Biochimica et Biophysica Acta, 727 (1983) 285–292
Elsevier Biomedical Press

BBA 71494

INTERACTION OF LANTHANIDE CATIONS AND URANYL ION WITH THE CALCIUM/PROTON ANTIPORT SYSTEM IN *MYCOBACTERIUM PHLEI*

NEERAJ AGARWAL and VIJAY K. KALRA *

Department of Biochemistry, University of Southern California, School of Medicine, Los Angeles, CA 90033 (U.S.A.)

(Received July 16th, 1982)

Key words: Lanthanide cation; Uranyl ion; Ca^{2+}/H^+ antiport; Ca^{2+} transport; Fluorescence; Membrane vesicle

Uranyl ions (UO_2^{2+}) and lanthanide cations (La^{3+}, Nd^{3+}, Sm^{3+}, Eu^{3+}, Tb^{3+} and Dy^{3+}) at 100–200 μM concentration inhibited active transport of Ca^{2+}, mediated by respiratory linked substrates as well as by ATP hydrolysis, without affecting respiration and membrane-bound ATPase activity, in inside-out membrane vesicles of *Mycobacterium phlei*. The extent of inhibition in the uptake of Ca^{2+}, mediated by ATP hydrolysis, increased with increase in ionic radii of these cations. Lanthanide cations did not dissipate the formation of a proton gradient, as measured by determining the effect either on the uptake of [^{14}C]methylamine or energy-linked quenching of the fluorescence of 9-aminoacridine. However, uranyl ion (UO_2^{2+}) caused reversal of the energy-linked quenching of 9-aminoacridine. UO_2^{2+} concentration yielding 50% of V_{max} ($S_{0.5}$) was approx. 15 μM. Kinetic studies revealed that inhibition in the uptake of Ca^{2+} was competitive with UO_2^{2+} while non-competitive with rare-earth metals. It is proposed that inhibition in the uptake of Ca^{2+} by uranyl ion occurs as a result of UO_2^{2+} transport into the interior of vesicles in exchange for protons, while lanthanide cations are not being transported but affect the binding of Ca^{2+} to the membrane, presumably to the Ca^{2+}/H^+ antiporter.

Introduction

Calcium is found in bacterial cells, however its distribution across the cell is unusual since its internal concentration is low when compared to the external environment. Recent studies have shown that bacteria contain a specific transport system for maintaining low levels of calcium within the cytoplasm [1,2]. We have previously reported the presence of active transport system for calcium, in inside-out membrane vesicles from *Mycobacterium phlei*, mediated by respiratory linked substrates as well as by ATP hydrolysis [3,4]. Studies show that uptake of calcium is energized by a protonmotive force [3] which, in inside-out membrane vesicles, has an orientation positive and acid interior. The uptake of calcium is inhibited by proton-conductors suggesting that a Ca^{2+}/H^+ antiport system is presumably responsible for the transport of calcium in *M. phlei* membrane vesicles, as has also been indicated for the transport of calcium in *Escherichia coli* [5,6] and *A. Vinelandi* [7]. In our laboratory [8] we have solubilized and partially purified the membrane protein(s) from *M. Phlei* which can mediate the translocation of calcium as has been demonstrated by reconstitution of calcium transport in proteoliposomes containing the partially purified carrier protein. However, the molecular mechanism of active transport of Ca^{2+} in *M. phlei* membrane vesicles is not clear since these membranes exhibit uptake of Ca^{2+} but do not contain ($Ca^{2+} + Mg^{2+}$)-ATPase as has

* To whom request for reprints should be addressed.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2 ethanesulfonic acid.

been shown in eukaryotic system.

In an effort to gain a molecular understanding of the mechanism of active transport of Ca^{2+} in membrane vesicles, studies were undertaken to determine whether lanthanide cations and uranyl ion which are isomorphic substitutes for Ca^{2+} in some biological systems [9] and competitive inhibitors of mitochondrial Ca^{2+} binding and transport [10] could be used as probes in *M. phlei* system. Moreover the trivalent lanthanides can be used as probes for assessing columbic interactions during the calcium transport process since they have very similar chemical properties and their ionic radii and free energies of hydration vary in graded sequential manner [10]. The results presented in this communication show that lanthanide cations and uranyl ion inhibit the uptake of Ca^{2+} in membrane vesicles of *M. phlei*, mediated either by respiratory linked substrates or by ATP hydrolysis, without affecting respiration and ATPase activity. Studies show that only UO_2^{2+} in contrast to lanthanide cations was capable of dissipation of proton gradient as measured by the quenching of fluorescence of 9-aminoacridine, indicating that the Ca^{2+}/H^+ antiport system transports UO_2^{2+} while lanthanide cations are not being taken up into the interior of the vesicles.

Materials and Methods

Preparation of membrane vesicles. *Mycobacterium phlei* ATCC 354, was grown and harvested as described by Brodie and Gray [11]. Membrane vesicles were prepared by sonication of cells as described by Brodie [12]. The membrane vesicles were suspended in 50 mM Tris acetate buffer (pH 8.0), containing 0.15 M KCl and 4 mM $MgCl_2$.

