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Ca^{2+} TRANSPORT MEDIATED BY A SYNTHETIC NEUTRAL Ca^{2+}-IONOPHORE IN BIOLOGICAL MEMBRANES

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Summary

The effect of a synthetic neutral ligand on the Ca^{2+} permeability of several biological membranes has been investigated. The ligand had been previously shown to possess Ca^{2+}-ionophoric activities in artificial phospholipid membranes. The neutral ionophore is able to transport Ca^{2+} across the membranes of erythrocytes and sarcoplasmic reticulum, when lipophilic anions such as tetraphenylborate or carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) are present, presumably to facilitate the diffusion of the charged Ca^{2+}-ionophore complex across the hydrophobic core of the membrane.

In mitochondria, the neutral ionophore promotes the active transport of Ca^{2+} in response to the negative membrane potential generated by respiration, in the presence of the specific inhibitor of the natural carrier ruthenium red.

Introduction

Ionophore molecules have been proved to be quite useful in the investigation of ion transport across the hydrophobic phase of natural membranes [1—4]. These compounds can selectively change the ionic permeability of a membrane and therefore could be used to mimic the properties of the ion carriers normally found in biological membranes.

In recent years a number of ionophores with a wide range of ion selectivity have been isolated or synthesized [1,5—7]. They belong to two different classes: those which bear no net electrical charge, and thus become charged when complexed with an ion, and those which bear a net electrical charge, and thus become neutral when complexed with an ion. Among the most recently

Abbreviations used: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

described specific ionophores of a particular interest are the antibiotics X-537 A and A23187, which display preference for Ca^{2+} and other divalent cations. The usefulness of specific Ca^{2+}-ionophores is obvious, since they provide a powerful tool in the study of the mechanism of the translocation of Ca^{2+} across biological membranes.

Both X-537 A and A23187 are charged molecules, which combine with Ca^{2+} with a 2 : 1 (carrier : cation) stoichiometry, which can change at low concentrations of the ionophore, thus yielding a neutral complex [8,9]. Clearly, then, they will promote the transmembrane translocation of Ca^{2+} in a concentration gradient, but will not respond to a membrane potential. The electrophoretic translocation of Ca^{2+} due to an electrical potential would, on the other hand, be promoted by specific neutral ionophores, much as is the case, for example, for the K^+ valinomycin system [10,11]. Two naturally occurring neutral Ca^{2+} ionophores, avanaciolide [12] and beauvericin [13] have been briefly studied in artificial and natural membranes (mitochondria and bacterial chromatophores), and shown to display a wide range of cation selectivity. A neutral ionophore has been recently synthesized [6] (Fig. 1), and shown to have a very high selectivity for Ca^{2+} in bulk phase membranes [14]. In recent studies, it has been shown that this ionophore is also able to transport Ca^{2+} across planar lipid membranes and single bilayer phospholipid vesicles, provided that uncouplers are also present in the medium. Their effect was tentatively attributed to the equilibration of H^+ across the membrane or to the counterbalance of the net positive charge of the ionophore · Ca^{2+} complex by penetrating in the dissociated form into the apolar domain of the membrane [15].

In the low dielectric constant of the artificial phospholipid membranes, a decrease in the selectivity of the ionophore towards Ca^{2+} and an increased preference for monovalent ions (Na^+, H_3O^+, K^+) has been observed. In this paper, the effect of the neutral Ca^{2+}-ionophore on several biological membranes has been investigated. The studies on mitochondria have been particularly interesting, since these organelles possess a carrier-mediated transport of Ca^{2+} driven by a negative membrane potential [16,17]. The results obtained have shown that the neutral Ca^{2+}-ionophore is able to substitute for the natural carrier in mitochondria to promote the active accumulation of Ca^{2+} when a

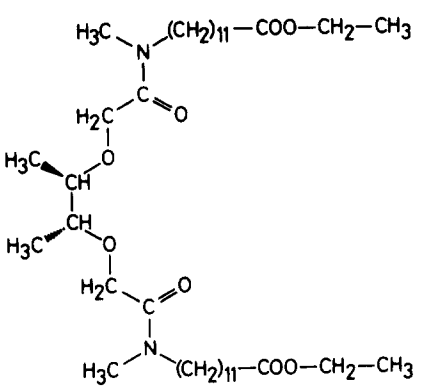

Fig. 1. Structure of the neutral synthetic Ca^{2+}-ligand.

negative potential inside the vesicle is available. The neutral ionophore is also effective in erythrocytes and sarcoplasmic reticulum.

