Polyamines and neomycin inhibit the purified plasma-membrane Ca\(^{2+}\) pump by interacting with associated polyphosphoinositides

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We investigated the effect of spermine, spermidine, putrescine and neomycin on the activity of the plasma-membrane Ca\(^{2+}\) pump and on its stimulation by negatively charged phospholipids and calmodulin. Millimolar concentrations of spermine and to a lesser extent of spermidine decreased the ATPase activity in the presence of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)), without affecting the stimulation by phosphatidylinositol 4-phosphate (PIP). Sub-millimolar concentrations of neomycin inhibited the stimulation of the ATPase by PIP and by PIP\(_2\). Neomycin was more effective at the higher concentrations of PIP and PIP\(_2\). We discuss that these findings are compatible with the hypothesis that PIP and PIP\(_2\) bind to the ATPase and that several of these molecules have to be available to stimulate the ATPase.

INTRODUCTION

Many of the known cellular effects of the polyamines are due to their polycationic nature [1]. The interaction of polyamines with biological membranes induces a variety of effects (reviewed in [2] and [3]), e.g. inhibition of the mobility of glycoproteins within the plane of the plasma membrane, promotion of membrane fusion and inhibition of the association of protein kinase C with membranes. Acidic phospholipids, especially PIP and PIP\(_{2}\), are the primary polyamine-binding sites in membranes [4]. Polyamines therefore exert strong effects on the polyphosphoinositide metabolism: spermine has been reported to inhibit phospholipase C-catalysed polyphosphoinositide hydrolysis, with a marked selectivity towards the stimulatory effect of guanosine 5'-[\(\gamma\)-thio]-triphosphate [1]. Polyamines further affect PI and PIP kinase activities [5, 6], and inhibit dephosphorylation of PIP and inositol trisphosphate [7]. All these effects are mostly due to an interaction of the positively charged groups on the polyamines with the negatively charged charged group of the polyphosphoinositides. Some of the polyamine effects are, however, unrelated to polyphosphoinositide metabolism [8, 9].

Negatively charged phospholipids increase the activity of the purified plasma-membrane Ca\(^{2+}\)-transport ATPase. This stimulatory effect is related to the number of negative charges on the lipids [10]. Because of the known strong interaction of polyamines with the polyphosphoinositides, it was of interest to investigate whether they also affect the stimulation of the ATPase by these lipids. We have furthermore compared their effect with that of neomycin, which is also a polycation, and whose interaction with PIP\(_2\) has been well characterized [11].

EXPERIMENTAL

The delipidated plasma-membrane Ca\(^{2+}\)-transport ATPase was purified from the antral part of pig stomach smooth muscle by calmodulin affinity chromatography and, unless otherwise indicated, re-activated by phospholipid mixtures at a ratio of 1 mg of phospholipid/1 mg of protein, as described previously [10]. The lipid mixtures normally contained either 20 % (w/w) negatively charged lipids (PI, PIP, PIP\(_2\), PS or PA) and 80 % PC, or 100 % PC, and were made in buffer [10].

A 10 \(\mu\)l portion of ATPase (corresponding to 2 \(\mu\)g of protein) was transferred to 590 \(\mu\)l of assay medium of the following composition: phosphoenolpyruvate 1.5 mM; pyruvate kinase 40 units/ml; lactate dehydrogenase 40 units/ml; imidazole/HCl (pH 6.9) 30 mM; MgCl\(_2\) 5.4 mM; KCl 100 mM; K-EGTA 0.5 mM; ATP 5 mM; NaN\(_3\) 5 mM; CaCl\(_2\) 0.5 mM (free Ca\(^{2+}\) concn. 6 \(\mu\)M); and NADH 0.26 mM. In some experiments calmodulin was added at a saturating concentration of 0.6 \(\mu\)M. The decrease in \(A_{340}\) was measured spectrophotometrically, and it varied linearly with respect to both time and protein concentration. The ATPase activity was first measured for 5 min. Thereupon 6 \(\mu\)l of a stock solution of spermine, spermidine, putrescine or neomycin sulphate (all from Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to the cuvette, and the ATPase activity was subsequently measured for another 5 min. The polyanine stock solutions were made with sterile water, to prevent metabolism of the polyamines by contaminating bacteria. In the controls, 6 \(\mu\)l of distilled water was added to the cuvette instead of the polyanine stocks and 6 \(\mu\)l of distilled water was added to the cuvette instead of the polyanine stocks and 6 \(\mu\)l of an equimolar K\(_2\)SO\(_4\) solution instead of neomycin sulphate. Protein was measured by the

Abbreviations used: (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase, (Ca\(^{2+}\) + Mg\(^{2+}\))-activated ATPase; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP\(_{2}\), phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid.