Trypsin treatment to activate ATPase in membrane vesicles. Unmasking of ATPase by trypsin treatment was carried out in a reaction mixture containing membrane vesicles (2 mg/ml) in 50 mM Hepes-KOH buffer (pH 7.5), 5 mM $MgCl_2$ and bovine pancreas trypsin (50 μg/mg membrane protein) for 10 min at 30°C. After 10 min the reaction was terminated by the addition of soybean trypsin inhibitor (100 μg/mg protein) then centrifuged at $105\,000 \times g$ for 45 min. The pellet was finally suspended in 50 mM Hepes-KOH containing 5 mM $MgCl_2$.

Assay of latent ATPase activity. ATPase activity in trypsin-treated membrane preparation was carried out in the presence of 4 mM $MgCl_2$ and 10 mM ATP at 30°C as described previously [13]. Unless otherwise indicated lanthanide cations and uranyl acetate were added 10 min prior to the addition of ATP in the reaction mixture. The inhibition of membrane-bound ATPase activity by *N,N'*-dicyclohexylcarbodiimide (DCCD) was assayed by the procedure described by Kalra and Brodie [14].

Assay of the calcium transport. Uptake of $^{45}Ca^{2+}$ in membrane vesicles and trypsin-treated vesicles was determined by filtration method on 0.45 μm Millipore filter as described previously [3,8]. Where indicated lanthanide cations and uranyl acetate were added prior to the initiation of uptake by the addition of $^{45}CaCl_2$ (50 μM).

Flow dialysis determination of the methylamine uptake, pH gradient. The uptake of [^{14}C]methylamine was measured in a flow dialysis apparatus as described by Ramos et al. [15]. The reaction mixture (0.85 ml) containing 50 mM Hepes-KOH buffer (pH 7.5), 5 mM $MgCl_2$, [^{14}C]methylamine (4.5 μM or 12.5 μM) and the trypsin-treated vesicles (1.5 mg protein) was stirred in the upper chamber and the uptake was initiated by the addition of 10 mM ATP. Buffer was pumped through the lower chamber at the rate of 6 ml/min with a peristaltic pump. Fractions of 2 ml were collected and assayed for radioactivity by liquid scintillation counting. Additions such as calcium chloride (1 mM) were made directly to the upper chamber. The control experiments, without ATP were run for each assay.

Measurement of the proton gradient by fluorescence method. Changes in transmembrane proton gradient (ΔpH) were estimated from the energy-linked quenching of 9-aminoacridine fluorescence essentially by the method of Schuldiner et al. [16] and Brey et al. [17]. The assay medium contained trypsin-treated vesicles (0.4 mg protein/ml), 50 mM Hepes-KOH (pH 7.4), 4 mM $MgCl_2$, 4 μM 9-aminoacridine in total volume of 2.5 ml. Unless otherwise indicated ATP was added at a concentration of 0.8 mM. Fluorescence measurements were carried out at 30°C using a Perkin-Elmer MPF-4 spectrofluorometer with excitation at 365 nm and emission at 451 nm.

Measurement of respiration. Rates of oxygen consumption in membrane vesicles were measured, in the presence of succinate (20 mM), with a clark-type oxygen electrode (YSI Model 53, oxygen monitor, Yellow Spring Instrument Co., Yellow Springs, OH).

Measurement of binding by equilibrium dialysis. The reaction mixture contained membrane vesicles (approx. 1 mg of Protein), 50 mM Hepes-KOH (pH 7.4), 5 mM $MgCl_2$, 40 μg of 2-*N*-nonyl-4-hydroxyquinoline-*N*-oxide and $^{45}Ca^{2+}$ (0.2 μM–2 μM) in a total volume of 0.5 ml. The incubation was carried out in the sample compartment of the equilibrium dialysis chamber (CRC Dialysis cells, Cleveland, OH). The lower compartment of the cell contained 50 mM Hepes-KOH buffer (pH 7.4) and 5 mM $MgCl_2$ [18,19]. The dialysis was performed under constant rhythmic shaking using Thermolyne Speci-mix (24 revolution/min) for 24 h with three changes of the dialysis fluid. After dialysis, an aliquot (0.1 ml) was removed from the sample compartment and the radioactivity was determined by liquid scintillation counting.

Protein measurement. Protein was estimated by the method of Lowry et al. [20] with bovine serum albumin as standard.

Materials. All radioactive chemicals were purchased from New England Nuclear. Europium chloride, terbium chloride, neodymium chloride, samarium chloride, dysprosium chloride were purchased from Aldrich Chemical Company. Lanthanum chloride was obtained from K and K Laboratories, Plainview, NY. Uranyl acetate was obtained from Polysciences Inc, Warrington, PA. All other reagents were of reagent grade purity and were purchased from Sigma.

