Biochimica et Biophysica Acta, 1023 (1990) 449–454
Elsevier

BBAMEM 74816

Ruthenium red and compound 48/80 inhibit the smooth-muscle plasma-membrane Ca^{2+} pump via interaction with associated polyphosphoinositides

Ludwig Missiaen, Humbert De Smedt, Guy Droogmans, Frank Wuytack, Luc Raeymaekers and Rik Casteels

Physiological Laboratory, K.U. Leuven, Leuven (Belgium)

(Received 14 September 1989)
(Revised manuscript received 16 January 1990)

Key words: Calcium pump; ATPase, $(Ca^{2+} + Mg^{2+})$-; Ruthenium red; Compound 48/80; Polyphosphoinositide; (Smooth muscle plasma membrane)

We will demonstrate that compound 48/80 and ruthenium red inhibit the smooth-muscle plasma-membrane Ca^{2+} pump by counteracting the stimulant effect of negatively charged phospholipids. Both substances did not affect the purified enzyme re-activated by pure phosphatidylcholine or phosphatidylinositol and measured in the absence of calmodulin, indicating that under these conditions they did not have a direct effect on the ATPase protein. Ruthenium red and compound 48/80 however inhibited the $(Ca^{2+} + Mg^{2+})$-ATPase in the presence of phosphatidylinositol 4-phosphate and especially phosphatidylinositol 4,5-bisphosphate. The $K_{0.5}$ for inhibition was 25 μM ruthenium red and 9 μg/ml of compound 48/80. The inhibition by ruthenium red developed slowly with half maximal inhibition occurring after about 75 s while that by compound 48/80 developed immediately within the time required for mixing. The efficacy of ruthenium red increased as the concentration of the acidic phospholipid increased, while no such cooperativity was observed for compound 48/80. Ruthenium red reduced the V_{max} for Ca^{2+} without affecting the affinity for Ca^{2+}, while compound 48/80 decreased both parameters. In conclusion, although ruthenium red and compound 48/80 affect the ATPase differently, both substances most likely inhibit the plasma-membrane Ca^{2+} pump by counteracting the stimulation by negatively charged phospholipids.

Introduction

Ruthenium red is a hexavalent polycationic dye which is known to inhibit the plasma-membrane Ca^{2+} pump [1–9]. This inhibition of the Ca^{2+}-transporting ATPase presents some specificity, since neither the basal Mg^{2+}-ATPase, nor the Na^+,K^+-ATPase are affected by ruthenium red [1]. Although the exact mechanism of inhibition by ruthenium red has not been described, it is assumed that it interacts at the cytoplasmic site of the Ca^{2+} pump [7] and that it does not interact at a site related to calmodulin [7]. It has been suggested that ruthenium red combines with the Ca^{2+} binding side because of its polycationic nature, although no experimental evidence for this has been presented [8].

Compound 48/80 is the product of the reaction of equimolar concentrations of formaldehyde and *p*-methoxy-*N*-methylphenylethylamine [10], and is a mixture of compounds which are both hydrophobic and polycationic. This substance is a potent inhibitor of the calmodulin-activated form of the plasma-membrane Ca^{2+} pump [11]. It recently became apparent that the binding of 48/80 constituents to calmodulin can not fully account for the inhibition of the enzyme and that also a direct interaction of 48/80 constituent(s) with the enzyme and/or the lipid portion of the membrane must occur [12,13].

Negatively charged phospholipids stimulate the purified plasma-membrane Ca^{2+} pump by increasing the V_{max} for Ca^{2+} and its affinity for Ca^{2+} and by slightly increasing the cooperativity for Ca^{2+}. This stimulant effect is related to the number of negative charges on the phospholipids [14]. In this report, arguments will be brought forward indicating that the polycations ruthenium red and compound 48/80 inhibit the

Abbreviations: $(Ca^{2+} + Mg^{2+})$-ATPase, $(Ca^{2+} + Mg^{2+})$-activated ATPase; PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylcholine.

