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Effect of spermine on the activity of synaptosomal plasma membrane Ca^{2+}-ATPase reconstituted in neutral or acidic phospholipids

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Abstract

The activity of purified plasma membrane Ca^{2+}-ATPase (PMCA) from pig brain was inhibited by spermine (a naturally occurring and highly abundant polycation in brain). The level of inhibition was dependent on the phospholipid used for reconstitution as well as on the intact or truncated state of the enzyme. An IC_{50} value of 12.5 mM spermine was obtained for both, the intact protein plus calmodulin and the trypsin-digested protein, reconstituted in phosphatidylcholine (PC). In the absence of calmodulin the intact Ca^{2+}-ATPase gave an IC_{50} of 27 mM. This form was more sensitive to spermine inhibition when it was reconstituted with phosphatidylserine (PS), showing an IC_{50} value of 2.5 mM spermine. However, the truncated form was less responsive to spermine inhibition, having an IC_{50} value of 12.5 mM. Spermine has no effect on the affinity of the PMCA for Ca^{2+} or ATP, but its effect on the protein is pH-dependent. It is suggested that spermine could bind to negatively charged residues on the ATPase with different accessibility, depending on the structural rearrangement of the protein. Further, when the protein is reconstituted in PS, spermine also binds to the lipid.

Keywords: Plasma membrane; Ca^{2+}-ATPase; Spermine

1. Introduction

The polycation spermine is present in many mammalian cells, where it usually is in close association with DNA. Spermine, as well as spermidine and its precursor putrescine, is an endogenous substance in nerve cells having a variety of effects within the CNS. These include effects in brain development [1], nerve growth and regeneration [2], brain tumours [3], regulation of neuronal ion channels [4], and modulation of several signalling proteins [5] and of neurotransmitter receptors in brain [6]. The mechanism of action of spermine at the molecular level is still controversial. Owing to its net positive charge, spermine shows some properties similar to those of inorganic cations such as Mg^{2+} or Ca^{2+}. However, the presence of positive charges, distributed at fixed lengths along a conformationally flexible carbon chain, confers special properties on the molecule; spermine does not have point-localized charges and is able to bridge between amino acid residues allowing specific interactions and functions.

The inhibitory effects of spermine on different Ca^{2+}-pumps have been studied, but the interactions responsible for its effects are a matter of controversy. Thus, Missiaen et al. [7] observed inhibition by spermine or spermidine of the plasma membrane Ca^{2+}-ATPase (PMCA) from pig stomach smooth muscle. The effect was attributed to a direct interaction of the positively charged polyamines with negatively charged polyphosphoinositides. By contrast, Hughes et al. [8] reported inhibition of the Ca^{2+}-ATPase from sarcoplasmic reticulum by direct interaction of spermine with the enzyme. We report here some novel features of spermine as an inhibitor of the purified synaptosomal PMCA. Furthermore, we have compared the inhibitory effect of the polyamine on both intact and trypsinized enzyme in order to identify protein binding domains important in the effects of polyamine. In the present work we show that spermine interacts with both the PMCA and the acidic phospholipid, phosphatidylserine (PS). The concentrations of spermine needed for inhibition are higher than the physiological

Abbreviations: PMCA, plasma membrane Ca^{2+}-ATPase; PC, phosphatidylcholine; PS, phosphatidylserine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid

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concentration of free spermine in the cell. Besides, our model system is just the purified protein isolated from its natural environment and reconstituted in one specific type of lipid. Thus, this is not a naturally physiological system. However, the lack of effective PMCA inhibitors and the fact that spermine inhibits the PMCA activity a substantial percentage made this polycation an interesting target for mechanistic studies on the enzyme.

2. Materials and methods

Spermine tetrahydrochloride was purchased from Fluka, the phospholipids PC type XI-E from egg yolk and PS from bovine brain, calmodulin from bovine brain and TPCK-trypsin were obtained from Sigma.