Results

Effects of rare-earth metals on Ca^{2+} uptake, mediated by ATP hydrolysis

Membrane vesicles of *M. phlei*, obtained by sonication, are mostly (85–90%) oriented inside out [21]. These membrane vesicles contain latent ATPase activity which can be unmasked by trypsin treatment. Following trypsin treatment of membrane vesicles, ATP caused active transport of calcium ions. As shown in Fig. 1, addition of La^{3+} and Nd^{3+} at 200 μM concentration to the mem-

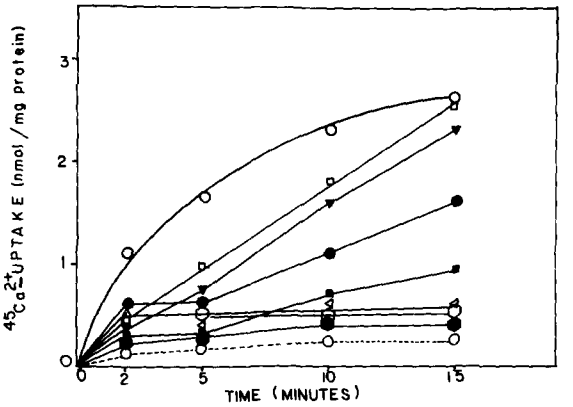

Fig. 1. Effect of lanthanides and uranyl ion on the uptake of Ca^{2+}. The reaction mixture (0.7 ml) contained trypsin-treated membrane vesicles (2 mg protein), 50 mM Hepes-KOH (pH 7.5) and 4 mM $MgCl_2$. The uptake was initiated by the addition of 1 mM ATP and 50 μM of $^{45}CaCl_2$. Where indicated lanthanide cation and uranyl acetate (pH 7.4) were added at a concentration of 200 μM, prior to the addition of radioactive calcium in the assay system. Samples of 0.1 ml were withdrawn at indicated intervals of time and the uptake of calcium was determined as described in Materials and Methods. O——O, $^{45}Ca^{2+}$ uptake, uptake of $^{45}Ca^{2+}$-in the presence of: Dy^{3+}, □——□; Tb^{3+}, ▲——▲; Eu^{3+}, ●——●; Sm^{3+}, ■——■; UO_2^{2+}, △——△; Nd^{3+}, ——— ; and La^{3+}, ——— . O------O, uptake of $^{45}Ca^{2+}$ in the absence of ATP. The results are shown for a typical experiment. Similar results were obtained in five separate experiments.

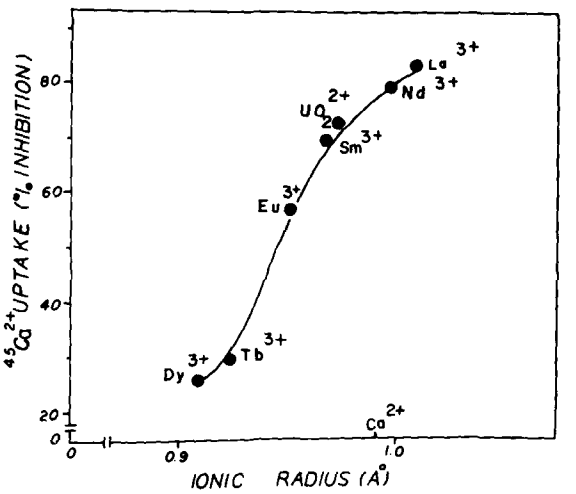

Fig. 2. Relationship of ionic radii of lanthanide cations and UO_2^{2+} to the inhibition in the active transport of Ca^{2+}. The data for the plot were obtained from the experiment described in Fig. 1. The uptake of calcium in the presence and absence of lanthanide cations and uranyl ion was determined at 15 min. Crystal ionic radii were obtained from CRC Handbook of Chemistry and Physics, 54th edn., p. F194, 1974.

brane vesicles resulted in 85% and 80% inhibition, respectively, in the uptake of Ca^{2+}. At 50 μM concentration, La^{3+} and Nd^{3+} exhibited 40% and 30% inhibition, respectively, in the uptake of Ca^{2+} (data not shown). The extent of inhibition of Ca^{2+} uptake by lanthanide cations (Fig. 2) was in the following order: $La^{3+} > Nd^{3+} > Sm^{3+} > Eu^{3+} > Tb^{3+} > Dy^{3+}$. Uranyl ion ($UO_2^{2+}$) which has an ionic radii similar to that of calcium ion, also caused significant inhibition (70%) in the uptake of Ca^{2+}.

Inhibition in the uptake of Ca^{2+}, driven by ATP hydrolysis, by members of the lanthanide family and uranyl ion increased as the ionic radii of these ions increased (Fig. 2). It is pertinent to mention that La^{3+} and Nd^{3+} which have crystal ionic radii of 1.016 and 0.995 Å, respectively, exhibited inhibition in the uptake of Ca^{2+} in *M. phlei* membrane vesicles, in contrast to respiration-dependent Ca^{2+} uptake in mitochondria wherein it has been shown [10] that inhibitory potency falls off as ionic radii of the lanthanide ions become larger or smaller than Ca^{2+} (ionic radii 0.990 Å).

