SO_4$ precipitation but was purified by affinity chromatography on Sepharose 4B Protein A [9]. PS4A7 was dissolved in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl), but PS1G3 was dissolved in 4 M guanidine-HCl owing to aggregation of the mAb in Tris-buffered saline, and was stored at −20°C. Prior to use, the guanidine-HCl was removed from 0.4-ml aliquots by 2-fold overnight dialysis at 4°C vs. 1 liter of 300 mM choline-Cl in 20 mM triethanolamine-HCl (pH 7.4), which reduced the guanidine-HCl concentration to maximally 1 mM as determined by the butanedione/naphthol/NaOH-procedure [10].

Antibody, which aggregated during dialysis, was removed by centrifugation and redissolved in guanidine-HCl, whereas the remaining two-thirds, which stayed in solution was used for inhibition studies on Na^+/K^+-ATPase. Protein in each fraction was determined by the Bio-Rad microassay (Bio-Rad, München, Germany) with bovine serum albumin as reference, but with a correction for 54% reduction in staining efficiency of IgG as compared to bovine serum albumin.

Antibody-antigen reactivity was tested on Na^+/K^+-ATPase via ELISA on microtiter plates, loaded with 0.5 μg per well in phosphate-buffered saline (PBS: 140 mM NaCl, 7.7 mM Na-phosphate (pH 7.4)). Blocking of aspecific binding was effected by rinsing with 1% gelatine in PBS, and followed by coating with the antibody, dissolved in PBS-0.05% (w/v) Tween. Antigenicity was brought to expression via reaction of the Na^+/K^+-ATPase/mAb complex with a second antibody (rabbit anti-mouse peroxidase), followed by several-fold washing with PBS-Tween. Visualisation of the antigenicity was accomplished by reaction of the conjugated peroxidase with 0.04% (w/v) H_2O_2 in citric acid-phosphate buffer (pH 6.0), containing 0.04% *o*-phenylenediamine. The reaction was run for 15 min at room temperature (22°C), then quenched by addition of 0.1 ml 4 M H_2SO_4 to 0.1 ml of the peroxidase reaction medium. Extinction at 492 nm was recorded by a Titertek Multiscan apparatus.

2.3. Assay of antibody effects on hydrolytic activities

Antibody effects were tested on overall Na^+/K^+-ATPase and related K^+-stimulated *p*-NPPase activity at room temperature in order to minimise dissociation of the antibody and to reduce inactivation of the enzyme during the 30-min preincubation period. Since the inhibition is strongly dependent on ionic strength, the Na^+/K^+-ATPase activity was assayed at 20 mM Na^+, 1 mM K^+, 1 mM ATP, 1.15 mM Mg^{2+} and 9.4 μg Na^+/K^+-ATPase per ml 25 mM triethanolamine-HCl (pH 7.0). Concentrations of PtdSer-antibody (added last to the preincubation medium, containing all the components mentioned above, except ATP) are given under Results. Since the antibody preparation contained choline-Cl, the same amount of choline-Cl ($\leq$ 30 mM) was added to the control. Following the 30-min preincubation of enzyme with antibody in a volume of 90 μl, hydrolysis was started by addition of 10 μl 10 mM ATP and stopped 10–15 min later by addition of 2 ml ice-cold 5% trichloroacetic acid and placement of the tubes on ice. Inorganic phosphate was determined in 1 ml aliquots according to the Malachite green staining procedure of Van Veldhoven and Mannaerts with $\Delta E_{610}/\Delta$ nmol ratios of $(6.85–7.4) \cdot 10^{-2}$ [11]. Background was corrected for by blanks in which the trichloroacetic acid was added prior to the ATP.

Half-maximally activating concentrations of Na^+, K^+ or MgATP were determined by varying the ligand under study, while keeping the others constant: Na^+ (2–20 mM) at 1 mM K^+, 1 mM ATP and 1.15 mM Mg^{2+}; K^+ (0.1–4 mM) at 20 mM Na^+, 1 mM ATP and 1.15 mM Mg^{2+}; ATP (0.1–1 mM) at 20 mM Na^+, 1 mM K^+ and Mg^{2+} (0.15 mM in excess of ATP [12]).

Graphical extrapolation to V_{max} was accomplished via Eady-Scatchard plots, and half-maximally activating concentrations and cooperativity coefficients via Hill plots. ATP concentrations, used for calculation in Eady-Scatchard and Hill plots were averages of initial and final concentrations.

The *p*-NPPase activity was assayed in a similar fashion. *p*-NPP was freed from Na^+ by passage over Dowex 50 W-X4 in the protonated form, followed by

neutralisation (pH 7.0) with triethanolamine. The medium contained 10 mM K^+, 5 mM *p*-NPP, 6 mM Mg^{2+}, ≤ 18 mM choline-Cl, 25 mM triethanolamine-HCl (pH 7.0) and 9.4 μg/ml Na^+/K^+-ATPase. Determination of the half-maximally activating K^+-concentration occurred at 1–10 mM K^+, keeping the other concentrations constant, while determination of the half-maximally activating *p*-NPP concentration occurred at 0.5–5 mM of the substrate under otherwise identical conditions. Blank subtraction was as for Na^+/K^+-ATPase, i.e., from samples with trichloroacetic acid added prior to the substrate.

2.4. Assay of antibody effects on partial reactions

Rb^+-occlusion

Rb^+-occlusion was carried out by the manual assay of Shani et al. [13], using 1 μg Na^+/K^+-ATPase per 50 μl assay medium, containing 25 mM triethanolamine-HCl (pH 7.5), 60 mM choline-Cl, and antibody (PS1G3) in concentrations (μg/ml) as indicated under Results. Following 30 min preincubation of enzyme and antibody at room temperature (22°C) 10 μl of $^{86}Rb^+$ in 25 mM triethanolamine-HCl (pH 7.5), was added to 40 μl of the preincubation medium, yielding the final concentrations as indicated under Results. Equilibration was given 10 min prior to addition of 0.5 ml ice-cold 200 mM sucrose and cation exchange chromatography at 0–4°C over Dowex 50 W-X8 in the triethanolamine-form (pH in the resin is 7.2). Blank radioactivity of samples containing no enzyme, but having passed an identical column, was subtracted.