2.1. Purification of pig brain PMCA

The PMCA protein was purified from pig brain as described by Salvador and Mata [9], using a calmodulin affinity column in the last step. The Ca^{2+}-ATPase was eluted from the column in 20 mM Hepes/KOH, pH 7.4, 130 mM KCl, 1 mM $MgCl_2$, 15% glycerol, 0.06% Triton X-100, and 2 mM EDTA and stored at −80 °C until use.

2.2. Ca^{2+}-ATPase activity

The Ca^{2+}-dependent ATPase activity was measured spectrophotometrically at 25 °C by using a coupled enzyme assay in a medium containing 50 mM Hepes/KOH, pH 7.4, 100 mM KCl, 5 mM Na_3N, 2 mM $MgCl_2$, 100 μM $CaCl_2$, enough BAPTA to give 3.16 μM free Ca^{2+}, 0.11 mM NADH, 0.42 mM phosphoenolpyruvate, 10 IU pyruvate kinase and 28 IU lactate dehydrogenase. Previously the chosen lipid, prepared in chloroform/methanol (7:3, v/v), was dried in to a thin film under N_2 atmosphere. The purified Ca^{2+}-ATPase (2.5 or 5 μg), containing 0.06% Triton X-100 in a volume of about 15–30 μl, was mixed with the lipid at a lipid/protein ratio of 5.30:1 (w/w) until clarity, incubated for 2 min at 25 °C and then diluted directly into the assay medium, in 1 ml final volume. A further 2 min incubation was done before starting the reaction by adding 1 mM ATP. The enzymatic activity was also assayed in the presence of 0.32 μg bovine brain calmodulin/ml. The spermine solution used in the activity assays was prepared in Hepes 100 mM, pH 7.4.

2.3. Trypsin digestion

The delipidated Ca^{2+}-ATPase (0.18 mg/ml) was treated with trypsin at a ratio of 10 mg ATPase/mg trypsin. The incubation was carried out on ice for the indicated times. The proteolytic fragments were separated by SDS/polyacrilamide gel electrophoresis, according to Laemmli [10] and stained with Coomassie blue. The Ca^{2+}-ATPase activity of 20 min treated samples was assayed as described above after a preliminary incubation for 4 min at 25 °C of 5 μg purified protein with the phospholipid indicated.

2.4. Protein determination

The protein content was measured by the Bradford method [11]. When the protein was used for gel electrophoresis, interference by Triton X-100 was eliminated before protein content determination, by a preliminary treatment of samples with BioBeads SM-2 (BioRad Laboratories).

3. Results

3.1. Effect of spermine on Ca^{2+}-ATPase activity of intact and trypsin-digested PMCA, in the presence of different phospholipids

The pig brain PMCA was purified by calmodulin affinity chromatography and eluted from the column in a buffer containing 0.06% Triton X-100 and glycerol instead of phospholipids [9]. Although the delipidated enzyme was inactive, it could be fully reactivated by phospholipid incubation, being the reactivation dependent of the phospholipid used for reconstitution [9]. Furthermore, the reconstituted protein could be inhibited by PMCA inhibitors [12], showing a Ca^{2+}/H^+ countertransport similar to other Ca^{2+}-ATPases and a net charge transfer [13]. The enzyme was digested with trypsin (Fig. 1), giving a 81 kDa fragment that remains stable at least after 20 min trypsin incubation. In a first set of experiments, we studied the effect of spermine on Ca^{2+}-ATPase activity. Intact or trypsin-digested PMCA was reconstituted with PC, in the presence or absence of calmodulin (Fig. 2). All the assays were performed at pH 7.4 and 25 °C.