Materials and Methods

Fresh red blood cells were obtained from the local blood bank and washed twice at room temperature with a medium containing, 130 mM KCl, 20 mM HEPES pH 7.4. Depletion of endogenous ATP was obtained as previously described [18].

The erythrocytes were then loaded with $CaCl_2$ by hemolysis in 5 volumes of 4 mM $MgCl_2$, 5 mM HEPES, pH 7.4, and 1 mM $CaCl_2$. After 2 min at room temperature the medium was made isotonic by adding a KCl solution, three more minutes of incubation were then allowed then the resealed cells were diluted at 0°C with 130 mM KCl, 20 mM HEPES, pH 7.4, and centrifuged at 10 000 × *g* for 5 min. Rabbit skeletal-muscle sarcoplasmic reticulum was prepared as previously described [19]. Mitochondria were prepared from rat livers as previously described [20]. Ca^{2+} was measured spectrophotometrically using the Ca^{2+}-indicator Arsenazo III, the change in absorbance were followed at 685—660 nm in a DW/2 Aminco spectrophotometer [21]. In some experiments, Ca^{2+} was followed using $^{45}Ca^{2+}$ and millipore filtration. Mitochondrial swelling measurements were carried out spectrophotometrically by determining the changes in absorbance at 540 nm. The ATPase activity of mitochondria was determined by following the proton changes during the reaction [22]. Protein concentration was determined with a biuret reaction.

The neutral ionophore was prepared as previously described (see ref. 46 in ref. 6). A23187 was obtained from Eli Lilly and Co., Indianapolis, Ind. U.S.A.; valinomycin was a gift of Dr. P.G. Heytler, Dupont, Wilmington, Del., U.S.A. All the solutions of ionophores and uncouplers were prepared in ethanol. Arsenazo III (BDH Chemical Ltd., Poole, U.K.) was decalcified prior to use as previously described [21]. All the other reagents used were analytical grade.

Results

Erythrocytes

The effect of ionophores on the Ca^{2+} permeability of red blood cells has been studied using cells depleted of endogenous ATP, to prevent the active translocation of Ca^{2+} by the membrane-bound Ca-Mg ATPase. As expected, (Fig. 2) a fast release of Ca^{2+} is induced by the charged antibiotic A23187, which readily exchanges Ca^{2+} for H^+. The neutral Ca^{2+}-ionophore, however, does not release Ca^{2+} unless the uncoupler anion FCCP, or the lipophilic anion tetraphenylborate (Fig. 2) are also added to the medium. Independent tests (erythrocytes loaded with [^{14}C]sucrose) have shown that the ligand does not induce a non-specific leakiness of the membrane. As will be discussed more fully below, the effect of FCCP is not related to its H^+ carrying ability, since valinomycin, in the presence of external K^+, does not induce release of Ca^{2+} from the erythrocytes (Fig. 2). The neutral ionophore induces Ca^{2+} release in a concentration range between 5 and 30 μM in the presence of lipophilic anions, but at higher concentrations a slow release of Ca^{2+} is induced also by the