The $E_1 + K^+ \rightleftharpoons E_2K$ transition

The $E_1 + K^+ \rightleftharpoons E_2K$ conformational transition was probed fluorimetrically via the K^+-induced fluorescence quench of the covalently FITC-labeled enzyme [14] or the non-covalently eosine-labeled enzyme [15]. Labeling with FITC (10 μM) of enzyme (0.9 mg/ml) has been carried out for 1.5 h at room temperature and pH 9.2 in the dark, the reaction stopped by dilution with Tris-EDTA (pH 7.5) and excess FITC removed by centrifugation as described by Karlish [14]. The fluorescence measurements were carried out at room temperature (22°C) in a reaction volume of 100 μl, containing FITC-enzyme (10 μg/ml), 40 mM triethanolamine-HCl (pH 7.0), 45 mM choline-Cl and antibody (PS1G3) as indicated under Results. Following 30 min preincubation of enzyme and antibody, 1 μl aliquots of K^+ (0.015–6.25 mM final concentration) were added. Correction was made for dilution (maximally 8%). Measurements were made in a Hellma 105.250.QS 100 μl cuvette with an OG-515 cut-off filter at 496 nm excitation (slit width 3 nm) and 516 nm emission (slit width 10 nm).

Fluorescence measurements, using 0.2 μM eosine and unlabeled enzyme (10 μg/ml) were made likewise but with a 540 nm cut-off filter. Excitation was at 530 nm (slit width 10 nm), emission at 560 nm (slit width 10 nm). Maximal fluorescence changes were determined from Eady-Scatchard plots and the $[K^+]_{0.5}$ values from Hill plots.

Phosphorylation and dephosphorylation reactions

The phosphorylation reaction was carried out for 5 s at room temperature (22°C) at 10 μg enzyme per ml and at 5 μM [γ-^{32}P]ATP (0.24–0.88 Ci/mmol) in 25 mM triethanolamine-HCl (pH 7.0), containing 0.1 mM Mg^{2+}, 15–30 mM choline-Cl, and 0.4–10 mM Na^+ in a volume of 100 μl. Phosphorylation took place following a preincubation period (30 min) of enzyme minus and plus antibody (PS1G3), the latter in concentrations as indicated under Results. Subsequently, 4–5 ml 5% trichloroacetic acid, containing 0.1 M H_3PO_4, was added and the precipitated protein filtered over 0.45 μm pore width cellulose nitrate filters (25 mm diameter) and washed three or four times with the stopping solution, then counted for ^{32}P. Blanks were subtracted in which stopping solution was added prior to the ATP. Maximal phosphorylation levels were determined by extrapolation from Eady-Scatchard plots and $[Na^+]_{0.5}$ values from Hill plots.

The dephosphorylation (3 s) reaction was preceded by a 5-s phosphorylation period as described above ($[Na^+] = 10$ mM). Then 9 vols of chase medium, containing all the components of the phosphorylation medium in the indicated concentrations, but without enzyme, antibody and radioactive ATP, but containing in addition non-radioactive ATP (21.7 μM) and K^+ (0–33.3 μM), was added. Then the trichloroacetic acid/phosphoric acid stopping medium was added and the samples were filtered and washed as described for the phosphorylation reaction. Blanks were prepared in which chase medium (containing in this particular case the radioactive ATP) and the phosphorylation medium (−[γ-^{32}P]ATP) were first mixed and the incorporation of radioactive phosphate stopped after 8 s by addition of the trichloroacetic acid/H_3PO_4 mixture. These blanks are indiscernible from those adding stopping-acid first and radioactive ATP later on. Hence, all blanks were averaged and the mean subtracted. The amount of dephosphorylation (3 s) was plotted in an Eady-Scatchard plot as percent decrease in steady-state phosphorylation level as function of the K^+ concentration. The latter was checked flame-photometrically in the phosphorylation and chase media. $[K^+]_{0.5}$ values were determined from Hill plots.

2.5. Preparation of phosphatidylserine and phosphatidylcholine liposomes

Phosphatidylserine and phosphatidylcholine liposomes were prepared as described in a previous publi-

cation [2] omitting cholesterol, and added from appropriate dilutions in 25 mM triethanolamine-HCl (pH 7.0). Na^+ was removed from PtdSer (diNa-salt) by passage of a solution in glacial acetic acid over Dowex 50 W-X4 (H^+-form), followed by rotatory evaporation of the eluent, drying under vacuum over NaOH and redissolution of the residue in chloroform. The concentration of the phospholipids was assessed by determination of phospholipid phosphorus, using the method of Fiske and SubbaRow [16] in the modification of Broekhuyse [17], applying $H_2SO_4/HClO_4$ (5:1, v/v) as destructing agent.

2.6. Commercial sources

Sepharose 4B Protein A was from Pharmacia, Uppsala, Sweden; Bio-Rad Protein Reagent from Bio-Rad, München, Germany; gelatine and triethanolamine (free base) from Merck, Darmstadt, Germany; Tween (polyoxyethylene-sorbitan monolaurate), *o*-phenylenediamine, *p*-NPP (diNa-salt) and bovine serum albumin (Fraction V) from Sigma, St. Louis, MO, USA; ATP (diNa-salt) from Boehringer Mannheim, Mannheim, Germany; [γ-^{32}P]ATP (code PB 168: ~ 3 Ci/mmol) from Amersham Int., Little Chalfont, Bucks, UK; $^{86}Rb^+$ (0.23–1.21 Ci/mmol) from Du Pont NEN, Bad Homburg, Germany; cellulose nitrate filters from Schleicher & Schuell, Dassel, Germany; rabbit anti-mouse peroxidase from Dako A/S, Copenhagen, Denmark; Malachite Green from Aldrich, Bornem, Belgium; FITC from Baltimore Biological Lab., Baltimore, USA; eosine (yellow shade) from Gurr, BDH, Poole, England; Dowex 50 W-X4 (20–50 mesh) and 50 W-X8 (50–100 mesh) from Fluka AG, Buchs, Switzerland; guanidine-HCl and choline-Cl from Janssen Chimica, Geel, Belgium, and phospholipids (phosphatidylserine from bovine brain, phosphatidylcholine from egg) from Avanti Polar Lipids, Alabaster, AL, USA. All further chemicals were of analytical grade and predominantly derived from Merck, Darmstadt, Germany.

