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EFFECT OF INHIBITORS ON THE SIGMOIDICITY OF THE CALCIUM ION TRANSPORT KINETICS IN RAT LIVER MITOCHONDRIA

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Summary

The kinetic plot (initial rate of Ca^{2+} transport versus concentration) of mitochondrial Ca^{2+} transport is hyperbolic in a sucrose medium. The plot becomes sigmoidal in the presence of competitive inhibitors of Ca^{2+} binding to low affinity sites of the membrane surface such as Mg^{2+} and K^+. The plot also becomes sigmoidal in the presence of Ba^{2+}. Ba^{2+} is a competitive inhibitor of both Ca^{2+} transport and Ca^{2+} binding to the low affinity sites. The K_i for the inhibition of Ca^{2+} transport by Ba^{2+} increases in the presence of K^+ and Mg^{2+}, which suggests a competition for the low affinity sites between the cations. The plot is still hyperbolic in the presence of La^{3+}, which inhibits Ca^{2+} transport competitively. Ruthenium red which is a pure non-competitive inhibitor of mitochondrial Ca^{2+} transport, does not affect the shape of the kinetic plot. These results indicate that the surface potential, which depends on the ions bound to the low affinity sites, determines whether the kinetics of Ca^{2+} uptake in mitochondria is sigmoidal or hyperbolic.

Introduction

A sigmoidal plot is obtained when the initial rate of mitochondrial Ca^{2+} transport is plotted against the free Ca^{2+} concentration [1—3]. These results have been interpreted to be due to cooperativity in the Ca^{2+} transport system. The sigmoidal kinetics imply that mitochondrial Ca^{2+} translocation would be very slow at the low (10^{-6} M) Ca^{2+} concentrations in the cytosol. This clearly would influence the interpretation of the role of mitochondria in the regulation of cytosolic Ca^{2+}. Ca^{2+} is bound to energy-independent binding sites with low

Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-(5-phenyloxazolyl-2)-benzene; EGTA, ethyleneglycol bis(α-aminoethylether)-*N,N'*-tetraacetic acid.

affinity at the outer surface of the mitochondrial membrane [4—6]. The amount of these sites is about 20 nmol/mg protein [5] and they are competitively inhibited by $Mg^{2+} > K^+ > H^+$ [7—9] and are mainly phospholipid in nature [7—10]. These binding sites have in some models been considered to be involved in the transport of Ca^{2+} [8,11,12]. A number of inhibitors of Ca^{2+} transport have been used in order to gain more insight into the mechanism of transport. La^{3+} and related rare earth cations inhibit mitochondrial Ca^{2+} transport in a competitive manner [13—17], while Ruthenium red is a commonly used noncompetitive inhibitor [16,18,19]. Their K_i is very low (about 0.03 μM), compared to the Ca^{2+} concentrations commonly used for Ca^{2+} transport measurements. Ba^{2+} is taken up by mitochondria in a similar way to Ca^{2+} [17, 20] and thus would be a competitive inhibitor of Ca^{2+} transport.

The aim of this work was to study the effects of these inhibitors of Ca^{2+} binding and transport on the transport kinetics.

Methods and Materials

Rat-liver mitochondria were prepared from young male rats (Sprague-Dawley) by a conventional procedure [21].

Ca^{2+} uptake was measured by using the EGTA quenching method essentially as described by Reed and Bygrave [16], in order to be able to distinguish between external binding and transport of Ca^{2+}. No Ca^{2+} buffers were used because the agents studied might affect the buffering. The buffers might also bind the inhibitors and thus decrease their active concentration. The mitochondria were removed from the medium either by Millipore filtration (pore size 0.6 μm) or rapid centrifugation. Energy-independent Ca^{2+} binding was measured as described before [22]. The ^{45}Ca was counted in a Packard Tricarb liquid scintillation spectrometer in Bray's solution (PPO, POPOP, naphthalene, ethylenglycol, dioxan and methanol). Three different reaction media were used: 0.25 M sucrose/20 mM Tris · Cl, pH 7.4 (sucrose medium). The same medium with 2 mM $MgCl_2$ (sucrose/Mg medium), and 130 mM KCl/20 mM Tris · Cl/2 mM $MgCl_2$, pH 7.4 (KCl/Mg medium).