Correspondence: R. Casteels, Physiological Laboratory, K.U. Leuven, Campus Gasthuisberg, B-3000 Leuven, Belgium.

purified and re-activated plasma-membrane Ca^{2+} pump from smooth muscle by interacting with negatively charged phospholipids or with inositide-dependent sites on the ATPase-protein.

Experimental

The delipidated plasma-membrane Ca^{2+}-transport ATPase was purified from the antral part of pig stomach smooth muscle using calmodulin-affinity chromatography and, unless otherwise indicated, re-activated by phospholipid mixtures at a ratio of 1 mg phospholipid per mg of protein, as described previously [14]. The lipid mixtures either consisted of PC only or 20% (w/w) of one of the negatively charged lipids (PtdIns, PtdIns4*P* or PtdIns(4,5)P_2) and 80% PC.

The assay medium for measuring the (Ca^{2+} + Mg^{2+})-ATPase activity had the following composition: phospho*enol*pyruvate, 1.5 mM; pyruvate kinase, 40 units/ml; lactate dehydrogenase, 40 units/ml; imidazole-HCl (pH 6.9), 30 mM; $MgCl_2$, 5.40 mM (free [Mg^{2+}], 1 mM); KCl, 100 mM; EGTA, 0.5 mM; ATP, 5 mM; NaN_3, 5 mM and NADH, 0.26 mM. The pH of the medium was adjusted to 6.9 at 37°C. 10 μl of ATPase (corresponding to 2 μg of protein) was transferred to 990 μl assay medium and the decrease of the light absorbance at 340 nm was measured spectrophotometrically at 37°C. The decrease in absorbance was found to vary linearly both with respect to time and protein concentration. In most experiments the free Ca^{2+} concentration was 10 μM. For the experiments in Fig. 4, Ca^{2+} was added in a cumulative way to obtain subsequently free Ca^{2+} concentrations of 0.10 μM, 0.18 μM, 0.30 μM, 0.56 μM, 1.0 μM and 3.0 μM. Calmodulin was not added in these experiments. The total amount of Ca^{2+} added to the cuvette was calculated with a computer program using the following decimal logarithms of the association constants: H-ATP, 6.49; H-HATP, 4.11; Ca-ATP, 3.78; Ca-HATP, 1.98; Mg-ATP, 4.00 and Mg-HATP, 2.06. The association constants for EGTA were: H-EGTA, 9.47; H-HEGTA, 8.85; Ca-EGTA, 10.97; Ca-HEGTA, 5.33; Mg-EGTA, 5.21 and Mg-HEGTA, 3.37 [15]. From the Ca^{2+} activation curves, the V_{max} for Ca^{2+} and the $K_{0.5}$ for Ca^{2+} were calculated by computerized nonlinear curve fitting using the Michaelis-Menten equation. The Hill-coefficient of these Ca^{2+}-activation curves indeed ranged around unity [14].

Protein concentrations were measured with the method of Lowry et al. [16], using serum albumin as a standard.

Phospho*enol*pyruvate, pyruvate kinase, lactate dehydrogenase and ATP were obtained from Boehringer (Mannheim, F.R.G.). NADH, PtdIns4*P* (from bovine brain, 98% pure, product No P-9638), PtdIns(4,5)P_2 (from bovine brain, 98% pure, product No P-9763), PC (from frozen egg yolk, 99% pure, product No P-4279), and compound 48/80 were all from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PtdIns was from Lipid Products (Redhill, U.K.). Ruthenium red was from Fluka AG (Buchs SG, Switzerland).

Results

The purification of the plasma-membrane Ca^{2+} pump from stomach smooth muscle in the absence of