3. Results

3.1. Antigenicity of Na^+/K^+-ATPase

Purified Na^+/K^+-ATPase contains 212 mol phospholipid per mol αß-protomer of 147 kDa of which 13.1% or 28 mol is PtdSer [18]. So one may expect a positive reaction between a PtdSer monoclonal antibody and Na^+/K^+-ATPase, which appears to be the case (Fig. 1). The virtually guanidine-free dialysed preparation displayed a 2-fold lower antibody titer ($K_{0.5}$ = 64 ng/well) as compared to the guanidine-HCl

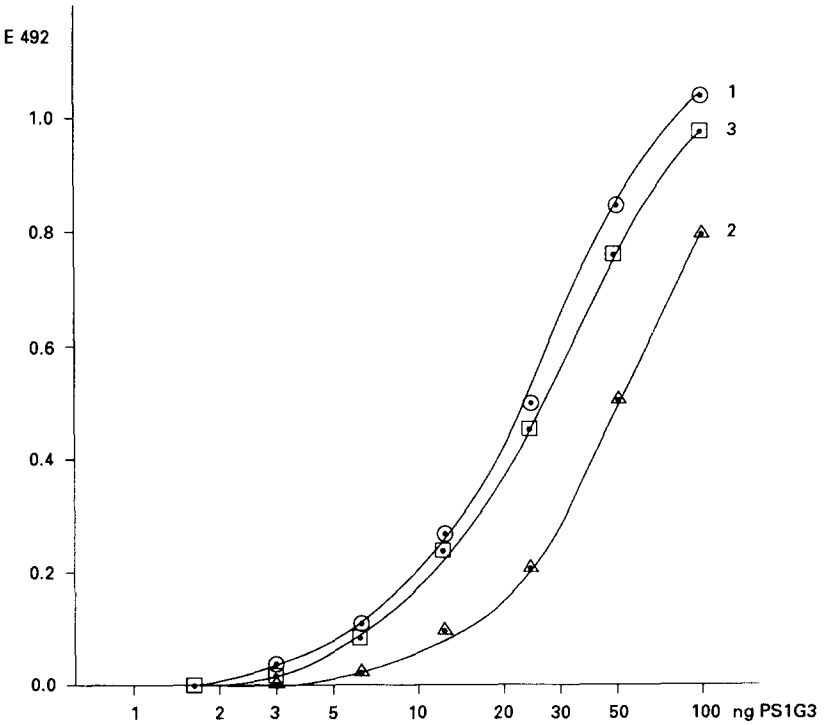

Fig. 1. ELISA of mAb (PS1G3) with Na^+/K^+-ATPase (0.5 μg per well). Shown is the conjugated rabbit anti-mouse peroxidase staining as a function of the antibody titer. The theoretical maximal level was determined via Scatchard analysis and the half-maximally saturating amounts via Hill plots. The three different preparations were: (1) PS1G3 stock in 4 M guanidine-HCl; (2) dialysed PS1G3 in triethanolamine-buffered 0.3 M choline-Cl; (3) dialysed, but aggregated PS1G3, redissolved in 4 M guanidine-HCl. For all three preparations the maximal staining level E_{492} = 1.2 was the same but the half-maximally saturating amounts were (1) 30 ng, (2) 64 ng and (3) 36 ng, respectively.

dispersed PS1G3 ($K_{0.5}$ = 30–36 ng/well), which may be explained by the chaotropic action (disaggregating effect) of guanidine-HCl. In a later batch the dialysed mAb had not lost antigenicity ($K_{0.5}$ = 32.5 ng/well). Guanidine-HCl is inhibitory to Na^+/K^+-ATPase, however [19], so we had to remove it from the mAb and be satisfied with an occasionally lower titer than in the presence of the hydrogen-bonding disruptive agent.

Total mAb binding, as determined by extrapolation of Scatchard plots, was the same for each preparation and corresponded to E_{492} = 1.2 in Fig. 1. Since the ratio of PtdSer/147 kDa protomer is 28:1 as described above, saturation that occurred at 0.9–1.7 mol antibody/mol of ATPase is equivalent to 6–12% of the total PtdSer assuming a ratio of 2 mol PtdSer per mol antibody.

3.2. Inhibition of hydrolytic activities by the monoclonal PtdSer-antibodies

Inhibition of Na^+/K^+-ATPase by monoclonal anti-PtdSer only occurred to any appreciable extent (40–50%) by the IgG (PS1G3, Fig. 2) at an estimated I_{50} value of 22.5 μg/ml (i.e., 0.15 μM). Inhibition by the PtdSer-specific IgM (PS4A7) in the same protein range (0–50 μg/ml) was minimal (about 10%), possibly due to steric hindrance of the appreciably bulkier protein (molecular mass 900 kDa vs. 150 kDa for the IgG). Inhibition of the *p*-NPPase activity by PS1G3 was stronger (about 70%), probably due to an increased K^+/substrate antagonism (Fig. 4A) and of higher affinity (I_{50} ~ 5 μg/ml = 0.03 μM). Obviously, strongest (94%) but of lower affinity (I_{50} = 24 μM) is the inhibition by ouabain of overall ATP hydrolysis, shown for comparison (Fig. 2).

Maximal inhibition by PS1G3 occurred at a level of 0.2 μM, corresponding to antibody binding to only 31% of the available PtdSer (binding stoichiometry 1:2). This amount is 2.6-fold higher than the maximal binding observed in ELISA, possibly due to the lower accessibility of the enzyme to antibody in the latter condition.