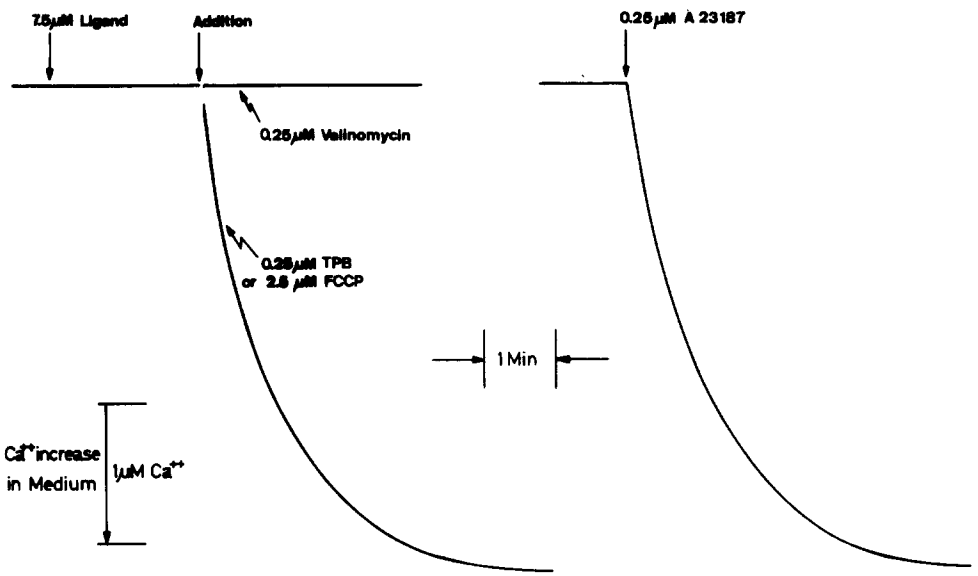

Fig. 2. Spectrophotometric determination of the release of Ca^{2+} from erythrocytes mediated by Ca^{2+}-ionophores. The medium contained: 130 mM KCl, 20 mM HEPES/KOH pH 7.4, 50 μM Arsenazo III, and 10 μl of Ca^{2+}-loaded erythrocyte cells in a final volume of 1 ml. The assay was carried out at 25°C as described in Materials and Methods. TPB, tetraphenylborate.

ionophore alone. A23187 is more efficient than the neutral ionophore, since it promotes the same rate of release at concentrations 5—10 times lower.

Sarcoplasmic reticulum

The neutral Ca^{2+} ionophore increases the Ca^{2+} permeability also in the membrane of sarcoplasmic reticulum vesicles. After activation of the sarcoplasmic reticulum Ca^{2+}-pump with ATP, Ca^{2+} is accumulated in the vesicles and is maintained inside until the added ATP is all hydrolyzed (Fig. 3). As was the case with erythrocytes, no release of Ca^{2+} was observed when the neutral ionophore was added alone. However, the subsequent addition of the lipophilic anion FCCP induced a rapid loss of the accumulated Ca^{2+} from the vesicles (the order of the addition could be reversed). Since some ATP was still present in the medium after the uptake, the release measured during the experiment was presumably underestimated, since it was the result of the release of Ca^{2+} induced by the Ca^{2+}-ionophore, minus the re-uptake of Ca^{2+} promoted by the Ca^{2+}-pump. Therefore, concentrations of ionophore slightly higher than in erythrocytes had to be used in these experiments in order to obtain a release which was faster than the active uptake.

Mitochondria

Coupled mitochondria maintain a membrane potential, negative inside, of about 180 mV, which is generated by the transfer of electrons along the respiratory chain [23]. Mitochondria, therefore, are an ideal system to study the effect of the neutral ionophore, since in their case Ca^{2+} should be expected