Reagents: FCCP (carbonylcyanide *p*-trifluoromethoxy phenylhydrazone) was obtained from Dr. P.G. Heytler and Ruthenium red from BDH Chemicals Ltd, Poole, England. The Ruthenium red was recrystallized accoring to Luft [23] before use.

Results

Effect of Mg^{2+} and K^+ on the Ca^{2+} transport kinetics

A hyperbolic plot is obtained when mitochondrial Ca^{2+} uptake is measured in the sucrose medium at +5° C and the initial rate is plotted against the Ca^{2+} concentration. In the presence of Mg^{2+} the kinetic plot becomes sigmoidal. In the KCl/Mg medium the sigmoidicity is still more pronounced (Fig. 1). This effect is of interest because neither Mg^{2+} nor K^+ is translocated by rat-liver mitochondria under these conditions, but both are competitive inhibitors of the binding of Ca^{2+} to the low affinity sites [5,7—9].

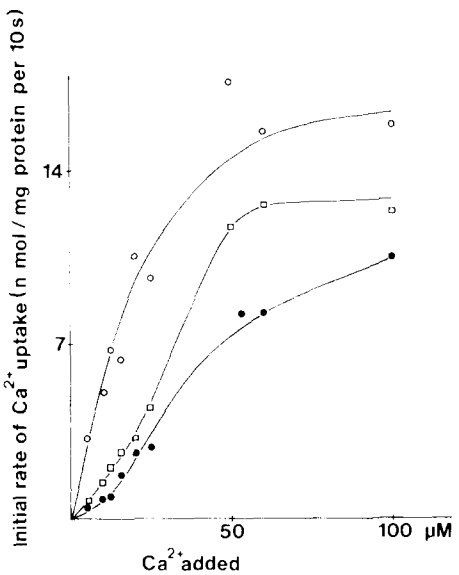

Fig. 1. Effect of Mg^{2+} and $K^+ + Mg^{2+}$ on mitochondrial Ca^{2+} transport kinetics. Mitochondria (0.5 mg protein/ml) were incubated 10 s in the presence of 10 mM succinate 5 μM rotenone and $^{45}CaCl_2$ (5—100 μM) at +5° C before quenching by an addition of 1 mM EGTA. Media: sucrose (○———○), sucrose/Mg (○———□) and KCl/Mg (●———●). After quenching the mitochondria were removed by millipore filtration and the radioactivity remaining in the filters after washing with cold medium was counted.

Effect of Ba^{2+} on the Ca^{2+} transport kinetics and on Ca^{2+} binding to the low affinity sites

Ba^{2+} is translocated into mitochondria in a similar way as Ca^{2+} [17,21]. It also induces similar respiratory stimulation [21]. Thus Ba^{2+} may be expected to inhibit mitochondrial Ca^{2+} transport competitively. Fig. 2 shows the effect of Ba^{2+} on the Ca^{2+} transport kinetics. In the sucrose medium the plot becomes

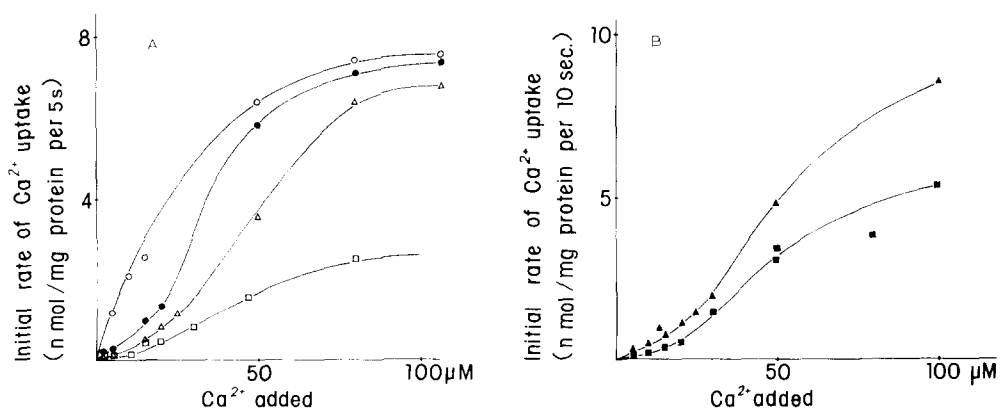

Fig. 2. Effect of Ba^{2+} on mitochondrial Ca^{2+} transport kinetics. Experimental conditions as in Fig. 1. Incubations made in (A) the sucrose medium (○———○) containing 25 μM $BaCl_2$ (●———●) and 50 μM $BaCl_2$ (△———△), 100 μM $BaCl_2$ (□— — —□), (B) in the KCl/Mg medium (▲———▲) containing 100 μM $BaCl_2$ (■———■). Mitochondrial protein 1 mg/ml.