Inhibition was dependent on enzyme concentration, as well as on ionic strength. Thus, it seems that the dependence of inhibition on enzyme concentration is due, to a large extent, to the antibody/antigen ratio and this is apparent by the 50% reduction in inhibition when the ratio is decreased by doubling the enzyme concentration. Fig. 3 shows the effect of ionic strength on the inhibition of Na^+/K^+-ATPase activity by PS1G3. Despite a 3-fold increase in the mAb concentration (from 31 to 93 μg/ml) the inhibition reduced to nil, mainly due to a reduction of the control activity by

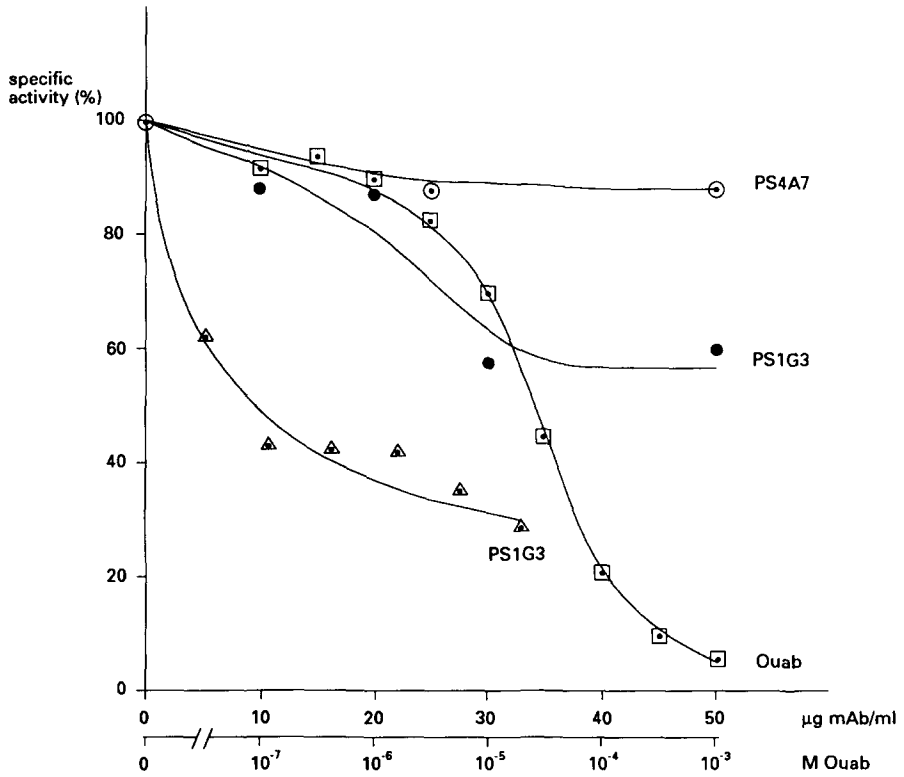

Fig. 2. Monoclonal PtdSer-antibody inhibition of overall Na^+/K^+-ATPase and *p*-NPPase activity. Meaning of symbols: (○), (●), residual ATPase activity; (Δ), residual *p*-NPPase activity; maximal inhibition (73%) was estimated by extrapolation of a plot of the percent inhibition/[I] vs. percent inhibition. For comparison, inhibition of the ATPase activity by ouabain (□) is shown.

choline-Cl above 60 mM, confirming earlier data of Robinson and Pratap [20]. In order to be able to demonstrate antibody inhibition it is therefore necessary to have the mAb in an as concentrated solution as possible (1 mg/ml thus far), and to keep the enzyme during the assay at the lowest practical level. We attained this goal by keeping the enzyme at about 10 μg/ml and choline-Cl at a concentration of $\leq$ 60 mM.

Inhibition was not affected by pH between 6.5 and 8.0, although Na^+/K^+-ATPase activity at pH 8.0 was 55% higher than at pH 6.5 (not shown). Extending the preincubation time of enzyme and PS1G3 from 0.5 to 4 h had neither any effect on the inhibition, but strongly reduced the activity of the control (e.g., by 65%). Hence, a 30-min preincubation period at room temperature was taken as sufficient for demonstrating maximal inhibition.

3.3. Effect of PS1G3 on $K_{0.5}$ and V_{max} values for ATPase and p-NPPase

Since the reduction in hydrolytic rate by the mAb feasibly could be due to increased $K_{0.5}$ values of substrate and activating ligands, we determined the half-maximally activating Na^+, K^+ and MgATP concentrations in the presence and absence of PS1G3. A concentration of the mAb (30–40 μg/ml), causing maximal inhibition (Fig. 2), was taken. It turned out that the kinetic affinity of the ATPase for its substrate and activating ligands was not affected, while interaction between the alkali cation activating sites was (Table 1). Actually, the Hill coefficient (n_H) for both Na^+ and K^+ declined by a full unit, i.e., for Na^+ from 3.0 to 1.6 and for K^+ from 1.6 to 0.8.

Inhibition of the *p*-NPPase activity by PS1G3, on the other hand, was accompanied by an increase in the $K_{0.5}$ value for K^+, which may become as high as 10-fold (Table 1). Here the Hill coefficient is hardly affected and the effect on n_H, if any, may be due to ligand exclusion [21]. At low concentrations of PS1G3 (about 10 μg/ml, Fig. 4A) the effect was merely on the $K_{0.5}$ value for K^+ (0.6 $\rightarrow$ 1.4 mM), whereas at higher concentrations (about 30 μg/ml, Table 1) an additional decrease of the V_{max} became apparent. In addition, the antibody caused an increased K^+/substrate antagonism (back-bending of the plot in Fig. 4A). A similar curvature can also be seen in the overall ATPase reaction, but then without PS1G3 (Fig. 4B), which has been attributed to an ATP/K^+ antagonism [22]. An alike antagonism has been reported for *p*-NPP and K^+ [23,24] with a K_i value for K^+ of 5 mM [24]. It appears to play no role in the overall Na^+/K^+-ATPase activity with PS1G3 present (Fig. 4B), nor in the *p*-NPPase activity without PS1G3 (Fig. 4A). This indicates