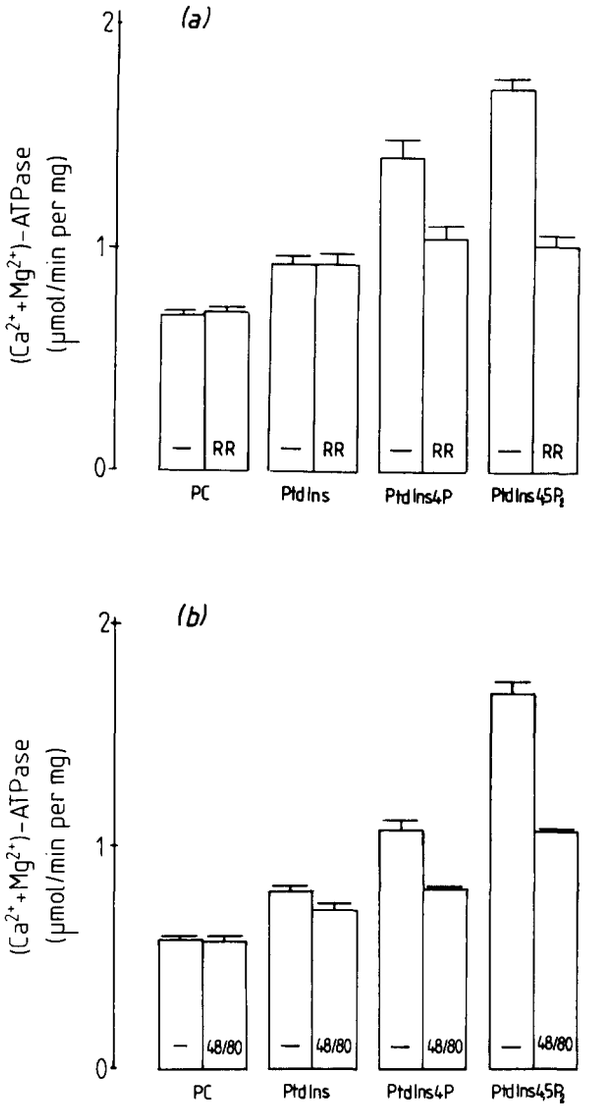

Fig. 1. Effect of ruthenium red and compound 48/80 on the (Ca^{2+} + Mg^{2+})-ATPase activity of the purified plasma-membrane Ca^{2+}-pump. The effects of 100 μM ruthenium red (panel a) and 100 μg/ml of compound 48/80 (panel b) on the (Ca^{2+} + Mg^{2+})-ATPase at 10 μM [Ca^{2+}] (μmol/min per mg of 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 pair of bars). The values in the presence of ruthenium red (RR) were obtained 5 min after adding the dye, those in the presence of compound 48/80 (48/80) were obtained immediately after adding this substance. The experiments in panel b were performed on another batch of enzyme as compared to those in panel a. The results are expressed as means ± S.E. (n = 5).

lipids yields an inactive ATPase that can be re-activated by adding phospholipids [14,17], as illustrated in Fig. 1. As reported previously, PC was the least potent lipid. The activity of the enzyme could be increased by substituting 20% of PC by PtdIns. The stimulant effect became more pronounced when PtdIns4*P* and PtdIns(4,5)P_2 were used to replace PC [14,18,19]. Also the effect of 100 μM ruthenium red and 100 μg/ml of compound 48/80 on the specific activity of the Ca^{2+}-transporting ATPase is represented in Fig. 1. Ruthenium red inhibited the ATPase in the presence of PtdIns4*P* (Fig. 1a). This inhibition was more pronounced in the presence of PtdIns(4,5)P_2. In the absence of negatively charged phospholipids, i.e., in the presence of pure PC, and also in the presence of 20% PtdIns, ruthenium red did not affect the enzyme activity. This finding allowed us to exclude any nonspecific effect of this compound on the coupled-enzyme ATPase assay. Compound 48/80 at a concentration of 100 μg/ml was without effect on the enzyme re-activated by PC or PtdIns (Fig. 1b), which again excluded an aspecific effect on the coupled-enzyme ATPase assay. The enzyme activities in the presence of PtdIns4*P* and especially of PtdIns4,5P_2 were significantly decreased. It should be stressed at this point that the ATPase assay medium was not supplemented with calmodulin. In separate experiments, 0.6 μM-calmodulin was found to increase the (Ca^{2+} + Mg^{2+})-ATPase re-activated by PC, from 672 ± 26 (n = 3) to 1168 ± 44 (n = 3) nmol/min per mg of protein. In the presence of 100 μg/ml of compound 48/80, calmodulin only increased the ATPase activity from 630 ± 38 (n = 3) to 652 ± 4 (n = 3) nmol/min per mg of protein. This finding confirms the already known calmodulin-antagonistic properties of this substance [11].