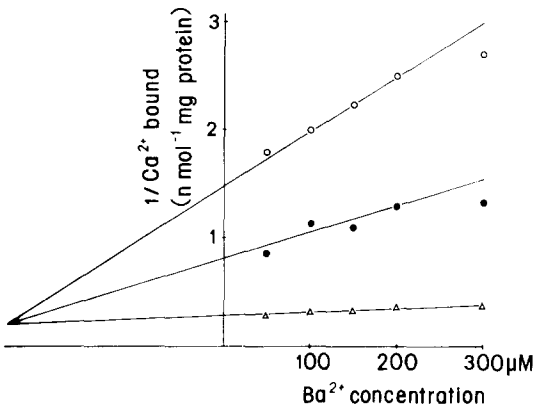

Fig. 3. Dixon plots for the inhibition of Ca^{2+} binding to the low affinity sites by Ba^{2+}. Mitochondria (5 mg protein/ml were incubated in the sucrose medium containing 5 μM FCCP and 1 μM Ruthenium red for 1 min. Thereafter $^{45}CaCl_2$ at a concentration of 25 μM (○———○), 50 μM (●———●) and 100 μM (△———△) was added and the incubation was continued for 1 min. The mitochondria were removed from the medium by centrifugation (14 000 × *g*) and samples were taken from the pellets for counting after solubilisation in 1 M formic acid.

sigmoidal in the presence of Ba^{2+} (Fig. 2a). In the KCl/Mg medium Ba^{2+} increases the sigmoidicity of the plot (Fig. 2b). In Fig. 2 it is also seen that Ba^{2+} inhibits Ca^{2+} transport more strongly in the sucrose medium as compared to the KCl/Mg medium. Dixon and Lineweaver-Burk plots (not shown) indicate that the K_i of Ba^{2+} varies between less than 10 μM at lower Ca^{2+} concentrations (10—30 μM), to about 70 μM, at higher Ca^{2+} concentrations (50—100 μM). In the KCl/Mg medium the K_i of Ba^{2+} is about 100 μM over the whole Ca^{2+} concentration range as calculated from Dixon plots. Due to the similar chemistry of Ba^{2+} and Ca^{2+} the former may be expected to inhibit all individual steps in the Ca^{2+} transport process. Fig. 3 shows Dixon plots for the inhibition of Ca^{2+} binding to the low affinity sites by Ba^{2+}.

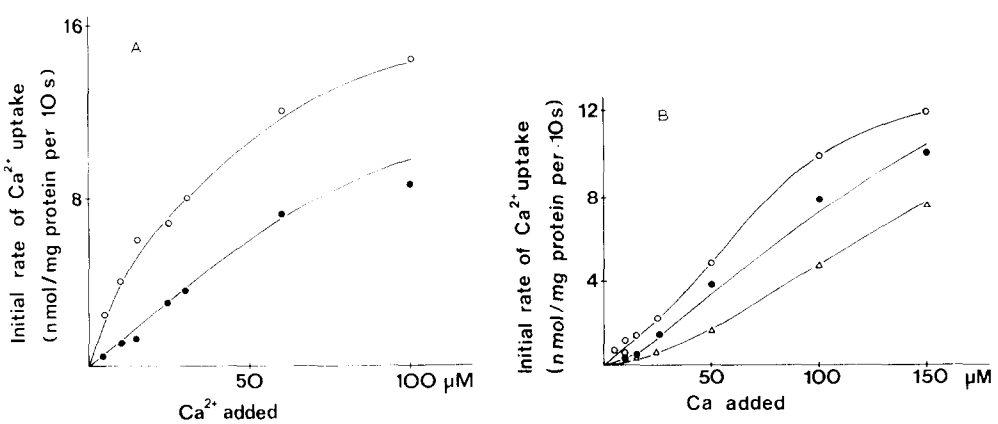

Fig. 4. Effect of La^{3+} on mitochondrial Ca^{2+} transport kinetics. Experimental conditions as in Fig. 1. Incubations were made in (A) the sucrose medium (○———○) containing 0.06 μM $LaCl_3$ (●———●), (B) the KCl/Mg medium (○———○) containing 0.03 μM (●———●) and 0.06 μM $LaCl_3$ (△———△). Mitochondrial protein 1 mg/ml in (A) and 0.5 mg/ml in (B).