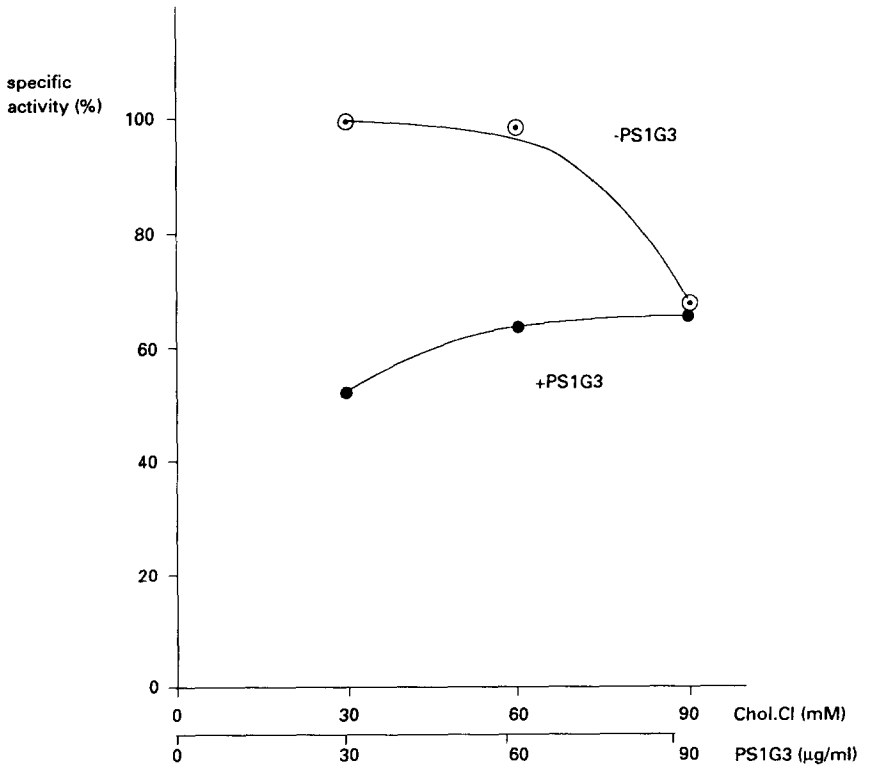

Fig. 3. Specific Na^+/K^+-ATPase activity as function of ionic strength. Presented is specific activity in the absence and presence of PS1G3 (31–93 μg/ml). Ionic strength is provided by choline chloride in concentrations as indicated. The 100% specific activity is equal to 55 μmol(mg protein)$^{-1}$ h^{-1}.

Table 1
Effects of PS1G3 on the kinetic parameters of Na^+/K^+-ATPase

	Na^+/K^+-ATPase activity	
	−PS1G3	+PS1G3
$[Na^+]_{0.5}$ (mM)	1.8 ±0.15	2.4 ±0.4
n_H Na^+	3.0 ±0.4	1.6 ±0.3
V_{max} ratio (+/−PS1G3)		0.61 ±0.01
$[K^+]_{0.5}$ (mM)	0.35 ±0.05	0.35 ±0.05
n_H K^+	1.6 ±0.1	0.8 ±0.1
V_{max} ratio (+/−PS1G3)		0.50 ±0.005
$[MgATP]_{0.5}$ (mM)	0.085 ±0.015	0.105 ±0.005
n_H MgATP	1.5 ±0.3	1.5 ±0.2
V_{max} ratio (+/−PS1G3		0.65 ±0.12
	p-NPPase activity	
	−PS1G3	+PS1G3
$[K^+]_{0.5}$ (mM)	0.63	6.4
n_H K^+	0.9	1.4
V_{max} ratio (+/−PS1G3)		0.43

Kinetic parameters were determined via Eady-Scatchard and Hill plots. PS1G3 was present in maximally inhibitory concentrations (31–40 μg/ml). Data ±S.E.M. are averages of duplicate determinations.

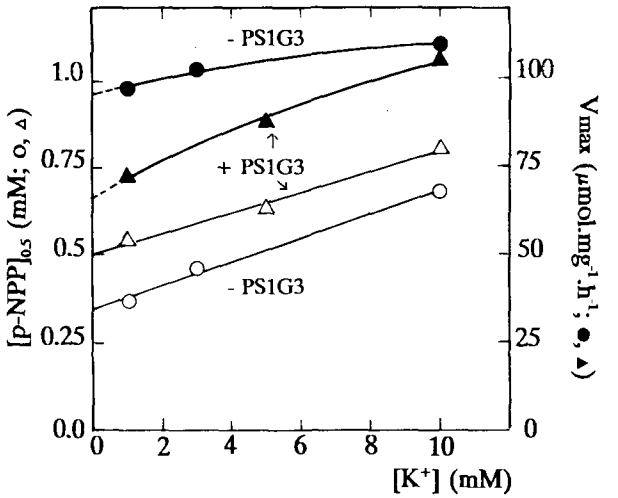

Fig. 5. Effects of K^+ and the antibody PS1G3 on kinetic parameters of the p-nitrophenyl phosphatase reaction. PS1G3 was 17.4 μg/ml. Shown are the effects on the half-maximally activating *p*-NPP concentration (○, Δ) and the extrapolated V_{max} values (●, ▲). Not shown are the effects on the Hill coefficients, which range at the indicated $[K^+]$ between 1.0 and 1.24 (−PS1G3) and between 1.84 and 2.07 (+PS1G3). The $[p\text{-NPP}]_{0.5}$ values +and −PS1G3 run almost parallel with direction angles of 0.029 and 0.034, respectively.

that PS1G3 increased the $K_{0.5}$ for *p*-NPP as well. This has been verified in the experiments of Fig. 5, which demonstrate (open symbols) that PS1G3 increases the $K_{0.5}$ for *p*-NPP and inhibits the *p*-NPPase activity competitively towards $[K^+]$ (closed symbols). The extrapolated values for V_{max} (dashed lines) indicate that very low $[K^+]$ would suffice if substrate/K^+ antagonism were absent. What is not demonstrated in this figure is the increase in apparent cooperativity between substrate activation sites upon addition of antibody (legend of Fig. 5).