The time course of the inhibitory action of 100 μM ruthenium red on the enzyme reactivated by a mixture of 20% PtdIns(4,5)P_2 and 80% PC is illustrated in Fig. 2. Half-maximal inhibition occurred after about 75 s,

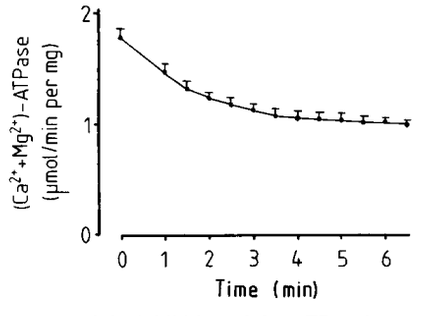

Fig. 2. Time course of the inhibition of the ATPase by ruthenium red. The (Ca^{2+} + Mg^{2+})-ATPase, measured at 10 μM [Ca^{2+}] and expressed in μmol/min per mg of protein, is given on the ordinate. The abscissa represents the time, in min, after addition of 100 μM ruthenium red. The ATPase was re-activated by 20% PtdIns(4,5)P_2, balanced by PC. The results are expressed as means ± S.E. (n = 5).

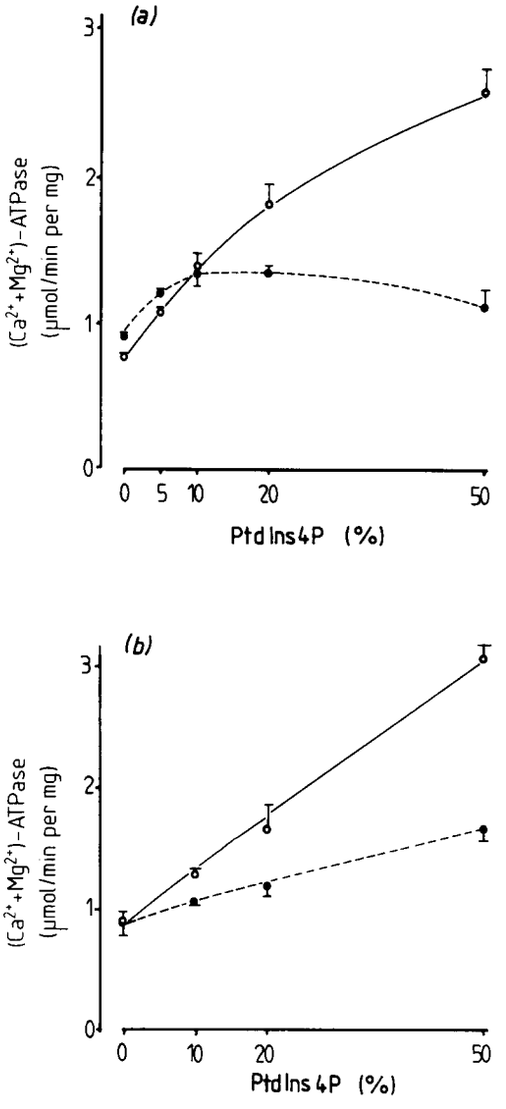

Fig. 3. Effect of ruthenium red and compound 48/80 on the stimulation of the plasma-membrane (Ca^{2+} + Mg^{2+})-ATPase by PtdIns4*P*. The ATPase was activated by adding 1 mg of lipid per mg of ATPase. When the amount of PtdIns4*P* increased, the amount of PC was proportionally reduced. The abscissa represents the percent PtdIns4*P* (w/w) relative to the total amount of lipid. The ordinate represents the (Ca^{2+} + Mg^{2+})-ATPase activity at 10 μM [Ca^{2+}] (μmol/min per mg of protein) in the absence (○) and in the presence (●) of 100 μM ruthenium red (panel a) or 100 μg/ml of compound 48/80 (panel b). The results are given as means ± S.E. (n = 4).

and a steady-state inhibition was only reached after 5 min. The effects of ruthenium red on the specific activities of the (Ca^{2+} + Mg^{2+})-ATPase (Fig. 1) were therefore measured 5 min after adding this dye. In contrast, the inhibition induced by compound 48/80 occurred within the time required for mixing.