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method of Lowry et al. [12], with serum albumin as a standard.

RESULTS AND DISCUSSION

The purification of the plasma-membrane Ca²⁺-transporting ATPase in the absence of lipids yields an inactive ATPase that can be re-activated by adding phospholipids [10]. PC is not very efficient in that respect. The specific activity in this condition was 1.02 ± 0.08 (n = 15) μmol/min per mg of protein. Fig. 1 illustrates that the activity of the enzyme can be increased by replacing 20% of PC by PI. This stimulatory effect became more pronounced when PIP, and especially PIP₂, was used to replace PC [10].

![Graph](image)

**Fig. 1. Effects of spermine and neomycin on the (Ca²⁺ + Mg²⁺)-ATPase activity of the purified plasma-membrane Ca²⁺ pump**

The effects of 5 mM-spermine and of 5 mM-neomycin on the (Ca²⁺ + Mg²⁺)-ATPase activity at 6 μM-Ca²⁺ (μmol/min per mg of protein) are illustrated. The ATPase was activated by different phospholipid mixtures (either pure PC, or a mixture of 80% PC and 20% of the acidic phospholipid indicated below each bar). The results are expressed as means ± S.E.M. for the numbers of observations given above each bar.

Spermine is a naturally occurring polycationic substance that has four positive charges at pH 6.9 and that therefore interacts strongly with PIP₂ [4]; 5 mM-spermine decreases the activity of the ATPase in the presence of 20% PIP₂, and 5 mM-neomycin, which has six positive charges, also decreased the ATPase activity in the presence of 20% PIP and almost completely abolished the stimulation by PIP₂. A similar, although less pronounced, effect was also observed for 5 mM-spermidine, which has only three positive charges, whereas the same concentration of putrescine with its two positive charges had no significant effect (results not shown).

In the absence of negatively charged phospholipids, i.e. in the presence of pure PC, 5 mM-spermine increased the ATPase activity by a factor of 1.2. The cause of this
Table 1. Effects of spermine and neomycin on (Ca^{2+}+Mg^{2+})-ATPase activity of the purified plasma-membrane Ca^{2+} pump

The effects of 5 mM-spermine and of 5 mM-neomycin on the (Ca^{2+}+Mg^{2+})-ATPase activity (μmol/min per mg of protein) at 6 μM-Ca^{2+} are illustrated. The ATPase was activated by a mixture of 80% PC and 20% of the acidic phospholipid as indicated, or by 0.6 μM-calmodulin in the presence of 100% PC. The results are expressed as means ± S.E.M. for the numbers of observations given in parentheses.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Control</th>
<th>Spermine</th>
<th>Neomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS</td>
<td>1.98 ± 0.10 (15)</td>
<td>1.92 ± 0.04 (4)</td>
<td>1.93 ± 0.09 (3)</td>
</tr>
<tr>
<td>PA</td>
<td>1.80 ± 0.08 (15)</td>
<td>1.68 ± 0.10 (4)</td>
<td>1.49 ± 0.05 (3)</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>2.34 ± 0.05 (18)</td>
<td>2.28 ± 0.07 (6)</td>
<td>2.28 ± 0.18 (3)</td>
</tr>
</tbody>
</table>

The effects of spermine and neomycin had no effect in the absence of negatively charged phospholipids. The activity of the enzyme in the presence of PC as the reconstituting lipid can also be increased by supplementation of the assay medium with 0.6 μM-calmodulin, or by replacing the PC by other negatively charged lipids, such as PS and PA (Table 1). Neomycin or spermine (both 5 mM) did not affect the stimulation of the ATPase by PS and calmodulin, and only partially inhibited the stimulation by PA. Spermidine or putrescine (5 mM) had no significant effect on the PS-, PA- or calmodulin-stimulated ATPase activity (results not shown).