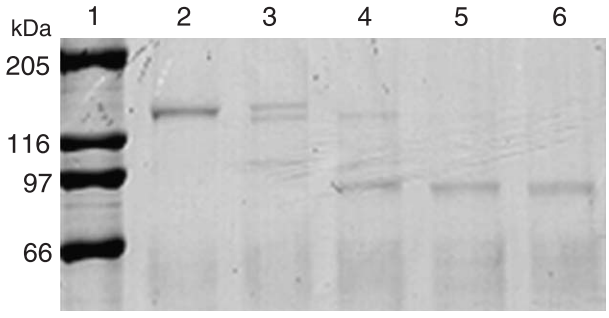


Fig. 1. SDS-electrophoresis of the intact and trypsin digested pig brain PMCA. The electrophoresis was performed according to Laemmli [10] in a 7.5% gel, loading 5 μg of protein per lane. The ATPase was incubated on ice with trypsin at a ATPase/trypsin ratio of 10:1 (w/w) and the reaction was stopped by addition of 2 mM PMSF. Lane 1: relative molecular mass standards. Lane 2: undigested delipidated ATPase. Lanes 3, 4, 5 and 6: proteolytic fragments of the ATPase digested for 1, 5, 10 and 20 min, respectively.

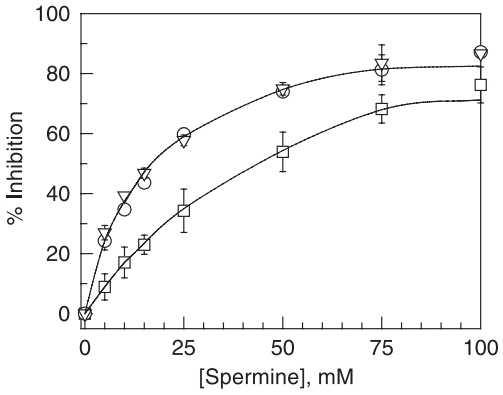


Fig. 2. Effect of spermine on Ca^{2+}-ATPase activity of native and trypsin-digested PMCA in the presence of PC and calmodulin. The enzyme activity was measured as indicated in Section 2 using delipidated protein (5 μg/ml) reconstituted with PC (□), PC plus 0.32 μg/ml calmodulin in the assay medium (○), or trypsin-digested protein in the presence of PC (▽). Activities were measured after subsequently addition of ATP and spermine to the assay medium. Data are means ± S.D. (bars) of four experiments from different preparations.

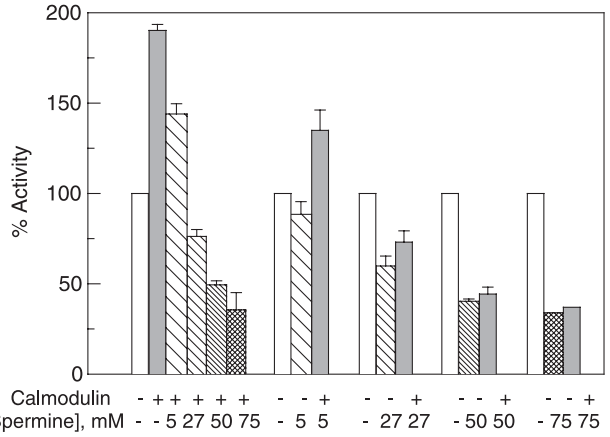


Fig. 3. Competitive effect of spermine and calmodulin on Ca^{2+}-ATPase activity reconstituted in PC. Activities of the delipidated protein (2.5 μg/ml) reconstituted with 26.5 μg/ml of PC were measured after subsequent addition of calmodulin and spermine as indicated. Data are means ± S.D. (bars) of three experiments from different preparations.