Fig. 3a illustrates the inhibitory effect of ruthenium red on the ATPase activity in the presence of different concentrations of PtdIns4*P*. In the absence of the dye, the gradual substitution of PC by PtdIns4*P* increased the ATPase activity dose-dependently, as shown previ-

ously [14]. In the presence of ruthenium red, PtdIns4*P* only increased the ATPase activity up to a concentration of 10%, while higher concentrations of the lipid did not further stimulate the enzyme. The inhibition of the ATPase by 100 μM ruthenium red is therefore characterized by a peculiar dependency on the PtdIns4*P* concentration whereby the inhibitory effect was absent at concentrations below 10% of this lipid. The inhibition at 20% was relatively small and became more pronounced at 50% PtdIns4*P*. Fig. 3b illustrates the effect of compound 48/80 at a concentration of 100 μg/ml on the (Ca^{2+} + Mg^{2+})-ATPase activity as a function of the concentration of PtdIns4*P*. In contrast with the effect of ruthenium red, compound 48/80 inhibited the enzyme equally well at 10% as at 50% PtdIns4*P*. From the data in Fig. 3b, it was calculated that the increase of the ATPase activity induced by 10% PtdIns4*P* over the value obtained in the absence of this lipid, was inhibited by 62%. The stimulation of the ATPase by 50% PtdIns4*P* was inhibited by 65%. Therefore no cooperative behaviour of the inhibition of the enzyme by compound 48/80 towards the concentation of PtdIns4*P* could be observed.

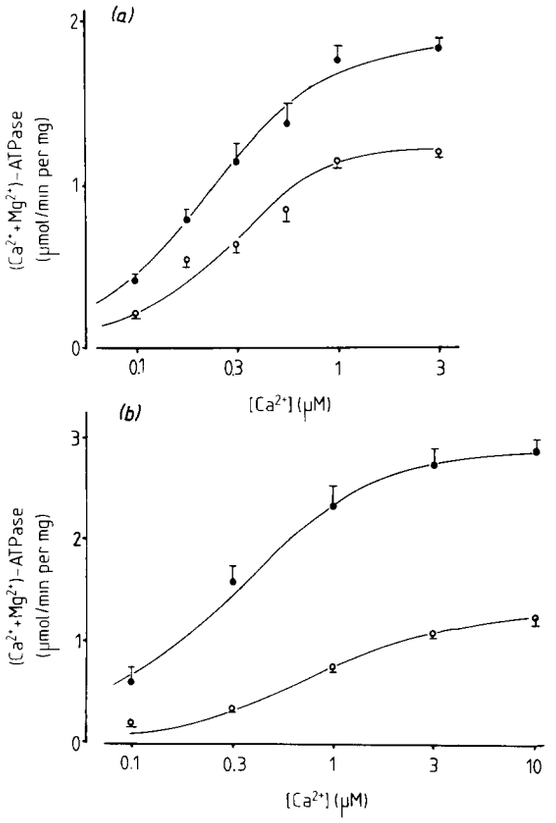

Fig. 4. Effect of ruthenium red and compound 48/80 on the Ca^{2+}-activation curves of the Ca^{2+} pump. The (Ca^{2+} + Mg^{2+})-ATPase activity (in μmol/min per mg of protein) is expressed on the ordinate as a function of the concentration of free Ca^{2+} (μM) given on the abscissa, either in the absence (●) and in the presence (○) of 100 μM ruthenium red (panel a) or 100 μg/ml of compound 48/80 (panel b). The ATPase was re-activated by 20% PtdIns4*P* (in panel a) and by 50% PtdIns4*P* (in panel b). The results are expressed as means ± S.E. (n = 4).