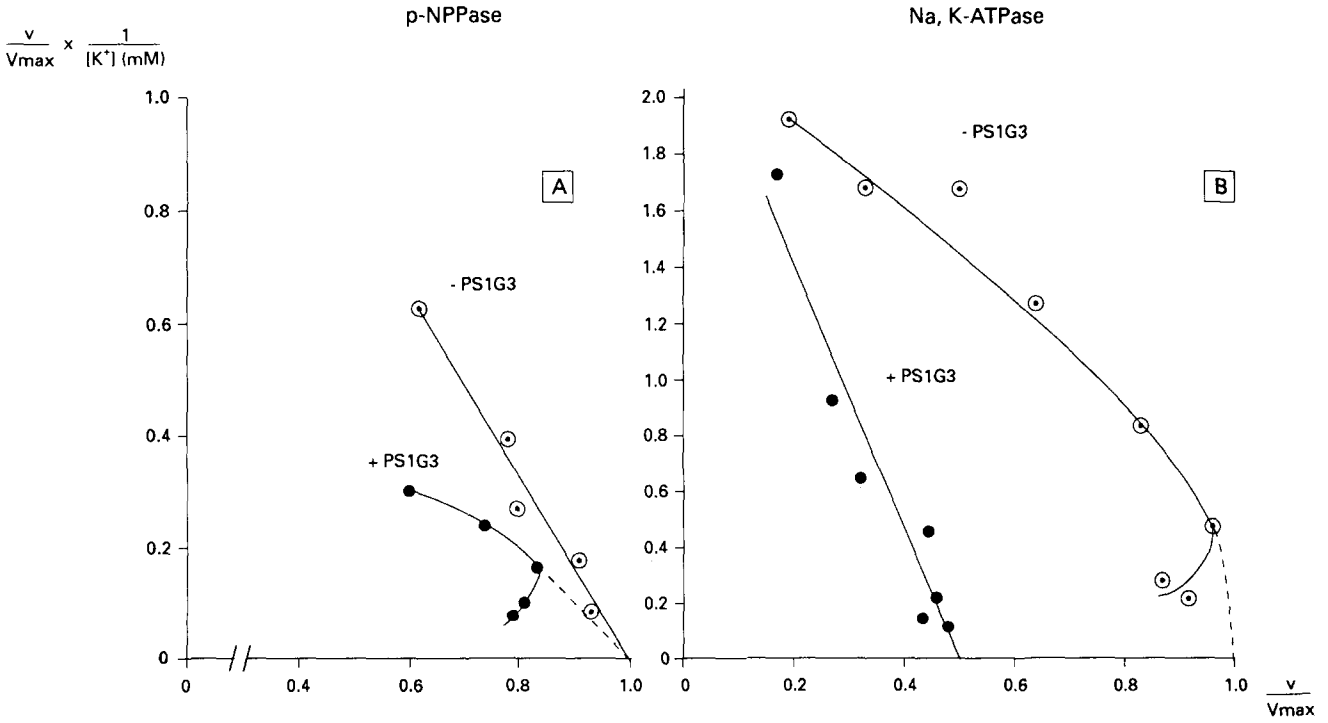

Fig. 4. Eady-Scatchard plots of *p*-NPPase (A) and Na^+/K^+-ATPase activity (B) in dependence on the K^+ concentration with (●) and without (○) PS1G3. PS1G3 is 11 μg/ml in A and 31 μg/ml in B.

3.4. Effects of PS1G3 on partial reactions

The $E_1 + K^+ \rightleftharpoons E_2K$ conformational transition and Rb^+-occlusion

Since the $K_{0.5}$ values for substrate and activator (K^+) in the *p*-NPPase reaction significantly increased in the presence of PS1G3, the idea came up that other K^+ (or K^+-analogue, like Rb^+) dependent reactions, supposedly involved in the phosphatase reaction, would be inhibited.

One such a reaction is the $E_1 + K^+ \rightleftharpoons E_2K$ conformational transition, which can be traced by the K^+-induced fluorescence quench of the FITC- or eosine-labeled enzyme. If PS1G3 would interfere with (slow down) this transition, it might enhance the $[K^+]_{0.5}$ value for the transition. It did not (Table 2). The only effect that can be seen in the case of the non-covalently eosine-labeled enzyme is a reduction in ΔF_{max} (not observed in the FITC-enzyme). It was not so much due to a reduction in the initial eosine fluorescence but rather to an increased final eosine fluorescence level.

On the other hand, the Rb^+ occlusion was strongly inhibited by PS1G3, via a reduction of Rb^+ affinity at the lower mAb concentrations and in addition by a reduction in binding capacity at the higher mAb concentrations (Fig. 6). The inhibition was fairly abrupt, with the onset taking place at the higher antibody to enzyme ratios as occuring in the overall Na^+/K^+-ATPase activity (Fig. 2). The quite lower I_{50} for inhibition of the *p*-NPPase could mean that a different set of PtdSer molecules with a higher affinity for the mAb is involved in the phosphatase reaction, e.g., a high-affinity set in substrate binding (Fig. 5) and a set of lower affinity in cation occlusion (Fig. 6).

Phosphorylation and dephosphorylation

Although we obtained for the counter-clockwise running reaction steps of the *p*-NPPase a pretty good idea about the localisation of PS1G3 inhibition in the cation occlusion step, this did not hold as yet for the Na^+/K^+-ATPase reaction steps, running in forward direction. To the easier to determine reaction steps belong the ($Na^+ + Mg^{2+} + ATP$)-dependent phosphorylation and subsequent K^+-dependent dephosphorylation reactions. Only the former of these appears to be inhibited, i.e., in a non-competitive fashion and to the same extent (about 46%, Table 3) as the overall Na^+/K^+-ATPase (Table 1).

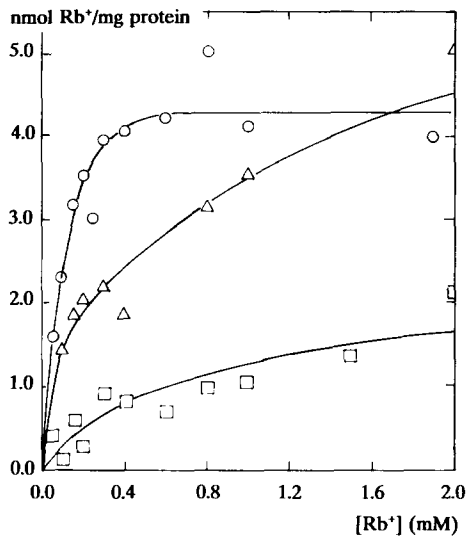

Fig 6. Inhibition of Rb^+-occlusion by PS1G3. Concentrations (μg/ml) of PS1G3 were: 34 (○), 40 (Δ) and 48 (□) leading to extrapolated values for maximal Rb^+ binding (nmol/mg protein) of 4.3, 5.0 and 2.0, respectively. Corresponding concentrations of Rb^+ giving half-maximal saturation are 0.08, 0.44 and 0.61 mM, respectively. The plot of the control, lacking antibody, is omitted for clarity because of near coincidence with the curve for 34 μg/ml PS1G3 (○). Control values were: maximal Rb^+-binding 5 nmol/mg protein with half-maximal binding at 0.07 mM Rb^+.