Neomycin did not inhibit the ATPase in the presence of PC, PI, PS and calmodulin (Fig. 1, Table 1). This finding argues against the possibility that an interaction between ATP and neomycin [13] could be involved in the observed inhibition of the ATPase in the presence of PIP_{2}.

Because spermine exerted its greatest inhibitory effect on the PIP_{2}-stimulated enzyme, we have determined a dose–response relationship for spermine in the presence of PIP_{2}. Fig. 2 illustrates that the threshold for inhibition was 0.1 mM-spermine, and that the effect became more pronounced at concentrations in the millimolar range. Although the free concentration of spermine in the cytoplasm is not known with any certainty, the total cellular concentration of polyamines usually ranges from 0.5 to 1 mM [6], and a total concentration of up to 5 mM has been reported in some cells [14]. From a study of the electrophoretic mobility of multilamellar vesicles formed from mixtures of PC and PIP_{2}, it was concluded that a considerable fraction of PIP_{2} molecules (about 40% at a surface potential of -30 mV) bind spermine at a concentration of 10 μM of this polyol [15]. Although no data exist on the amount of polyamines bound to the internal leaflet of the plasma membrane, it is not impossible that membrane-associated polyamines could play a role in the regulation of the plasma-membrane Ca^{2+} pump via interaction with polyphosphoinositides.

A dose–response relationship for inhibition of the PIP_{2}-stimulated enzyme by neomycin (Fig. 2) illustrates that the threshold for inhibition was 10 μM. The effect increased up to concentrations within the millimolar range. This concentration range for inhibition of the ATPase is two orders of magnitude higher than the concentrations at which neomycin binds to pure PIP_{2} vesicles [11]. Such a difference could indicate that the PIP_{2} molecules complexed by neomycin are associated with proteins [11], or, in our case, are associated with the Ca^{2+}-pump protein. As a consequence, there could be competition for the PIP_{2} molecules between the polyamines and positively charged sites on the proteins.

Fig. 3(a) illustrates the inhibition by neomycin of the ATPase activity in the presence of different concentrations of PIP. PIP increased the ATPase activity dose-dependently, as shown previously [10]. However, the inhibition of the ATPase activity by neomycin showed a peculiar dependence on the PIP concentration. The inhibitory effect of neomycin was small at 5% PIP, but it increased at higher PIP concentrations, such that the PIP-stimulation curve became bell-shaped in the presence of neomycin. One possible explanation of this result is that neomycin decreases the total amount of lipid surrounding the ATPase, thereby decreasing its activity.
Because in these experiments the total amount of added lipids was kept constant at 1 mg/mg of ATPase, an increase in PIP was balanced by a decrease in PC. Neomycin could therefore decrease the total amount of lipids to a higher degree at higher PIP concentrations. However, this explanation is unlikely, since the ATPase activity reached a maximum at 0.5 mg of phospholipid/mg of ATPase (results not shown). The experimental concentration of 1 mg of phospholipid/mg of ATPase is thus a supramaximal concentration, and, even after complexing of 50% of the surrounding lipids, the ATPase will still be maximally activated. Furthermore, we have also studied the effect of neomycin on an ATPase preparation supplemented by a constant amount of 1 mg of PC/mg of ATPase and with PIP added on top of this fixed amount of PC, instead of replacing PC by PIP. This procedure yielded a curve very similar to that of Fig. 3(a) (results not shown). A result essentially similar to that seen with PIP was obtained with PIP₂ (Fig. 3b). Neomycin inhibited the PIP₂ stimulation of the enzyme by 21 ± 9% \((n = 3)\) in the presence of 5% PIP₂ and by 80 ± 4% \((n = 3)\) in the presence of 20% PIP₂. The nearly complete inhibition was observed at a much lower concentration of PIP₂, as compared with PIP, probably because PIP₂ is more effective in stimulating the ATPase than is PIP [10].

The peculiar dependence of the inhibition by neomycin on the PIP and PIP₂ concentration can be explained if it is assumed first that the binding of more than one polypehosphoinositide molecule is needed to stimulate the ATPase, and secondly that at high concentrations of these lipids the binding of the polycation to the lipids could be enhanced by a co-operative effect of the protein-bound PIP and PIP₂ molecules thereby locally a high charge density would be created.

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REFERENCES