Effect of rare-earth metals on membrane functions mediated by ATP hydroylsis

We determined whether lanthanide cations exerted their inhibitory effect on the uptake of Ca^{2+}, mediated by ATP hydrolysis, through the inhibition of proton translocating ATPase, as has been observed for the inhibition in the uptake of Ca^{2+} and ATPase activity by vanadate ion in *M. phlei* membrane vesicles [22]. Lanthanide cations (La^{3+} and Nd^{3+}) as well as UO_2^{2+} which exhibit maximal inhibition in the uptake of Ca^{2+} did not affect membrane-bound ATPase activity (data not shown). Moreover, these cations did not affect ATPase activity even in the presence of high (15–20 mM) concentrations of Mg^{2+} (data not shown) which has been shown previously [22] to be required for eliciting the inhibitory effect of vanadate on membrane-bound ATPase activity.

The quenching of the fluorescence of 9-aminoacridine, reflecting uptake of protons and

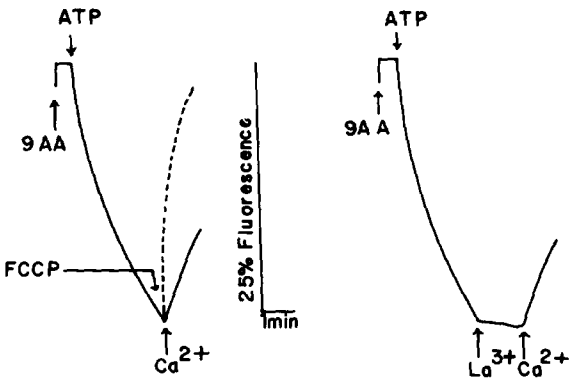

Fig. 3. Effect of La^{3+} on the formation of a proton gradient. The change in the fluorescence (365 → 451 nm) of 9-aminoacridine was measured in 2.5 ml of the reaction mixture containing membrane vesicles (0.8 mg protein/ml), 50 mM Hepes-KOH (pH 7.4), 5 mM $MgCl_2$ and 4 μM 9-aminoacridine. ATP (0.8 mM), $CaCl_2$ (40 μM), $LaCl_3$ (200 μM) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 8 μM) were added as indicated by the arrow.

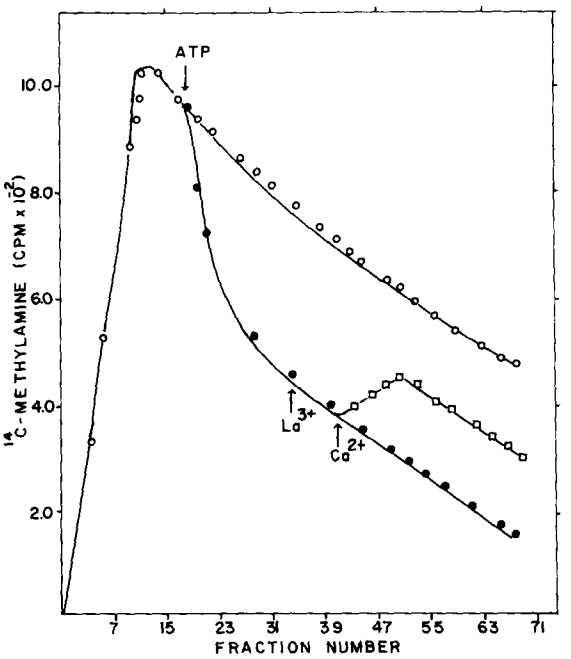

Fig. 4. Flow dialysis determination of ATP hydrolysis-induced uptake of ^{14}[C]methylamine. The reaction mixture (0.85 ml) containing 50 mM Hepes-KOH buffer (pH 7.5), 5 mM $MgCl_2$, 12.5 μM [^{14}C]methylamine and the trypsin-treated membrane vesicles (1.5 mg protein) was stirred in the upper chamber of the flow dialysis apparatus and the dialyzing buffer was pumped through the lower chamber at the rate of 6 ml/min. 2-ml fractions of dialysate were collected and the radioactivity in an aliquot (0.2 ml) of the dialysate was plotted against the fraction number. At the indicated fraction ATP (10 mM) or $CaCl_2$ (1 mM) or La^{3+} (200 μM) was added to the upper chamber. The results are shown for ATP (●———●) and without ATP (○———○). □———□, after adding $CaCl_2$ (1 mM).

formation of a proton gradient (ΔpH) in membrane vesicles was measured during ATP hydrolysis. As shown in Fig. 3, ATP hydrolysis in trypsin-treated vesicles produced significant quenching of 9-aminoacridine fluorescence. Addition of Ca^{2+} to the energized vesicles caused an immediate enhancement of fluorescence, reflecting efflux of the dye and, hence of protons (Fig. 3). Similarly, addition of proton conductor, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) caused a rapid enhancement of fluorescence, as a result of efflux of protons. However, addition of rare-earth metal ions i.e. La^{3+}, Nd^{3+} upto 200 μM concentration did not cause dissipation of ΔpH, indicating that the calcium/hydrogen antiport system does not catalyze exchange of any of these lanthanide cations for protons. Moreover, addition of lanthanide cations did not prevent dissipation of ΔpH by the subsequent addition of calcium ions (Fig. 3). It is pertinent to mention that in *Escherichia coli* membranes La^{3+} (50 μM) has been shown to abolish the response of ΔpH to Ca^{2+} [5].