When the delipidated enzyme in the intact form was reactivated in the presence of the zwitterionic phospholipid PC, the Ca^{2+}-ATPase activity was 0.89 ± 0.11 μmol mg^{-1} min^{-1}. Inhibition of the activity by 5 mM spermine was about 9%, a maximum inhibition of 73% being reached at 100 mM spermine. The IC_{50} was 27 ± 1 mM. In the presence of calmodulin, the enzyme activity of the intact form increased up to 1.69 ± 0.18 μmol mg^{-1} min^{-1}. Likewise, the presence of calmodulin increased the sensitivity to the spermine inhibition. Thus, 5 mM spermine produced 24% inhibition of the activity, and the IC_{50} value was 12.5 ± 1 mM. The Ca^{2+}-ATPase activity of the trypsin-digested enzyme reconstituted in PC was 1.50 ± 0.13 μmol mg^{1} min^{-1}. The inhibition by 5 mM spermine was 27% and the IC_{50} value was 12.5 ± 1 mM. As can be seen, the effects of spermine on the activities of the intact enzyme in the presence of calmodulin and of the truncated form are very similar, producing a maximum inhibition of 84%. In these experiments addition of spermine was done immediately after starting the reaction by ATP. Similar results were obtained when spermine was incubated for at least 10 min with the protein and the lipid in the assay medium, before triggering the reaction with ATP. A competition experiment between the effects of spermine and calmodulin showed that the percentage activation of ATPase activity by calmodulin decreased with increasing concentration of spermine in the reaction medium (Fig. 3). Spermine concentrations above 50 mM completely abolished activation of the pump by calmodulin. When a given concentration of spermine was added to the protein after its activation by calmodulin, the activity was similar to that obtained when calmodulin was added to the protein subsequently to spermine addition.

Fig. 4 shows the enzymatic activity data when the acidic phospholipid PS was used for reconstitution instead of PC. The activating effect of PS on the intact PMCA was greater than that observed with PC in the absence of calmodulin, and similar to the value found for PC in the presence of calmodulin. Thus, the Ca^{2+}-ATPase activity was 1.75 ± 0.24 μmol mg^{-1} min^{-1}. Spermine inhibited drastically the reconstituted intact ATPase, reaching a maximum 78% inhibition. The IC_{50} was 2.5 ± 0.5 mM. The activity of trypsinized PMCA reconstituted with PS in the absence of spermine was only 0.87 ± 0.12 μmol mg^{-1} min^{-1}. Interestingly, 2.5 mM spermine only reduced this activity to 0.83 ± 0.12 μmol mg^{-1} min^{-1}, the IC_{50} value being 12.5 ± 0.5 mM. The activity of the truncated protein was inhibited up to 53% by spermine. It should be noted that the effect of spermine was measured after 10 min incubation of the polycation with the protein and the lipid in the reaction

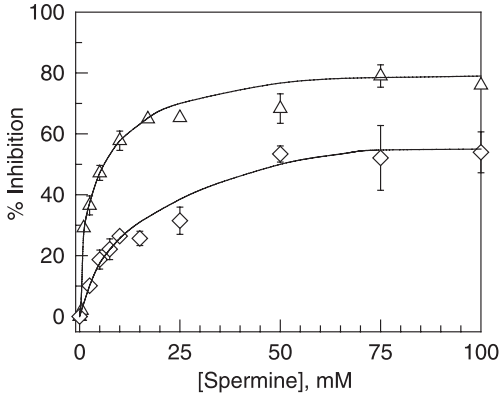


Fig. 4. Effect of spermine on Ca^{2+}-ATPase activity of native and trypsin-digested PMCA in the presence of PS. The ATPase activity was measured using 5 μg/ml of native delipidated protein reconstituted with PS (△) or trypsin-digested protein (◇) as described in Section 2. Spermine was incubated for 10 min with the protein and the lipid in the assay medium, before the reaction was started by ATP addition. Data are means ± S.D. (bars) of four experiments from different preparations.

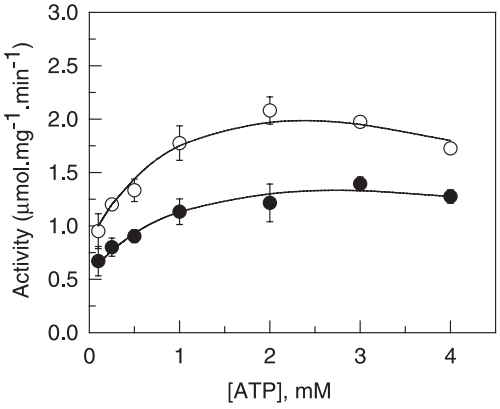


Fig. 5. Effect of spermine on the activity of the purified PMCA as a function of the concentration of ATP. The protein (2.5 μg/ml) was reconstituted with 26.5 μg/ml of PC and calmodulin (0.32 μg/ml) was added into the reaction medium that contained 5 mM $MgCl_2$ instead of the standard concentration. After 2 min incubation, activities were measured by subsequently additions of ATP (at the given concentrations) (○) and 12.5 mM of spermine (●). Data are means ± S.D. (bars) of four experiments from different preparations.

medium; without this incubation the response was not stable. The level of inhibition of the PMCA by spermine was independent of the protein concentration (data not shown).