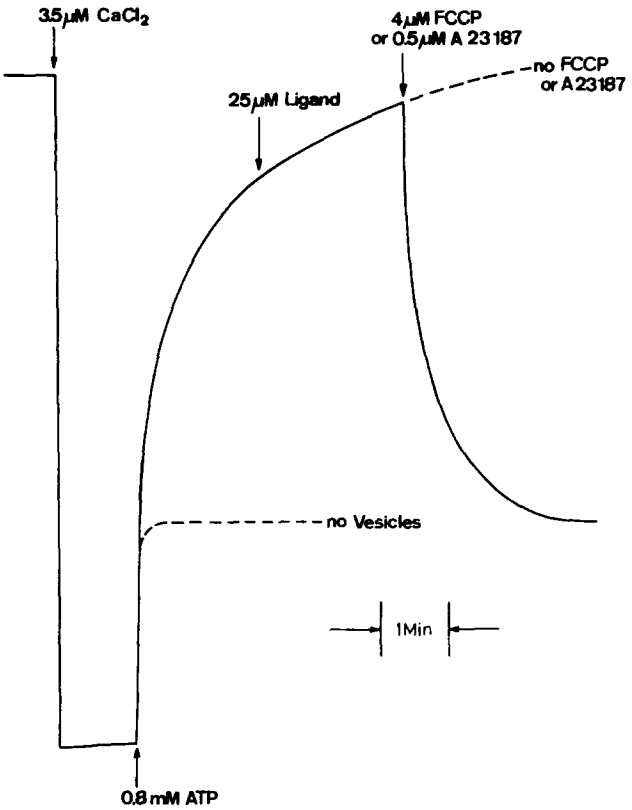

Fig. 3. Release of Ca^{2+} from sarcoplasmic reticulum mediated by the neutral ionophore. The reaction medium contained 50 mM KCl, 10 mM $MgCl_2$, 20 mM imidazole buffer, pH 7.2, 100 μM Arsenazo III and 0.3 mg membrane protein in a final volume of 1 ml, at 20° C. The accumulation of Ca^{2+} in the vesicles was induced with 0.8 mM K-ATP. Immediately after the latter addition, some Ca^{2+} disappeared from the medium, probably due to chelation by ATP.

to be transported inside in response to the negative potential. Fig. 4 shows that the neutral Ca^{2+}-ionophore is indeed able to promote the transport of Ca^{2+} into energized mitochondria by substituting for the natural carrier, which was blocked with the specific inhibitor ruthenium red, (mitochondria had been preincubated with the respiratory inhibitor rotenone, to allow the discharge of the endogenous Ca^{2+}, to promote the uptake of larger amounts of Ca^{2+}). As shown in the figure, and as is the case in normal mitochondria in which the natural carrier operates, the accumulated Ca^{2+} could be discharged with inhibitors of respiration or uncouplers, which dissipate the membrane potential. The amount of ionophore needed to obtain maximal uptake of Ca^{2+} was about 40 μM. At higher concentrations of the ionophore a slight decrease of the uptake was sometimes observed. The maximal amount of Ca^{2+} that can be accumulated in the presence of the neutral ionophore (Fig. 5) is about 60 nmol/mg protein, i.e., a smaller amount than can be accumulated in mitochondria when the natural carrier is operating in the absence of permeant anions (80—120 nmol/mg protein) [24].

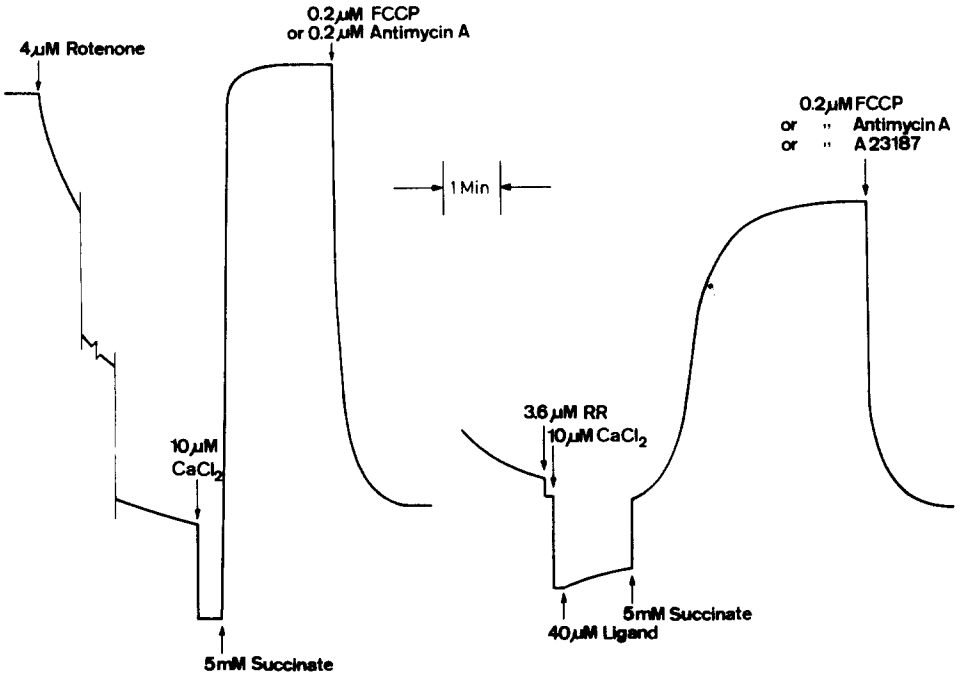

Fig. 4. Active uptake of Ca^{2+} by mitochondria in the presence of the neutral ionophore. The movements of Ca^{2+} were followed using Arsenazo III. The medium contained: 210 mM mannitol, 70 mM sucrose, 10 mM Tris · HCl, pH 7.2, 40 μM Arsenazo III preincubated in the medium for 8 min to allow complete release of the endogenous Ca^{2+}. Temperature, 25° C.