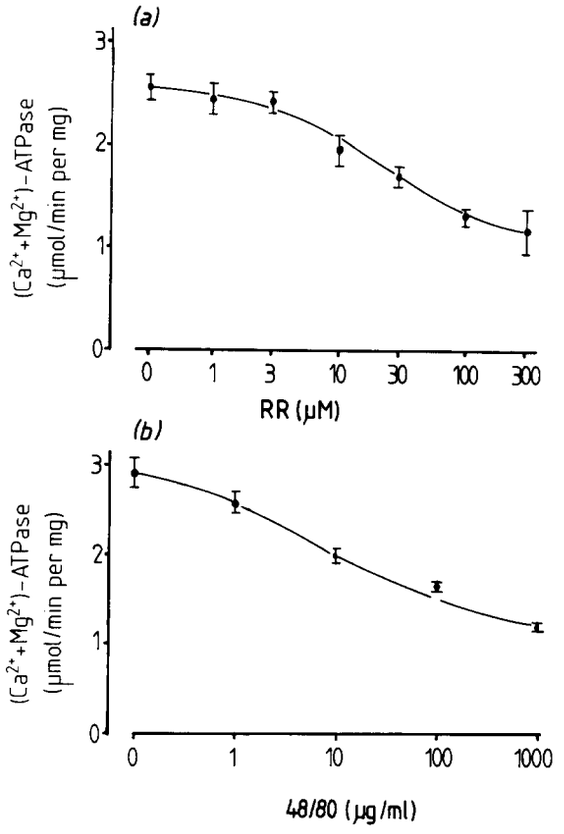

Fig. 5. Dose-response relationship representing the interaction of ruthenium red and compound 48/80 with PtdIns4*P*. The delipidated ATPase was re-activated by addition of a lipid mixture containing 50% PtdIns4*P* and 50% PC. The effect of different concentrations of ruthenium red (panel a) and of different concentrations of compound 48/80 (panel b) (given on the abscissa) on the (Ca^{2+} + Mg^{2+})-ATPase at 10 μM -[Ca^{2+}] (given in the ordinate in μmol/min per mg of protein) is represented. The results are expressed as means ± S.E. (n = 6).

In order to further specify to which extent ruthenium red and compound 48/80 affect the kinetic parameters of the plasma-membrane Ca^{2+} pump, the (Ca^{2+} + Mg^{2+})-ATPase activity of the enzyme at different concentrations of free Ca^{2+} was determined (Fig. 4). In the absence of ruthenium red, Ca^{2+} increased the ATPase activity in the presence of 20% PtdIns4*P* dose-dependently with a V_{max} of 1.86 ± 0.08 (n = 5) μmol/min per mg of protein, and a $K_{0.5}$ of 0.28 ± 0.04 (n = 5) μM Ca^{2+}. 100 μM ruthenium red decreased the V_{max} to 1.13 ± 0.04 (n = 5) μmol/min per mg of protein without however affecting the affinity for Ca^{2+} (0.26 ± 0.06 (n = 5) μM Ca^{2+}). Because the inhibitory effect at 50% PtdIns4*P* was much more pronounced than at 20%, it was of interest to investigate the effect of the dye on the kinetic parameters at 50% PtdIns4*P*. Ruthenium red decreased the V_{max} from 2.62 ± 0.15 (n = 3) to 1.11 ±

0.06 ($n = 3$) μmol/min per mg of protein. Half-maximal activation of the enzyme occurred at 0.21 ± 0.05 ($n = 3$) μM Ca^{2+} in the absence and at 0.29 ± 0.06 ($n = 3$) μM Ca^{2+} in the presence of 100 μM ruthenium red. This difference in Ca^{2+} affinity was not statistically significant. Fig. 4b represents the effect of 100 μg/ml of compound 48/80 on the Ca^{2+}-activation curve of the smooth-muscle membrane Ca^{2+}-ATPase, re-activated by 50% PtdIns4*P*. In the absence of this compound, Ca^{2+} increased the ATPase activity dose-dependently with a V_{max} of 3.03 ± 0.10 ($n = 4$) μmol/min per mg of protein, and a $K_{0.5}$ of 0.34 ± 0.07 ($n = 4$) μM Ca^{2+}. Compound 48/80 (100 μg/ml) decreased the V_{max} to 1.39 ± 0.08 ($n = 4$) μmol/min per mg of protein. In contrast to the effect of ruthenium red, the affinity for Ca^{2+} was appreciably decreased by compound 48/80 (from 0.34 ± 0.07 ($n = 4$) to 0.86 ± 0.05 ($n = 4$) μM Ca^{2+}).