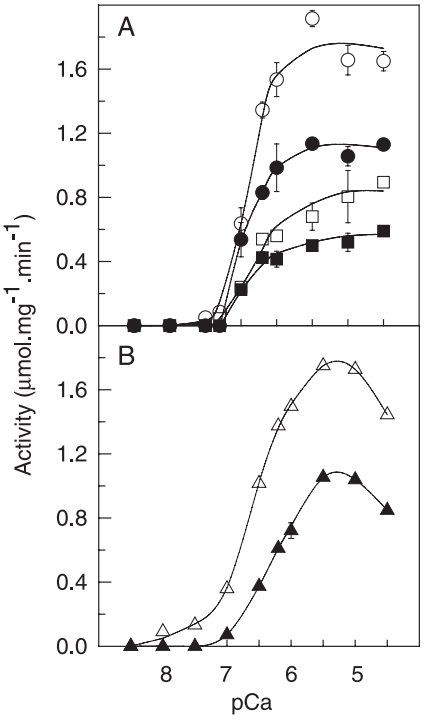


Fig. 6. Effect of spermine on the Ca^{2+} dependence of ATPase activity. The protein (2.5 μg/ml) was reconstituted as described in Section 2, in PC (A), or in PS (B). The Ca^{2+} dependence of the ATPase activity was measured for the protein alone (empty symbols) or in the presence (filled symbols) of spermine at the IC_{50} concentrations of 27, 12.5 or 2.5 mM, for PC (□■), PC plus 0.32 μg/ml of calmodulin (○●) or PS (△▲), respectively. Data are means ± S.D. (bars) of four experiments from different preparations.

3.2. Influence of spermine on the ATP and Ca^{2+} dependencies of ATPase activity

The ATP dependence of Ca^{2+}-ATPase activity in the presence or absence of 12.5 mM spermine, equivalent to the IC_{50}, is shown in Fig. 5. The protein was reconstituted in PC, and calmodulin was added into the reaction medium. It can be seen that the level of inhibition caused by spermine did not change significantly with the concentration of ATP, varying from 42.4 ± 4.6% at 0.1 mM ATP to 35.4 ± 2.0% at 4 mM ATP. Similar results were found in the absence of calmodulin or when PS was used for protein reconstitution (not shown).

Fig. 6 shows the effect of spermine on ATPase activity, measured as a function of Ca^{2+} concentrations, in the presence of PC or PC plus calmodulin (A) or PS (B). The spermine concentration used in each experiment corresponded to the IC_{50} calculated in Figs. 2 and 4. Spermine did not have any large effect on Ca^{2+} affinity, at any concentrations of spermine tested.