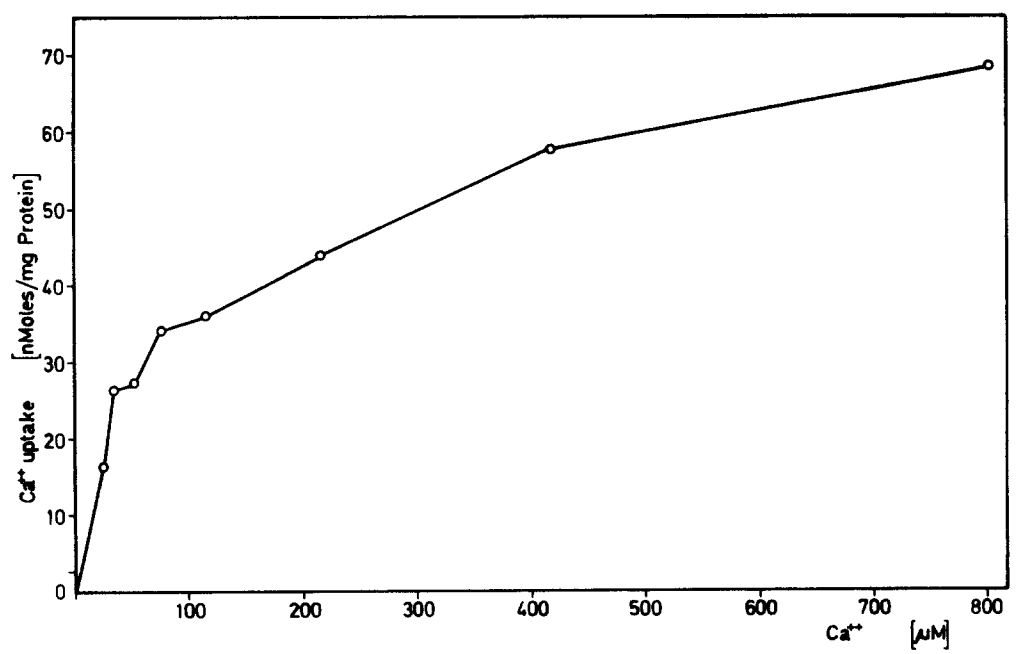

Fig. 5. Ca^{2+} uptake in mitochondria at different Ca^{2+} concentrations. The medium was the same as in Fig. 4, without Arzenazo III. The mitochondria were preincubated with rotenone for 10 min at 25° C, before the addition of $^{45}CaCl_2$ and succinate. Aliquots were withdrawn 3 min after the addition of succinate.