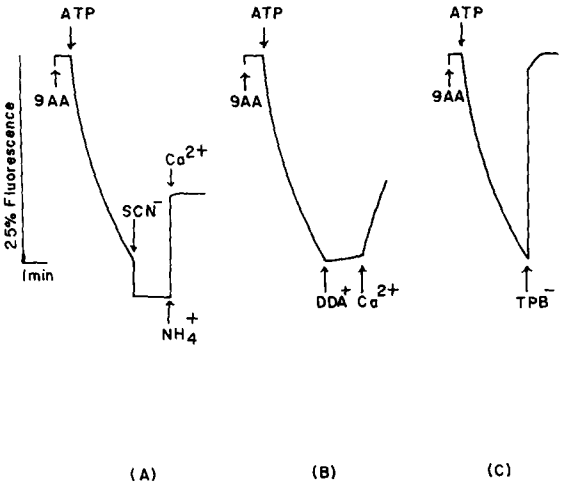

Fig. 5. Effect of SCN^- on the formation of a proton gradient driven by ATP hydrolysis. The change in fluorescence of 9-aminoacridine was measured in 2.5 ml of the reaction mixture containing 50 mM Hepes-KOH (pH 7.4), 5 mM $MgCl_2$, 4 μM 9-aminoacridine and trypsinized membrane vesicles (0.8 mg protein/ml) as described under Materials and Methods. Sodium thiocyanate (15 mM), NH_4Cl (60 mM) and calcium chloride (40 μM) were added as indicated by the arrows. Where indicated 0.8 mM ATP was added. (B) Dibenzyldiammonium ion (DDA^+), 200 μM; and (C) tetraphenylboron (TPB^-), 80 μM were added at the time indicated. Sodium thiocyanate (15 mM exhibited 50% quenching of the fluorescence in the absence of membrane vesicles. This nonspecific contribution to the quenching of fluorescence was substracted in the plot of Fig. 5A.

Qualitatively identical results were obtained by measurement of ^{14}[C]methylamine uptake, utilizing flow dialysis technique. Essentially, it was observed that the addition of calcium ion caused 40–50% efflux of methylamine, indicating outward movement of protons upon the addition of calcium (Fig. 4). However addition of La^{3+} (200 μM) did not cause efflux of methylamine, indicating that ΔpH was not affected by lanthanide cations (Fig. 4).

Membrane potential requirement for the uptake of Ca^{2+}

As shown in Fig. 5A addition of thiocyanate, a permeant anion, in the 9-aminoacridine fluorescence assay, resulted in a large increase in the quenching of fluorescence as a result of dissipation of membrane potential ($\Delta\psi$) through SCN^- influx. NH_4Cl was subsequently added to adjust ΔpH to the same level as before the addition of thiocyanate so that one could delineate the effect

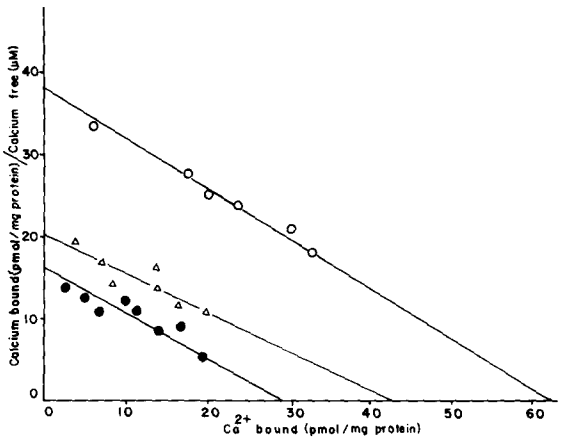

Fig. 6. Effect of $LaCl_3$ and uranylacetate on the binding of calcium to membrane vesicles. The reaction mixture contained 50 mM Hepes-KOH buffer (pH 7.4), 5 mM $MgCl_2$, membrane vesicles (1.5 mg protein), 40 μg of 2-*N*-nonyl-4-hydroxyquinoline-*N*-oxide (respiratory inhibitor) and $^{45}CaCl_2$ in a total volume of 0.5 ml. Binding assays were performed as described under Materials and Methods. $^{45}Ca^{2+}$ bindng to non-energized vesicles (○——○); in the presence of 200 μM $LaCl_3$ (●——●); and in the presence of uranyl 100 μM acetate (△——△).