Because ruthenium red exerted its greatest inhibitory effect on the ATPase in the presence of 50% PtdIns4*P*/50% PC, we have determined a dose-response relationship for the inhibition of the ATPase in the presence of this lipid mixture. Fig. 5a illustrates that the threshold for inhibition was 1 μM, and that the inhibitory effect progressively increased with a $K_{0.5}$ of 25 μM, to a maximal inhibitory effect at 300 μM of the dye. The dose-inhibition relationship for compound 48/80 is illustrated in Fig. 5b. Half maximal inhibition of the enzyme re-activated by 50% PtdIns4*P* occurred at 9 μg/ml.

Discussion

Ruthenium red is often considered as an inhibitor of the plasma-membrane Ca^{2+} pump [1–9]. Ruthenium red did not affect the ($Ca^{2+} + Mg^{2+}$)-ATPase re-activated by PC or by PtdIns, in contrast to its inhibition of enzyme stimulated by PtdIns4*P* and especially PtdIns(4,5)P_2. This suggests that this dye affects the activity of the purified plasma-membrane Ca^{2+} pump by interfering with the interaction between polyphosphoinositides and the Ca^{2+}-transporting ATPase. PtdIns(4,5)P_2 presents, depending on the pH, four to five and PtdIns4*P* three negative charges [20], and therefore an electrostatic interaction between positive charges of ruthenium red and the polyposphoinositides seems very likely. Since the polyphosphoinositides are located at the inner leaflet of the plasma membrane, our hypothesis would imply that ruthenium red must interact with the ATPase at the cytoplasmic face of the membrane, as has been suggested previously [8]. This action of ruthenium red resembles that of neomycin [19]. Evidence has been presented indicating that also this inhibition was due to the interaction of these strongly positively charged substances with associated polyphosphoinositides and not to a direct effect on the ATPase protein. A similar peculiar dependency of the inhibition of the ATPase by ruthenium red on the PtdIns4*P* concentration has also been observed for neomycin [19]. This finding strengthens our hypothesis that the binding of more than one polyphosphoinositide molecule is needed to stimulate the ATPase. We furthermore suggest that high concentrations of the lipid would create on the ATPase protein a high charge density and that this combination of negative charges would promote in a cooperative way the binding of the ruthenium-red molecule with its six positive charges.

Compound 48/80 is a mixture of compounds which are both hydrophobic and polycationic and which has been suggested to be a more selective anti-calmodulin agent than other anti-calmodulin drugs [11]. Recent evidence indicated that in addition to its anti-calmodulin action it also directly interacts with the Ca^{2+} pump [12,13]. However, both groups did not discriminate between a direct interaction of this substance with the enzyme or with surrounding phospholipids. The present experiments however provide convincing evidence that compound 48/80 prevents the stimulation of the Ca^{2+}-pump protein by negatively charged phospholipids.

The stimulant effect of negatively charged lipids on the plasma-membrane Ca^{2+} pump can be formally described as the result of an increased V_{max}, an increased affinity for Ca^{2+} and an increase of the cooperativity for Ca^{2+} [14,21–23]. The order of potency of the different phosphoinositide lipids to increase the V_{max} and the affinity for Ca^{2+} was as follows: PtdIns(4,5)P_2 > PtdIns4*P* > PtdIns, and this order is proportional to the number of negative charges on the lipid [14]. An interesting finding was that ruthenium red decreased the V_{max} Ca^{2+} without affecting the affinity, while compound 48/80 decreased both the V_{max} and the affinity. This finding indicates that both compounds affect the interaction between the polyphosphoinositides and the Ca^{2+} pump differently. Because ruthenium red selectively reduces the V_{max} of the ATPase, it could become a valuable tool for further exploring the regulation of the catalytic turnover of the enzyme.