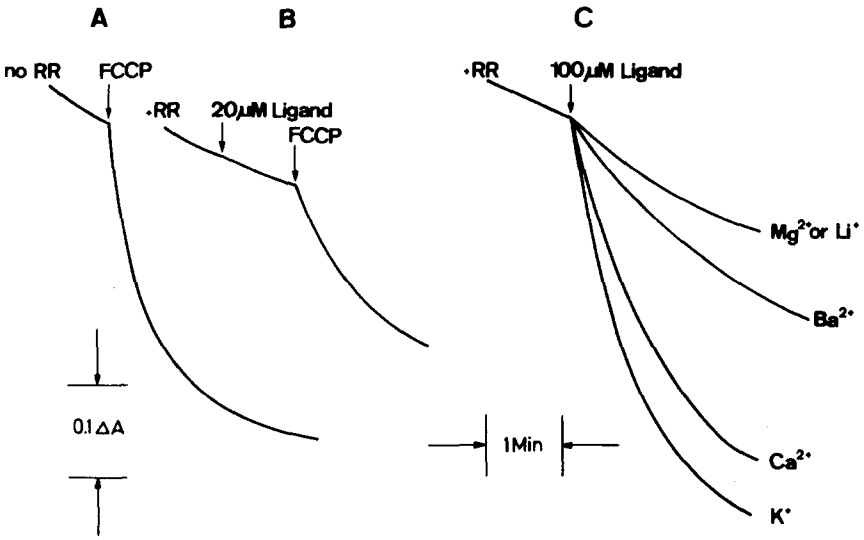

Fig. 6. Swelling of mitochondria in the presence of the neutral ionophore and different acetates. The medium contained the salts of divalent (80 mM) or of monovalent cations (120 mM), 5 mM HEPES/KOH, 5 μM rotenone, and 1.2 mg prot./ml. Final volume 2 ml. Ruthenium red (RR), when added, was 4 μM. Temperature 25°C. A, control experiment without added ionophore and Ca^{2+}-acetate; B, Ca^{2+}-acetate in the presence of low concentrations of the neutral ionophore, and ruthenium red; C, accetates in the presence of high concentrations of neutral ionophore, and ruthenium red.

Since some of the selectivity of the neutral ionophore was lost in artificial phospholipid bilayer membranes with respect to bulk phase membranes [15], the ability of the ligand to transport other cations was tested by studying the swelling of mitochondria in isotonic media of acetates of K^+, Ca^{2+} or other cations. In the experiment shown in Fig. 6, FCCP was also added to facilitate the penetration of acetate [25] and ruthenium red was added to block the natural Ca^{2+} carrier. Since swelling will only occur if salt accumulates inside mitochondrial space, the experiment clearly shows that the ionophore is able to transport both K^+ and Ca^{2+}. No conclusion can be drawn for the case of Na^+, since this cation can also be transported by mitochondria in exchange for H^+ [26], and therefore, swelling occurs also in the absence of the neutral ionophore and FCCP.

In addition to K^+ and Ca^{2+}, the neutral ionophore seems to be able to transport protons across the inner mitochondrial membrane. This is indicated by its ability to stimulate the mitochondrial ATPase, a phenomenon that requires the permeabilization of the inner membrane to protons (not shown).

Discussion

The transport of ions across lipid membranes mediated by neutral ionophores occurs via the formation of charged complexes, in which the charged ion is shielded by the hydrophobic countour of the ionophore molecules, and is thus allowed to penetrate into the hydrophobic domain of the membrane. The neutral Ca^{2+}-ionophore studied in this paper is apparently unable to shield very

efficiently the charge of the transported Ca^{2+}, at least when used at low concentrations. As a result, the diffusion of the complex through the hydrophobic phase of the membrane must be facilitated by the addition of lipopholic anions like tetraphenylborate or FCCP, which are known to diffuse into the membrane in the anionic form and, therefore, can counterbalance the positive charge in the ionophore · Ca^{2+} complex in the apolar domain of the membrane. The potentiating effect of FCCP could, in principle, be explained with the antiport of H^+, as mentioned before; however, if this were the correct interpretation, the antiport of K^+ promoted by valinomycin should have lead to the same potentiating effect, which was not the case. In addition, tetraphenylborate would not be expected to potentiate the effect of the neutral Ca^{2+}-ionophore, since it can not act as an H^+ carrier (it has a pK of <2, and, accordingly, it does not uncouple respiration in intact mitochondria).

In mitochondria, the electrochemical potential generated during the transport of electrons in the respiratory chain can drive the electrophoretic transport of Ca^{2+} in the presence of the neutral ionophore, under conditions in which the natural carrier is inhibited, and in this case tetraphenylborate is not needed. The transport of positive lipophilic ions into mitochondria in response to the negative membrane potential has been already reported [27]. As expected, the Ca^{2+} accumulated in the presence of the neutral ionophore can be discharged when the membrane potential is collapsed with uncouplers, or wich inhibitors of respiration.

Under normal conditions, at the steady state, the maximal net uptake of Ca^{2+} mediated by the natural carrier corresponds to about 120 nmol/mg protein [24]. In the presence of the artificial carrier, this value is considerably lower. This may be explained with the observation that the neutral ionophore can also transport protons and can, therefore, decrease the membrane potential across the inner mitochondrial membrane.

The results obtained on mitochondria are of interest also for what concerns the mechanism of action of ruthenium red. This polyvalent cation could inhibit Ca^{2+} uptake either by screening fixed negative charges on the mitochondrial surface or by blocking specifically the natural Ca^{2+} carrier. The fact that active Ca^{2+} transport in mitochondria can be induced by the synthetic ionophore in the presence of ruthenium red rules out the first possibility, and adds support to the concept of a specific interaction of the inhibitor with the natural Ca^{2+} carrier.