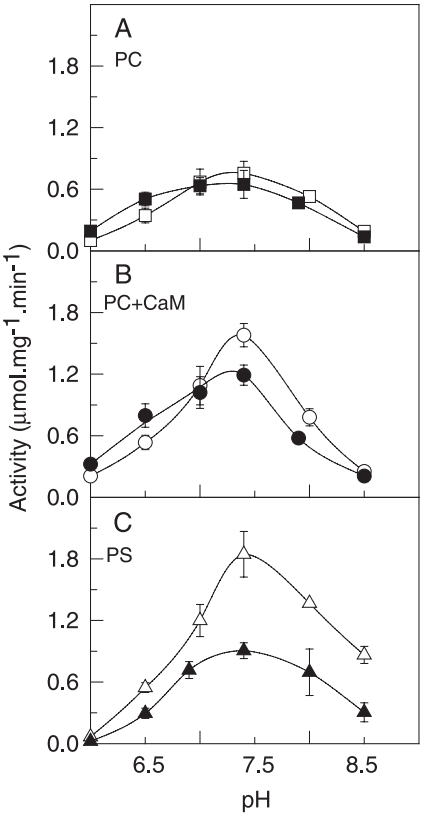


Fig. 7. Effect of pH on the dependence of ATPase activity by spermine in the presence of PC (□■), PC and 0.32 μg/ml of calmodulin (○●) or PS (△▲). The activity of 2.5 μg/ml of protein, reconstituted with the indicated lipid as described in Section 2, was measured in the absence (empty symbols) or presence (filled symbols) of 5 mM spermine. The following buffers were used in the assay media to adjust the pH: 50 mM MES/Tris, pH 6.0 and pH 6.5; 50 mM Hepes/KOH, pH 7.0 and pH 7.4; 50 mM Tris/HCl, pH 8.0; and 500 mM Tris/HCl, pH 8.5. Data are means ± S.D. (bars) of six experiments from different preparations.

3.3. Effect of spermine on the pH dependence of the ATPase

The activity of the ATPase is pH dependent in the absence or presence of calmodulin, in PC or PS (Fig. 7). Although the maximal activity is observed at about pH 7.4 in all systems, the effect of pH on the enzyme reconstituted in PS is much larger than that reconstituted in PC. Thus, the ratio of activity at pH 7.4/pH 6.0 was 25.45 ± 2.80 with PS, while values of 7.33 ± 0.62 and 7.59 ± 0.37 were obtained with PC or with PC and calmodulin, respectively.

The inhibition of the enzyme activity by spermine is also dependent on pH and the phospholipid used for reconstitution. Thus, inhibition by 5 mM spermine in the presence of PC or PC plus calmodulin (Fig. 7A and B) was only observed at pH values higher than 7.0. Interestingly, at pH values lower than 7.0 spermine stimulated the ATPase activity. The ratios of activity at pH 7.4/pH 6.0 were 3.32 ± 0.46 with PC and 3.69 ± 0.13 with PC and calmodulin. The pH effect on enzyme inhibition by 5 mM spermine was of higher magnitude when PS was used as the reconstituting lipid (Fig. 7C). Furthermore, in the presence of PS, ATPase activity was inhibited by spermine over the whole pH range tested, the activity ratio being at pH 7.4/6.0 of 25.88 ± 3.10. Similar profiles were obtained when the experiments were performed at the IC_{50} values of spermine calculated from Figs. 2 and 4 (results not shown).

4. Discussion

The activity of purified pig brain PMCA was inhibited by spermine, a polycation present in brain, and the percentage of inhibition was dependent on the phospholipid used for reconstitution, on whether the protein was present in the native or truncated form, and on the presence of calmodulin.

When the intact ATPase was reconstituted in PC (Fig. 2), the inhibition of ATPase activity by spermine increased in the presence of calmodulin or when the protein was digested by trypsin. Calmodulin binds to a C-terminal region of the protein located about 40 residues downstream of the last transmembrane α-helix [14,15]; the C-terminal region acts as an autoinhibitory domain in the absence of calmodulin. The domain interacts intramolecularly with two large cytosolic loops, involving the phosphorylation site and the ATP binding site [16–18].