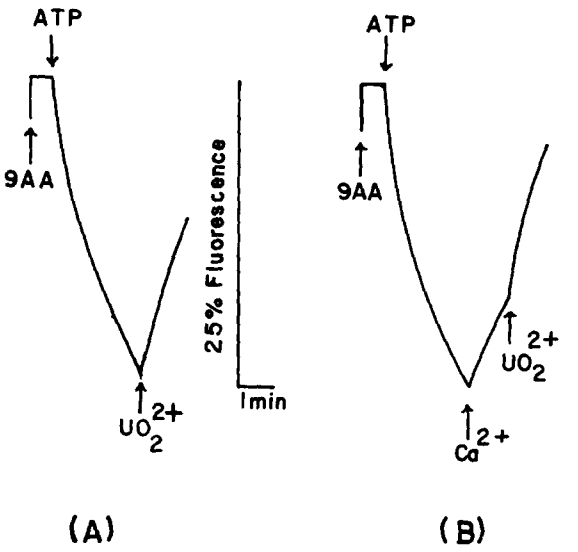

Fig. 7. Effect of uranyl ion on the formation of a proton gradient, driven by ATP hydrolysis. The experimental details were similar to those described under legends to Fig. 3. (A) UO_2^{2+} (50 μM) was added at the indicated time interval. (B) As indicated by the arrows, Ca^{2+} (40 μM) and UO_2^{2+} (50 μM) were added.

of Ca^{2+} on ΔpH determination, in the presence and absence of $\Delta\psi$, with a constant ΔpH. As shown (Fig. 5A) addition of Ca^{2+}, in the presence of thiocyanate ion, in the reaction mixture did not cause reversal of the quenching of fluorescence suggesting that membrane potential is required for the calcium/hydrogen antiport system. Also, dibenzyldiammonium ion (DDA^+), which does not dissipate membrane potential ($\Delta\psi$) in *M. phlei* membrane vesicles, did not affect the dissipation of ΔpH induced by Ca^{2+} (Fig. 5B). However, addition of tetraphenyl boron (TPB^-) which inhibits active transport of Ca^{2+} and dissipates $\Delta\psi$ [3] also dissipated ΔpH (Fig. 5C).

Effect of lanthanide cations on the affinity of binding/transport of Ca^{2+} in membrane vesicles

Since lanthanide cations did not dissipate ΔpH, therefore it is likely that lanthanide cations are not being transported into the inside-out membrane vesicles of *M. phlei*. Therefore, studies were undertaken to determine whether lanthanide cations inhibited the uptake of Ca^{2+} by binding to the Ca^{2+} binding/carrier protein in the membrane. Binding of Ca^{2+} to membrane vesicles under non-energized condition was determined by equilibrium dialysis procedure. Scatchard plots of Ca^{2+} binding to respiratory inhibited *M. phlei* membrane vesicles (Fig. 6) revealed one class of Ca^{2+} binding sites with an apparent K_d of $6.0 \cdot 10^{-7}$ M and binding sites corresponding to 63 pmol per mg protein. Addition of La^{3+} caused reduction in the number of binding sites to 29 pmol per mg protein and also reduced the affinity of Ca^{2+} to membrane vesicles (apparent K_d in the presence of La^{3+} was $1.82 \cdot 10^{-6}$ M). La^{3+} (200 μM) inhibited the uptake of Ca^{2+} by decreasing the V_{max} from 330 p mol/mg protein to 66 p mol/mg protein without affecting the K_m (33.3 μM) (Data not shown).

Effect of uranyl ion on 9-aminoacridine fluorescence

Since UO_2^{2+} inhibited the uptake of Ca^{2+}, mediated by ATP hydrolysis, without affecting ATPase activity, studies were undertaken to determine whether it affected ΔpH formation. As shown in fig. 7A, addition of UO_2^{2+} caused reversal of quenching of fluorescence of 9-aminoacridine, induced by ATP hydrolysis, indicating efflux of protons. Addition of UO_2^{2+} subsequent to the

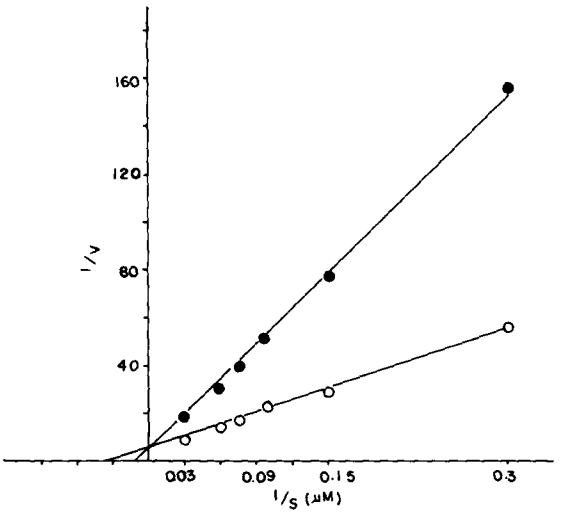

Fig. 8. Lineweaver-Burk plot of calcium uptake in the presence of uranyl acetate. The reaction mixture and the assay procedures were similar to those described in Fig. 1. Initial rates of uptake were determined at 30 s. Uranyl acetate was added at a concentration of 100 μM. In the absence (○———○) and presence (●———●) of UO_2^{2+}. The plots were obtained using a programmable HP calculator attached to plotter. Correlation coefficient R values was 0.992.