Table 2
Lack of effect of PS1G3 on the $E_1 + K^+ \rightleftharpoons E_2K$ equilibrium, using the covalently FITC-labeled Na^+/K^+-ATPase or the non-covalently eosine-labeled Na^+/K^+-ATPase and probing the K^+-dependent fluorimetric quench

FITC enzyme				Eosine enzyme		
PS1G3 (μg/ml)	$[K^+]_{0.5}$ (mM)	n_H	ΔF_{max} (%)	$[K^+]_{0.5}$ (mM)	n_H	ΔF_{max} (%)
0	0.06	1.02	42	0.05	1.00	20.0
8.7	0.04	0.94	43	0.07	1.13	20.2
14.5	0.07	1.11	43	0.06	1.09	13.4
29	0.08	0.99	40	0.08	1.14	12.2
43.5	0.03	0.82	40	0.07	1.04	11.8

Meaning of abbreviations: n_H, slope of the Hill plot or Hill coefficient; $[K^+]_{0.5}$, K^+-concentration yielding the half-maximal quench; ΔF_{max} (%), the maximal K^+-dependent fluorescence quench as percent of total fluorescence.

Table 3
Effects of PS1G3 on the Na^+-activated, ATP-dependent steady-state phosphorylation and the K^+-induced 3-s dephosphorylation

	Phosphorylation reaction	
	−PS1G3	+PS1G3
$[Na^+]_{0.5}$ (mM)	0.46 ± 0.18	0.28
n_H Na^+	1.2 ± 0.16	1.4 ± 0.2
EP_{max} ratio (+/−PS1G3)		0.54 ± 0.09
	Dephosphorylation reaction	
	−PS1G3	+PS1G3
$[K^+]_{0.5}$ (μM)	5.3 ± 2	7.0 ± 3.6
n_H K^+	1.8 ± 0.6	1.4 ± 0.5
$-EP_{max}$ ratio (+/−PS1G3)		1.0

PS1G3 is 30–40 μg/ml. Meaning of abbreviations: n_H, Hill coefficient; $[Na^+]_{0.5}$ and $[K^+]_{0.5}$, concentration of Na^+ or K^+ giving a half-maximal effect; EP max, the maximal steady-state phosphorylation level, -EP max, the maximal (100%) 3-s reduction in phosphorylation level upon addition of K^+.

3.5. PtdSer / PS1G3-antagonism in the inhibition reaction

Since PS1G3 is PtdSer-specific, its inhibition on the hydrolytic activity of Na^+/K^+-ATPase should be released by addition of PtdSer and not by addition of other phospholipids. We confined ourselves to the comparison of phosphatidylserine and phosphatidylcholine using *p*-NPPase as a measure of activity, because of its stronger inhibition by antibody than Na^+/K^+-ATPase. Fig. 7 shows that PtdSer can relieve, but not totally abolish, the inhibition exerted by PS1G3. It is clear that phosphatidylcholine cannot. Residual inhibition, occurring with excess PtdSer, may mean that the PtdSer-mAb complex still is inhibitory, but to a lesser extent than the mAb itself. The concentration of liposomal PtdSer which has a maximal effect (60 μM, Fig. 7) is several-fold higher than the mAb present (about 0.2 μM). This can only mean that PS1G3 has a higher affinity for endogenous PtdSer than for the exogenously added phospholipid, which may be due to the degree of organisation of PtdSer within the membrane.

During these assays we became aware of the fact that added PtdSer stimulated the *p*-NPPase activity by about 50%, a stimulation that was not shared by phosphatidylcholine or by EDTA in the same concentration range (0–0.2 mM). This means that the stimulatory effect of PtdSer in all probability is not due to complexation of an inhibitory heavy metal cation [25]. Similar data on stimulation by PtdSer have been found by Lehotsky et al. for porcine red cell membrane (Ca^{2+} + Mg^{2+})-ATPase and interpreted by them as a poising of the balance between enzyme conformations [26].

4. Discussion

4.1. The involvement of PtdSer in enzyme activity

The data on the inhibition of Na^+/K^+-ATPase by a monoclonal antibody to PtdSer once more, and this time even more specifically, hint at the involvement of PtdSer in the reaction mechanism of the title enzyme. Previous data [1] on the inhibitory action of ethylene diamine (Eda) could be traced to an identical site of action, i.e., PtdSer in the plasma membrane [2]. Some of the features of Eda and of PS1G3 are comparable, others are not:

(1) Each ligand inhibits Na^+/K^+-ATPase and *p*-NPPase activity but in the case of PS1G3 the inhibition is partial.

(2) Each ligand inhibits the Na^+-activated phosphorylation, but not the K^+-activated dephosphorylation. In the case of Eda the inhibition is competitive towards Na^+; PS1G3 inhibits Na^+-activation non-competitively.