In conclusion, the inhibitory effect of ruthenium red and compound 48/80 differs in the way of interaction with the phospholipids: the inhibitory effect exerted by compound 48/80 was due to a reduction of the V_{max} and a decrease of the Ca^{2+} affinity, while ruthenium red did not affect the latter parameter. The inhibition of the enzyme by ruthenium red was cooperative towards PtdIns4*P*, while this was not the case for compound 48/80.

Acknowledgements

This work was supported by the FGWO, Belgium. We thank Mr. J. Cuypers and Mrs. I. Willems for the

technical assistance. L.M. is Senior Research Assistant of the National Fund of Scientific Research, Belgium.

References

1 Watson, E.L., Vincenzi, F.F. and Davis, P.W. (1971) Biochim. Biophys. Acta 249, 606–610.
2 Sarkadi, B., Szász, I., Gerlóczi, A. and Gardos, G. (1977) Biochim. Biophys. Acta 464, 93–107.
3 Raess, B.U. and Vincenzi, F.F. (1980) Mol. Pharmacol. 18, 253–258.
4 Sarkadi, B., Szász, I. and Gardos, G. (1980) Biochim. Biophys. Acta 598, 326–338.
5 Sarkadi, B. (1980) Biochim. Biophys. Acta 604, 159–190.
6 Schuurmans Stekhoven, F. and Bonting, S.L. (1981) Physiol. Rev. 61, 1–76.
7 Hinds, T.R., Raess, B.U. and Vincenzi, F.F. (1981) J. Membr. Biol. 58, 57–65.
8 Schatzmann (1982) in Membrane Transport of Calcium (Carafoli, E., ed.), pp. 41–108, Academic Press, New York.
9 Garahan, P.J. (1986) in The Ca^{2+}-pump of plasma membranes (Rega, A.F. and Garrahan, P.J., eds.), pp. 127–164, CRC Press, Boca Raton.
10 Baltzly, R.L., Buck, J.S., De Beer, E.J. and Webb, F.J. (1949) J. Am. Chem. Soc. 71, 1301–1305.
11 Gietzen, K., Adamczyk-Engelmann, P., Wuthrich, A., Konstantinova, A. and Bader, H. (1983) Biochim. Biophys. Acta 736, 109–118.
12 Di Julio, D., Hinds, T.R. and Vincenzi, F.F. (1989) Biochim. Biophys. Acta 981, 337–342.
13 Rossi, J.P.f.C. and Rega, A.F. (1989) Biochim. Biophys. Acta 996, 153–159.
14 Missiaen, L., Raeymaekers, L., Wuytack, F., Vrolix, M., De Smedt, H. and Casteels, R. (1989) Biochem. J. 263, 687–694.
15 Martell, A.E. and Smith, R.J. (1982) Critical Stability Constants, Plenum Press, New York.
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
17 Vrolix, M., Raeymaekers, L., Wuytack, F., Hofmann, F. and Casteels, R. (1988) Biochem. J. 255, 855–863.
18 Missiaen, L., Droogmans, G., De Smedt, H., Wuytack, F., Raeymaekers, L. and Casteels, R. (1989) Biochem. J. 262, 361–364.
19 Missiaen, L., Wuytack, F., Raeymaekers, L., De Smedt, H. and Casteels, R. (1989) Biochem. J. 261, 1055–1058.
20 Van Paridon, P.A., De Kruijff, B., Ouwerkerk, R. and Wirtz, K.W.A. (1986) Biochim. Biophys. Acta 877, 216–219.
21 Niggli, V., Adunyah, E.S. and Carafoli, E. (1981) J. Biol. Chem. 256, 8588–8592.
22 Choquette, D., Hakim, G., Filoteo, A.G., Plishker, G.A., Bostwick, J.R. and Penniston, J.T. (1984) Biochem. Biophys. Res. Commun. 125, 908–915.
23 Enyedi, A., Flura, M., Sarkadi, B., Gardos, G. and Carafoli, E. (1987) J. Biol. Chem. 262, 6425–6430.