addition of Ca^{2+} caused further reversal of quenching of fluorescence (Fig. 7B). Analysis of the kinetic data of the plot of the initial rate of fluorescence enhancement as a function of uranyl ion concentration revealed that the UO_2^{2+} concentration caused partial dissipation of ΔpH yielding 50% V_{max} ($S_{0.5}$) at approx. 15 μM (data not shown). It is pertinent to mention that Mn^{2+}, Sr^{2+} and Ba^{2+} caused partial dissipation of ΔpH (data not shown). Since uranyl ion, in contrast to lanthanide cations caused reversal of quenching of fluorescence it appears that site of binding of UO_2^{2+} on the membrane or Ca^{2+} carrier protein appears to be different than that of lanthanides. This is substantiated by the observation that UO_2^{2+} acts as competitive inhibitor for the uptake of Ca^{2+} (Fig. 8). Studies also showed that UO_2^{2+} (100 μM) decreased the affinity of binding Ca^{2+} to the membrane ($K_d = 2.15 \cdot 10^{-6}$ M) and also caused a reduction in the number of Ca^{2+} binding sites to 43 pmol/mg protein (Fig. 6).

Discussion

The results presented in this study show that lanthanide cations and uranyl ion whose ionic radii (crystal) range from 0.908 Å (Dy^{3+}) to 1.016 Å (La^{3+}) inhibit the active transport of Ca^{2+} (0.990 Å), mediated either by hydrolysis of ATP or driven by respiration. Inhibitory potency of these cations increased with an increase in their ionic radii. This pattern of inhibition is somewhat different compared to rat-liver mitochondria wherein it has been shown that inhibitory potency falls as the ionic radii of lanthanide ions become larger or smaller than Ca^{2+} [23]. The lanthanide cations and uranyl ions while inhibiting active transport of Ca^{2+} in inside-out membrane vesicles of *Mycobacterium phlei* did not affect respiration or ATPase activity. It is pertinent to mention that La^{3+} (20 μM) has been shown to inhibit both (Ca^{2+} + Mg^{2+})-ATPase activity and uptake of Ca^{2+} in sarcoplasmic reticulum [24]. Previous studies from our laboratory [22] have shown that vanadate ion (52 μM) inhibited the uptake of Ca^{2+} by dissipation of ΔpH formation as a consequence of inhibition in F_1-F_0-ATPase activity.

We determined the effect of lanthanide cations and uranyl ion on dissipation of proton gradient by studying their effect on the energy-linked quenching of the fluorescence of 9-aminoacridine. The fluorescence of such amines is quenched by the formation of a ΔpH, acidic inside, although the extact relation between the degree of fluorescence quenching and ΔpH is not clear [25,26]. The quenching of fluorescence of aminoacridines has been used as a qualitative measure of ΔpH and in elucidation of Ca^{2+}/H^+, Na^+/H^+ and K^+/H^+ antiport systems in *Escherichia coli* by Rosen and his coworkers [17,27]. Our studies showed that lanthanide cations (50–200 μM) did not cause the reversal of quenching of fluorescence or dissipation of ΔpH as has been observed for the effect of Ca^{2+} in *M. phlei* membrane vesicles. Moreover, Ca^{2+} could dissipate ΔpH in the presence of lanthanide cations. These results thus indicate that lanthanide cations are not transported into the interior of the vesicles in exchange for protons via the Ca^{2+}/H^+ antiport system. However, uranyl ion caused reversal of the quenching of fluorescence of 9-aminoacridine similar to that observed with Ca^{2+}, suggesting that UO_2^{2+} species are presumably transported into the interior of vesicles in exchange for protons. These results thus indicate that the mechanism of inhibition of Ca^{2+} uptake by La^{3+}, Nd^{3+} and UO_2^{2+} is different although the ionic radii of La^{3+} (1.016 Å), Nd^{3+} (0.995 Å) and UO_2^{2+} (0.97 Å) are closer to that of Ca^{2+} (0.990 Å).

Studies of Ca^{2+} binding to membrane vesicles of *M. phlei* under non-energized condition, indicate that binding of calcium exhibits single class of binding sites. The affinity sites for Ca^{2+} had a dissociation constant K_d, $0.6 \cdot 10^{-6}$ M and bound 63 pmol Ca^{2+} per mg of protein. It is pertinent to mention that Shamoo and Yeng [28] observed high (9.5 μM) and low (33 μM) affinity binding sites for Ca^{2+} in calf heart mitochondria. Results presented in our study show that lanthanum and uranyl ion both decrease the affinity of Ca^{2+}, K_d $1.82 \cdot 10^{-6}$ M and $2.15 \cdot 10^{-6}$ M, respectively, to the membrane, and also reduce the number of binding sites to 30 pmol and 43 pmol per mg protein, respectively. Furthermore, lanthanide cations were non-competitive inhibitors for the uptake of Ca^{2+} while UO_2^{2+} showed competitive