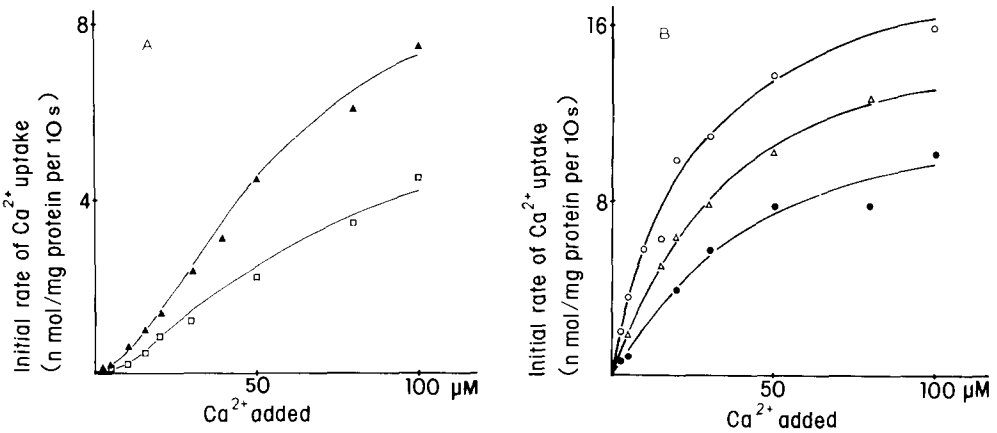

Fig. 5. Effect of Ruthenium red on mitochondrial Ca^{2+} transport kinetics. Experimental conditions as in Fig. 1. Incubations were made (A) in the KCl/Mg medium (▲———▲) containing 0.06 μM Ruthenium red (□———□) or (B) in the sucrose medium (○———○) containing 0.03 μM (△———△) or 0.06 μM Ruthenium red (●———●).

Effect of La^{3+} on the Ca^{2+} transport kinetics

In the sucrose medium the kinetic plot is still hyperbolic in the presence of La^{3+} (Fig. 4a). In the KCl/Mg medium the shape of the plot becomes slightly more sigmoidal in the presence of La^{3+} (Fig. 4b). Dixon plots (not shown) for the inhibition of Ca^{2+} transport by La^{3+} indicate that the K_i of La^{3+} in the sucrose medium does not differ significantly from that in the KCl/Mg medium. Due to the very low concentration used (0.03—0.06 μM) La^{3+} ought not to affect Ca^{2+} binding to the low affinity sites significantly.

Effect of Ruthenium red on the Ca^{2+} transport kinetics

Ruthenium red does not affect the shape of the kinetic plot to any significant degree. It is equally potent at all Ca^{2+} concentrations used. This is shown in Fig. 5. Dixon plots (not shown) also indicate that Ruthenium red is an equally potent inhibitor during the slow phase (15 μM) and during the steep rise of the kinetic plot (50 μM) and that the K_i in the sucrose medium does not differ significantly from the K_i in the KCl/Mg medium. Because the K_i for Ruthenium red is in the same range as that for La^{3+} [16] it is not expected to interact with the low affinity binding of Ca^{2+}.

Discussion

The results of this work show that a hyperbolic kinetic plot (initial rate versus concentration) is obtained for Ca^{2+} transport in rat-liver mitochondria when suspended in a sucrose medium. This holds for a Ca^{2+} concentration range of 5—100 μM. In the presence of Mg^{2+} the plot becomes sigmoidal. This effect is enhanced when both Mg^{2+} and K^+ are present. Since both Mg^{2+} and K^+ inhibit Ca^{2+} binding to the low affinity sites their effects on calcium transport may well result from an interference with Ca^{2+} binding to these sites. K^+ alone does not change the shape of kinetic plots significantly (submitted for publication),