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References

1 Pressman, B.C. (1976) Annu. Rev. Biochem. 45, 501—530
2 Reed, P.W. and Lardy, H.A. (1972) J. Biol. Chem. 247, 6970—6977
3 Pressman, B.C., Harris, E.J., Jagger, W.S. and Johnson, J.H. (1967) Proc. Natl. Acad. Sci. U.S. 58, 1949—1956
4 Heaton, G.M. and Nicholls, D.G. (1976) Biochem. J. 156, 635—646

5 Truter, M.R. (1976) in The Regulation of Intracellular Calcium (Duncan, C.J., ed.), pp. 19—40, Cambridge University Press, Cambridge
6 Amman, D., Bissig, R., Cimerman, Z., Fiedler, U., Guggi, M., Morf, W.E., Oehme, M., Osswald, H., Pretsch, E. and Simon, W. (1976) in Ion and Enzyme Electrodes in Biology and Medicine (Kessler, M. et al., eds.), pp. 22—37, Urban and Schwarzenberg, Munchen
7 Reed, P.W. (1972) Fed. Proc. 31, 432 Abs.
8 Pfeiffer, D.R., Reed, P.W. and Lardy, H.A. (1974) Biochemistry 13, 4007—4014
9 Hyono, A., Hendriks, Th., Deamen, F.J.M. and Bonting, S.L. (1975) Biochim. Biophys. Acta 389, 34—64
10 Moore, C. and Pressman, B.C. (1964) Biochem. Biophys. Res. Commun. 15, 562—567
11 Chappell, J.B. and Haarhoff, K.N. (1967) in Biochemistry of Mitochondria (Slater, E.C., Kaniuga, Z. and Wojtczak, L., eds.), pp. 75—91, Academic Press, New York
12 Harris, E.J. and Wimhurst, J.M. (1974) Arch. Biochem. Biophys. 162, 426—435
13 Prince, R.C., Crofts, A.R. and Steinrauf, L.K. (1974) Biochem. Biophys. Res. Commun. 59, 697—703
14 Amman, D., Güggi, M., Pretsch, E. and Simon, W. (1975) Anal. Lett. 8 (10), 709—720
15 Vuilleumier, P., Gazzotti, P., Carafoli, E. and Simon, W. (1977) Biochim. Biophys. Acta 467, 12—18
16 Mela, L. (1968) Fed. Proc. 27, 828 Abs.
17 Rottenberg, H. and Scarpa, A. (1974) Biochemistry 13, 4811—4817
18 Schatzmann, H.J. (1973) J. Physiol. 235, 551—569
19 Carvalho, A.P. and Mota, A. (1971) Arch. Biochem. Biophys. 142, 201—212
20 Schneider, W.C. (1957) in Manometric Techniques (Umbreit, W.W., Burris, R. and Stauffer, J.F., eds.), Burgess, Minneapolis, Minnesota
21 DiPolo, R., Requena, J., Brinley, Jr., F.J., Mullins, L.J., Scarpa, A.and Tifferet, T. (1976) J. Gen. Physiol. 67, 433—467
22 Nishimura, M., Ito, T. and Chance, B. (1962) Biochim. Biophys. Acta 59, 177—182
23 Mitchell, P. (1966) in Chemiosmotic Coupling and Oxidative and Photosynthetic Phosphorylation, Glynn Research Ltd., Bodmin
24 Drahota, Z., Carafoli, E., Rossi, C.S., Gamble, R.L. and Lehninger, A.L. (1965) J. Biol. Chem. 240, 2712—2720
25 Singer, M.A. and Bangham, A.D. (1971) Biochim. Biophys. Acta 241, 687—692
26 Mitchell, P. and Moyle, J., (1967) Biochem. J. 105, 1147—1162
27 Bakeeva, L.E., Grinius, L.L., Jasaitis, A.A., Kuliene, V.V., Levitsky, D.O., Liberman, E.A., Severina, I.I. and Skulachev, V.P. (1970) Biochim. Biophys. Acta 216, 13—21