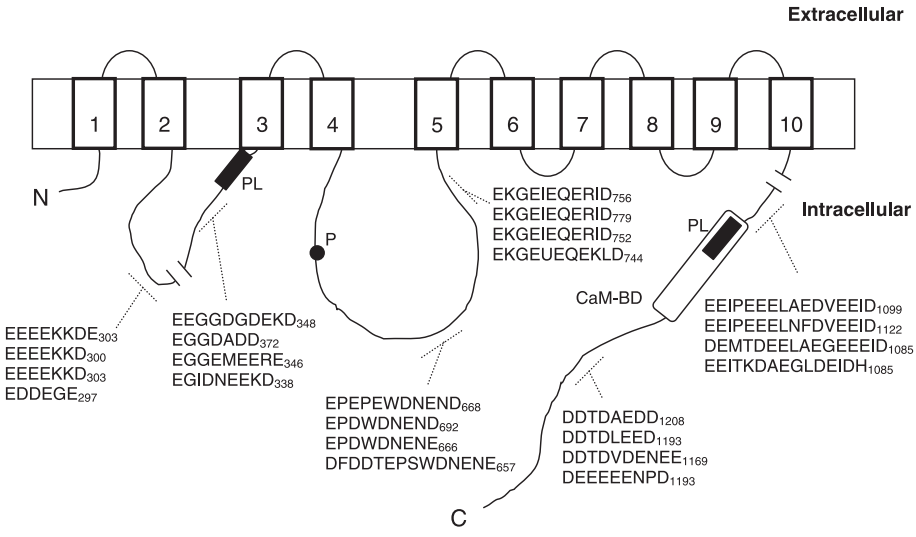
The regulatory mechanism is similar to that found in other Ca^{2+}-calmodulin-dependent enzymes [17]. Thus, alike to a study done with the crystal structure of the Ca^{2+}/calmodulin-dependent protein kinase I in its inhibitory state [19], the calmodulin binding domain interacts (probably through positively charged residues) with multiple sites (likely negative charged residues) in the catalytic core, hindering Ca^{2+} access and ATP binding in the absence of calmodulin, and thus lowering the activity of the protein. In this conformation, spermine does not substantially affect the activity of the ATPase at concentrations below 5 mM. This could be explained by an occlusion of most of its binding sites in the protein by the C-terminal autoinhibitory tail, containing the calmodulin-binding domain.

When calmodulin is added into the reaction medium, it binds with high affinity to the ATPase, opening the autoinhibitory domain and stimulating the PMCA activity. In this state the activity was more sensitive to 5 mM spermine, reaching a threefold inhibition with respect to that in the absence of calmodulin. The higher inhibition of ATPase activity produced by spermine in the presence of calmodulin strongly suggests that the effect of spermine is mediated by its binding to negatively charged residues of the protein that now are more accessible to the polycation in the open structure. These residues could be in the two large cytosolic loops. There are only a few regions in all the PMCA isoforms that are enriched in negatively charged amino acid residues, E and D, and which could therefore act as targets for spermine binding (See Scheme 1). The C-terminal region cannot provide a binding site for spermine because when removed by trypsin treatment (Fig. 2), the effect of spermine upon the ATPase activity of the remaining 81 kDa fragment does not change with respect to the inhibition produced with the intact protein in the presence of calmodulin.

Spermine seems to interfere with the activating effect of calmodulin (Fig. 3). At low concentrations, spermine could partially open the autoinhibitory domain of the protein and bind to just a few residues, producing a small effect on the activity. The subsequent addition of calmodulin completely would then displace the domain, allowing the binding of more spermine molecules to the protein. In such state, the activity reached by the ATPase is similar to that obtained when the same concentration of spermine is added to the protein already activated by calmodulin. At high concentrations of spermine and in the absence of calmodulin, the molecules of the polycation could completely displace the C-terminal autoinhibitory domain, giving access to all possible binding sites, so that the effect of spermine on ATPase activity would be the same in the presence of calmodulin as following removal of the autoinhibitory domain by trypsin.

The molecule of spermine has four positive charges at physiogical pH, distributed at fixed distances of 1.6 nm along a flexible carbon chain [20]. Thus one molecule could bind up to four negatively charged amino acid residues located in one or the two large cytosolic loops, bridging these loops and then blocking access of substrate to the protein. Because of its polycation structure, spermine could also mimic the effects of Ca^{2+} and Mg^{2+}, acting as an antagonist of these cations in the reaction cycle.