possibly because of the sites having a lower affinity for K^+ or because of K^+ having only one charge. Neither Mg^{2+} nor K^+ is translocated by the mitochondria under the experimental conditions. Some workers [1,2] have reported sigmoidal Ca^{2+} transport kinetics also in a sucrose medium. However, they used ATP as a Ca^{2+} buffer and were thus able to measure Ca^{2+} transport at very low free Ca^{2+} concentrations. The sigmoidicity of these plots was apparent at free Ca^{2+} concentrations near 1 μM. Thus it appears possible that Mg^{2+} and K^+ do not actually change the hyperbolic kinetics to sigmoidal but may shift the point at which the sigmoidicity occurs to higher Ca^{2+} concentrations. The use of Ca^{2+} buffers in this work was not possible due to interaction of the used inhibitors with the buffer.

The sigmoidal kinetics have previously (Vinogradov and Scarpa, ref. 3) been ascribed to a cooperative transport mechanism where binding of two Ca^{2+} to a carrier would be required for translocation. They measured Ca^{2+} transport in a KCl/Mg medium using the murexide technique and obtained a result very similar to ours (Fig. 1). They also reported a change from sigmoidal to hyperbolic Mn^{2+} transport kinetics in the presence of Ca^{2+}. However, measurement of cation transport with the murexide technique in the presence of other cations that also interact with murexide is questionable. The results of this work show that Ba^{2+}, which like Mn^{2+} is translocated by mitochondria, does not change the kinetics of Ca^{2+} transport from sigmoidal to hyperbolic. If anything Ba^{2+} enhances the sigmoidicity of the kinetic plot (see Fig. 2b). Ca^{2+}, Mn^{2+} and Ba^{2+} could of course be translocated by different mechanisms. However, this appears highly unlikely in view of the many similarities in their mode of transport [20]. Ba^{2+} is a competitive inhibitor of Ca^{2+} transport. It is of interest that Ba^{2+} changes the Ca^{2+} transport kinetics from hyperbolic to sigmoidal like Mg^{2+}. It is also of interest that the K_i of Ba^{2+} for Ca^{2+} transport increases in the KCl/Mg medium as compared to the sucrose medium. This would suggest a competition between the cations (K^+, Mg^{2+} and Ba^{2+}) for the low affinity sites. The inhibition of Ca^{2+} binding to the low affinity sites by Ba^{2+} confirms this.

The results reported here indicate that an unspecific alteration of the surface of the membrane might determine the shape of the kinetic plot. The surface potential is determined by the charge density and hence presumably by the ions bound at nonspecific sites. Spermine, a tetravalent polyamine, known to decrease the negative surface charge of mitochondria significantly [24], also changes the shape of kinetic plots in a similar way to Mg^{2+} (submitted for publication), which gives further evidence in favour of the above interpretation. Moreover it has recently been demonstrated [25] that transport kinetics of ions may mimic the kinetics of allosteric mechanisms (sigmoidal kinetics) when the surface has the same charge as the translocated ion. Our results indeed suggest that the surface properties of the mitochondrial membrane have great influence on the Ca^{2+} transport kinetics. Mg^{2+} and spermine also decrease the activation energy of Ca^{2+} transport significantly, whereas neither affects that of respiration [26] suggesting that not only the charge but also the mobility of the polar head-groups of phospholipids at the surface of the mitochondrial membrane might be of importance in the kinetics of Ca^{2+} transport.

La^{3+} and ruthenium red, which are inhibitory at very low concentrations (K_i about 0.04 μM), do not affect the shape of the kinetic plot significantly.

This also is consistent with the above proposal that the surface properties of the mitochondrial membrane might have great influence on the Ca^{2+} transport kinetics, because neither of these agents is expected to affect the surface charge significantly at the very low concentration used.

It is concluded that Ca^{2+} transport in mitochondria involves at least two steps. First, the movement of the cation to the surface of the membrane with or without subsequent binding to the unspecific low affinity sites, and secondly, the actual translocation process. The first step is strongly dependent on the surface potential and hence on the number and charge of ions bound to the surface sites as K^+ and Mg^{2+}.