inhibition. It appears that lanthanide cations inhibit the uptake by binding to sites away from the Ca^{2+} binding site, while inhibition by uranyl ion occurs as a result of uptake of uranyl ion via Ca^{2+} carrier protein, since UO_2^{2+} reduces the number of binding sites of Ca^{2+} as well as dissipates formation of ΔpH. Although we do not have direct proof for the transport of UO_2^{2+}, it appears likely that this species is being transported. In whole cells of *Pseudomonas aeruginosa*, uranyl acetate at a concentration of 1 mM has been shown to inhibit the oxidation of glucose, alanine and citrate presumably by inhibiting the uptake of these nutrients [29]. There is a contribution of $\Delta\psi$ to the uptake of Ca^{2+}, since dissipation of membrane potential by the addition of thiocyanate, a permeant anion, caused inhibition in the reversal of quenching of fluorescence of 9-aminoacridine induced by Ca^{2+}. Brey and Rosen [5] also observed requirement for membrane potential in the uptake of Ca^{2+} in *E. coli* membrane vesicles and suggested a stoichiometry of $H^+ : Ca^{2+} > 2$. Although, we have not directly measured stoichiometry it appears that one can draw similar conclusion regarding stoichiometry of $H^+ : Ca^{2+} > 2$ in the uptake of Ca^{2+} in *M. phlei* membrane vesicles.

Acknowledgement

This work was supported by NIH-grant AI-05637. We thank Dr. Suresh C. Sikka for his help in setting up fluorescence assay. The technical assistance of Mr. Morris Rehn is acknowledged. We thank Donna Kopitcke for typing the manuscript.

References

1 Silver, S. (1978) in Bacterial Transport (Rosen, B.P., ed.), pp. 221–234, Marcel Dekker, New York
2 Deves, R. and Brodie, A.F. (1981) Mol. Cell. Biochem. 36, 65–84
3 Kumar, G., Deves, R. and Brodie, A.F. (1979) Eur. J. Biochem. 100, 365–375
4 Kumar, G., Kalra, V.K. and Brodie, A.F. (1979) Arch. Biochem. Biophys. 198, 22–30
5 Brey, R.N. and Rosen, B.P. (1979) J. Biol. Chem. 254, 1957–1963
6 Tsuchiya, T. and Takeda, K. (1979) J. Biochem. 85, 943–951
7 Bhattacharya, P. and Barnes, E.M., (1976) J. Biol. Chem. 251, 5614–5619
8 Lee, S.H., Kalra, V.K. and Brodie, A.F. (1979) J. Biol. Chem. 254, 6861–6864
9 Gomez-Puyou, A., Tuena De Gomez-Puyou, M., Becker, G. and Lehninger, A.L. (1972) Biochem. Biophys. Res. Commun. 47, 814–819
10 Tew, W.P. (1977) Biochem. Biophys. Res. Commun. 78, 624–630
11 Brodie, A.F. and Gray, C.T. (1956) J. Biol. Chem. 219, 853–862
12 Brodie, A.F. (1959) J. Biol. Chem. 234, 398–404
13 Higashi, T., Kalra, V.K., Lee, S.H., Bogin, E. and Brodie, A.F. (1975) J. Biol. Chem. 250, 6541–6548
14 Kalra, V.K. and Brodie, A.F. (1971) Arch. Biochem. Biophys. 147, 653–659
15 Ramos, S., Schuldiner, S. and Kaback, H.R. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1892–1896
16 Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70
17 Brey, R.N., Beck, J.C. and Rosen, B.P. (1978) Biochem. Biophys. Res. Commun. 83, 1588–1594
18 Catterall, W.A. and Pederson, P.L. (1972) J. Biol. Chem. 247, 7969–7976
19 Lee, S.H., Kalra, V.K., Ritz, C.J. and Brodie, A.F. (1977) J. Biol. Chem. 252, 1084–1091
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
21 Brodie, A.F., Kalra, V.K., Lee, S.H. and Cohen, N.S., (1979) Methods Enzymol. 55, 175–200
22 Yoshimura, F. and Brodie, A.F. (1981) J. Biol. Chem. 256, 12239–12242
23 Lehninger, A.L., Reynafarje, B., Vercesi, A. and Tew, W.P. (1978) Ann. N.Y. Acad. Sci. 307, 160–176
24 Abramson, J.J. and Shamoo, A.E. (1980) Ann. N.Y. Acad. Sci. 358, 322–323
25 Rottenberg, H., and Lee, C.P. (1975) Biochemistry 14, 2675–2680
26 Rottenberg, H. (1979) Methods Enzymol. 55, 547–569
27 Beck, J.C. and Rosen, B.P. (1979) Arch. Biochem. Biophys. 194, 208–214
28 Shamoo, A.E. and Yeng, A.Y. (1979) Ann. N.Y. Acad. Sci. 307, 235–237
29 Eagon, R.G. and Asbell, M.A. (1969) J. Bacteriol. 97, 812–819