Effects of spermine are different for the ATPase reconstituted in PC and in PS. The mechanism of PMCA activation by acidic phospholipids is unknown. However, the presence of two separate phospholipid binding sites for acidic phospholipids has been demonstrated in the pump by functional studies using synthetic peptides [21,22] and a



Scheme 1. Two-dimensional model of the PMCA. The 10 putative transmembrane α-helices are indicated by the numbered boxes. Black boxes: phospholipid-binding sites (PL); empty box: calmodulin-binding domain (CaM-BD). P: phosphorylation site; =: point of cleavage by trypsin, giving the 81 kDa fragment. The dashed lines indicate regions enriched in negatively charged amino acid residues. The sequences of these regions are given for PMCA isoforms 1, 2, 3 and 4, according to Refs. [24–27].

proteolyzed Ca^{2+}-ATPase lacking the calmodulin-binding domain, this domain being one of the two lipid-binding regions [22] (see Scheme 1). Therefore, its removal by trypsin digestion of the PS reconstituted protein, under the described conditions, decreased the activity of the digested protein. Interestingly, this fragment was less sensitive to spermine inhibition than the intact protein. Considering that the digested protein lacks an N-terminal fragment of 315 amino acid residues that carries a region enriched in acidic residues (see Scheme 1) in all PMCA isoforms, we propose that these residues could also be involved in spermine binding. Their removal by trypsin makes the protein less sensitive to inhibition by spermine. The affinity for spermine is higher for the ATPase in PS than in PC (Figs. 2 and 4). This could reflect a conformational change of the enzyme in PS to a state with a higher affinity for spermine. However, an additional lipid–spermine interaction must also be considered, and that is an electrostatic interaction between spermine and the negatively charged phospholipid head-groups, preventing the potent activating effect of PS on PMCA activity. This interaction is weaker in the digested protein (Fig. 4) because of the removal of a lipid binding site.

The presence of spermine had no significant effect on the ATP dependence of the ATPase activity (Fig. 5), suggesting that spermine did not affect the affinity of the ATPase for ATP. The Ca^{2+} dependence of inhibition of the Ca^{2+}-ATPase is also unaffected by spermine (Fig. 6), suggesting that spermine has no effect on the affinity of the phosphorylated ATPase for Ca^{2+}. Similarly, spermine had no effect on the affinity of the Ca^{2+}-ATPase of sarcoplasmic reticulum for ATP or Ca^{2+} [8].

The pH dependence of the effect of spermine (Fig. 7) also suggests a direct interaction between PMCA and spermine. The p*K* values for PS are 3.6 for the carboxylic group and 0.5 for the phosphate group, whereas the p*K* values for PC are lower than 2 for the phosphate group [23]. This means that these lipids are not affected by protonation changes over the pH range used in our experiments, and a direct effect of spermine on the protein seems more likely. The pH dependence shown in Fig. 7 could be explained by competition between H^+ and spermine (4+) for interaction with negatively charged residues on the protein. When PMCA is reconstituted in the presence of PC, spermine increased the ATPase activity at pH values lower than 7.0, even when calmodulin was present (Fig. 7A and B). The cause of this effect is not clear. It could be that at acidic pH, the rate limiting step is stimulated by spermine, whereas at alkaline pH the rate limiting step is a different step, inhibited by spermine. Alternatively, at acidic pH spermine in a fully protonated state could bind to a stimulatory site to which it cannot bind at alkaline pH, where the spermine will be present in a partially protonated state. In PS the situation is simpler since spermine inhibits activity at all pH values (Fig. 7C).

In conclusion, effects of spermine on PMCA activity are likely to follow from both a direct interaction with the protein and an indirect effect mediated via acidic lipids. The spermine–protein interaction depends on: (i) the lipid used for protein reconstitution, (ii) the native or truncated state of the protein, and (iii) addition of calmodulin to the protein reconstitued in PC. All these factors involve a different structural rearrangement of the protein. Thus, in each state a different number of negatively charged residues is accessible for spermine binding, altering the inhibition effect of the spermine. The spermine–lipid interaction may also be present when an acidic phospholipid is used for reconstitution. Some spermine molecules could interact with

the head-groups of the lipid affecting their interaction with the protein. Nevertheless, the spermine–protein interaction must be important because the effect of spermine is different on the truncated and the intact protein reconstituted in the same lipid.

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