It is interesting to speculate that changes in the electrical surface properties of the mitochondrial membrane (by control of the binding constants for K^+, Mg^{2+} etc.) may control the kinetics of Ca^{2+} transport also in vivo. A change from sigmoidal to hyperbolic Ca^{2+} uptake kinetics would have profound effects on the translocation rate especially at the low intracellular Ca^{2+} concentrations. Recently it has been demonstrated that Ca^{2+} transport in smooth muscle mitochondria obeys essentially hyperbolic kinetics [27,28,29] even in the presence of K^+ and Mg^{2+}. Thus it appears possible that the surface of these mitochondria might have different properties from those of liver and heart. It has also been shown that muscle mitochondria from various aquatic arthropods show a large variety of energy independent Ca^{2+} binding characteristics, i.e. variations in binding constants and number of binding sites for Ca^{2+} [30]. This suggests that the surface properties of mitochondrial membranes might vary significantly between different tissues and species.

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References

1 Bygrave, F.L., Reed, K.C. and Spencer, T. (1971) Nat. New Biol. 230, 89
2 Spencer, T. and Bygrave, F.L. (1973) Bioenergetics 4, 347–362
3 Vinogradov, A. and Scarpa, A. (1973) J. Biol. Chem. 248, 5527–5531
4 Rossi, C., Azzi, A. and Azzone, G.F. (1967) J. Biol. Chem. 242, 951–957
5 Reed, K.C. and Bygrave, F.L. (1974) Biochem. J. 142, 555–566
6 Chappell, J.B., Cohn, M. and Greville, G.D. (1963) in Energy-Linked Functions of Mitochondria (Chance, B., ed.), pp. 219–231, Academic Press, New York and London
7 Scarpa, A. and Azzi, A. (1968) Biochim. Biophys. Acta 150, 473–481
8 Scarpa, A. and Azzone, G.F. (1968) J. Biol. Chem. 243, 5132–5138
9 Jacobus, W.E. and Brierley, G.P. (1969) J. Biol. Chem. 244, 4995–5004
10 Scarpa, A. and Azzone, G.F. (1969) Biochim. Biophys. Acta 173, 78–85
11 Saris, N.-E.L. (1972) in Biochemistry and Biophysics of Mitochondrial Membranes, pp. 641–652, Academic Press, New York and London
12 Azzone, G.F., Massari, S., Rossi, E. and Scarpa, A. in Mitochondria Structure and Function (Ernster, L. and Drahota, Z., eds.), pp. 301–318, Academic Press, New York and London
13 Mela, L. (1967) Fed. Proc. Fed. Amer. Sci. Exp. Biol. 26, 456
14 Mela, L. (1968) Arch. Biochem. Biophys. 123, 286–293
15 Mela, L. (1969) Biochemistry 8, 2481–2486
16 Reed, K.C. and Bygrave, F.L. (1974) Biochem. J. 140, 143–155
17 Vainio, H., Mela, L. and Chance, B. (1970) Eur. J. Biochem. 12, 387–391
18 Moore, C.L. (1971) Biochem. Biophys. Res. Comm. 42, 298–305

19 Vasington, F.D., Gazzotti, P., Tiozzo, R. and Carafoli, E. (1972) Biochim. Biophys. Acta 256, 43—54
20 Drahota, Z., Gazzotti, P., Carafoli, E. and Rossi, C.S. (1969) Arch. Biochem. Biophys. 130, 267—273
21 Wikström, M.K.F. and Saris, N.-E.L. (1969) Eur. J. Biochem. 9, 160—166
22 Åkerman, K.E., Saris, N.-E.L. and Järvisalo, J.O. (1974) Biochem. Biophys. Res. Commun. 5, 801—807
23 Luft, J.H. (1971) Anat. Rec. 171, 347—368
24 Huunan-Seppälä, A.J. (1972) MD Thesis, University of Helsinki, Finland
25 Theunevet, A.P.R. and Borst-Pauwels, G.W.F.H. (1976) J. Theor. Biol. 57, 313—329
26 Åkerman, K.E.O. (1976) J. Bioenergetics, in press
27 Wikström, M., Ahonen, P. and Luukkainen, T. (1975) FEBS Lett. 56, 120—123
28 Batra, S. (1975) in Calcium Transport in Contraction on Secretion (Carafoli, E. et al., eds.), pp. 87—94, North-Holland Publishing Company
29 Vallieres, J., Scarpa, A. and Somlyo, A.P. (1975) Arch. Biochem. Biophys. 170, 659—669
30 Honnappa, G.V., Sulochana, R.H. and Jayaraman, J. (1975) J. Bioenergetics 7, 149—159