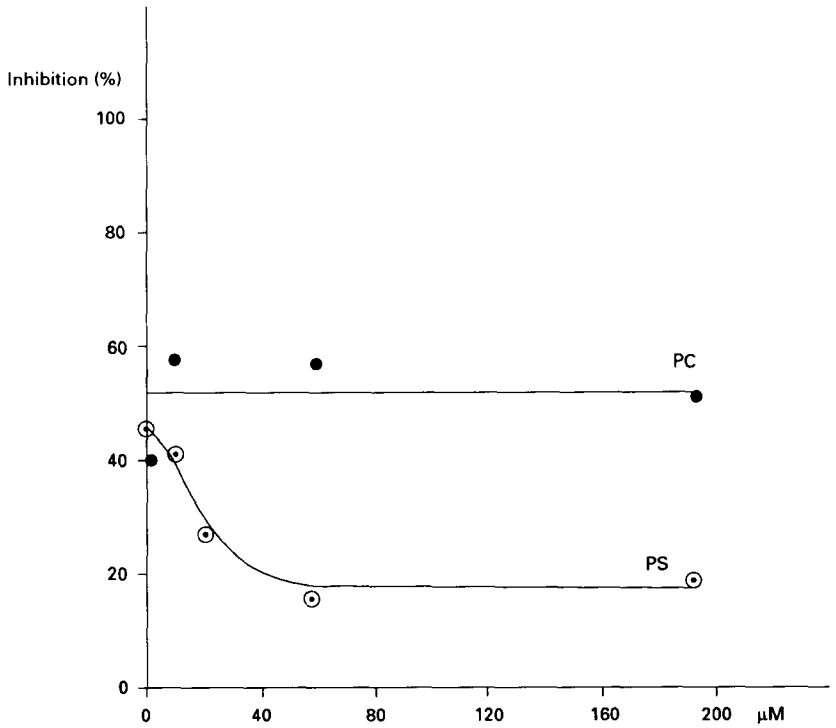

Fig. 7. PtdSer/PS1G3 antagonism in the inhibition of *p*-NPPase. Concentration of PS1G3 was 33 μg/ml and the concentration of phospholipid as indicated on the abscissa. The effect of phosphatidylcholine (PC) is shown for comparison. Specific activity without PS1G3 and PtdSer (= PS) or PC was 102–121 μmol(mg protein)$^{-1}$ h^{-1}. In the presence of increasing PtdSer the specific activity rose to a maximum of 194 μmol(mg protein)$^{-1}$ h^{-1}; the specific activity in the presence of PC (−PS1G3) averaged at 113 ±2.5 μmol(mg protein)$^{-1}$ h^{-1}.

(3) Each ligand inhibits the K^+-activation of the *p*-NPPase competitively and the related Rb^+-occlusion (Eda may even be occluded itself [27], PS1G3 obviously not). Eda poises the $E_1 + K^+ \rightleftharpoons E_2K$ to the left [28], but PS1G3 does not appear to influence this transition.

We do not think that a tightly bound antibody can be easily removed from its epitope by competition with a small cation at the same site. Antibody purification via an antigen column has taught us that it takes molar concentrations of the cation to do this. Therefore, we think the so-called competitive inhibition toward K^+ in the phosphatase reaction (and Rb^+ in the occlusion) is conformational and exerted from a site different from, but may be giving access to, the occlusion pocket. The insensitivity of the $E_1 + K^+ \rightleftharpoons E_2K$ transition to PS1G3, in contrast to Rb^+ occlusion, suggests that binding of K^+ and its concomitant conformational change to E_2K is outside the membrane from where the ions are transferred to the occlusion site. This is in agreement with the concept of Esmann and Skou [29], based on eosine-fluorescence kinetics, in which this transition precedes the occlusion.

The insensitivity of the K^+-dependent dephosphorylation to antibody (and Eda) may be connected with the asymmetric (cytosolic) localisation of PtdSer [30,31] and thus also of PS1G3. K^+ activates the dephosphorylation step at the extracellular side [32] whereas Na^+ activates phosphorylation at the intracellular side [33]. In agreement with the latter data Na^+-activation is inhibited, but non-competitively. Since arguments in favour of an identical localisation of Rb^+ and Na^+ occlusion have been adduced [34] one would expect a competitive inhibition of Na^+-activation as well. The absence of such competition indicates that Na^+ activates the phosphorylation reaction outside the occlusion cavity. In effect $[Na^+]_{0.5}$ of the steady-state phosphorylation may be governed by the $E_2 \rightleftharpoons E_1$ transition [35], which appears not to be influenced by the mAb.

Another apparent difference in response of the forward ATP-hydrolytic cycle and the backward running phosphatase activity to PS1G3 is a difference in cooperativity index or Hill coefficient for activating ligands. The mAb reduces interaction between Na^+ and K^+ sites in the forward running mode of the pump, while increasing interaction for *p*-NPP in the reverse hydrolytic mode. Usually, the backward running mode is accompanied with subunit association and the forward running mode with dissociation [4]. PS1G3 appears to strengthen this behaviour, although the decrease in cooperativity in the forward running mode also can be explained by a decrease in interaction between activation sites on one subunit. Since there exists probably one *p*-NPP binding site per α-subunit, PS1G3 might act by clamping two subunits together [21]. Stimulation of the *p*-NPPase by addition of PtdSer in the absence and presence of PS1G3 is another example of promoted subunit interaction, but being reversible [4].

4.2. The type of inhibition

A remarkable feature of PS1G3 inhibition is its partial inhibitory nature and the different apparent titer of antigenicity in free solution and on the solid matrix of the titer plate. The latter difference may be explained in part by the asymmetric localisation of PtdSer in the membrane, suggesting that half of the membranes will be directed with their PtdSer attached to the plate and thus inaccessible to PS1G3.

Partial occupancy of the affinity ligand in free solution, as emerging from inhibition studies may be caused by: (1) selection of certain classes of PtdSer by the enzyme for activity, (2) selection of certain classes of PtdSer by the antibody, (3) steric hindrance to further occupancy by the bulky mAb bound initially. It is evident that options (1) and (2) should coincide for PS1G3 to inhibit.

Examples of (1) and (2) have been verified experimentally. For instance, Na^+/K^+-ATPase only effectively interacts with phospholipids with melting points below that of stearoyl-, oleoyl-PtdChol (8.5°C, Pistorius and Schuurmans Stekhoven, unpublished results), which may constitute only 46 mol% [36] of the PtdSer in the membrane. Relevant to argument (2) is the finding that mAbs to PtdChol, raised in a similar way as PS1G3, demonstrate definitive preference to certain members of the family with unsaturated acyl chains [37] which are just the ones showing a tendency to interact with the enzyme (see also [38]). Whether PS1G3 shows any preference to a certain kind of PtdSer is not known at this stage of our research.

Partial inhibition as exerted by PS1G3 should be related to argument (3) or be genuine, i.e., the inhibitor/substrate-enzyme (EIS) complex is suboptimally active. Steric hindrance as an explanation for partial inhibition is exemplified by the low (10%) maximal inhibition on ATPase activity exerted by the 900-kDa IgM PS4A7, compared to 40–50% inhibition by the less bulky IgG (150 kDa molecular mass) PS1G3. On the other hand, if the EIS complex is suboptimally active, this would confirm that PtdSer is not indispensable, but favourable, for enzyme activity [18].

Conclusions

PS1G3 inhibits the Na^+/K^+-ATPase (clockwise reaction cycle) and its constituting Na^+-activated phosphorylation, as well as the *p*-NPPase (anticlockwise partial reaction) and its constituting alkali cation occlusion by complexation with PtdSer. The phospholipid may be part of the cation access channel in the membrane.

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