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STUDIES OF MITOCHONDRIAL CALCIUM MOVEMENTS USING CHLOROTETRACYCLINE

RAJYALAKSHMI LUTHRA and MERLE S. OLSON

Department of Biochemistry, College of Medicine, University of Arizona, Tucson, Ariz. 85724 (U.S.A.)

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SUMMARY

1. The association of calcium with isolated rat liver mitochondrial membranes under various metabolic conditions was monitored using the fluorescent chelate probe, chlorotetracycline. Chlorotetracycline fluorescence increased markedly during energized calcium uptake in the absence of a permeant anion. Uncoupler and a respiratory chain inhibitor caused a rapid decrease in chlorotetracycline fluorescence when added either before or after calcium. During calcium uptake experiments concentrations of calcium exceeding 100 μM caused a transient fluorescence increase followed by an extensive decrease in fluorescence.

2. Changes in the chlorotetracycline-associated fluorescence of the mitochondrial suspensions were correlated with the uptake of exogenous ^{45}Ca. A positive correlation was observed between fluorescence and energized ^{45}Ca uptake in the absence of permeant anions. Addition of the permeant anion, phosphate, caused an extensive decrease in chlorotetracycline fluorescence but an enhanced uptake of exogenous ^{45}Ca.

3. The interaction of endogenous mitochondrial calcium with the fluorescent chelate probe was studied under a number of experimental conditions using mitochondria labeled during preparation with ^{45}Ca. Endogenous ^{45}Ca was lost rapidly from the mitochondria upon treatment with uncoupler, antimycin A, and A23187. Potassium phosphate and EGTA had no effect on the endogenous calcium as measured by either the ^{45}Ca content of the mitochondria or the fluorescence of the probe.

4. Mitochondria treated with antimycin A lost most of their endogenous ^{45}Ca within 3 min; subsequent energization of the mitochondria resulted in a partial uptake of the released ^{45}Ca but caused nearly a complete return of the chlorotetracycline fluorescence to the original level. Addition of phosphate did not change the fluorescence level but resulted in an almost complete accumulation of the ^{45}Ca previously released.

5. Following this energized uptake of ^{45}Ca, EGTA, *p*-trifluoromethoxyphenyl hydrazone of carbonyl cyanide, A23187 and calcium chloride all caused a nearly

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Abbreviations: FCCP, *p*-Trifluoromethoxyphenylhydrazone of carbonyl cyanide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

complete loss of the ^{45}Ca from the mitochondria and, with the exception of calcium chloride, caused an extensive decrease in the fluorescence level. Hence, the apparent location and/or properties of the endogenous calcium in this rat liver mitochondrial system were altered significantly by manipulation of the energetic state of the mitochondrial membrane.

INTRODUCTION

Much of the previous literature devoted to calcium transport by isolated mitochondria has concerned the energy [1–4] and counter ion [2, 3, 5] dependency of this process and the probable existence of a carrier-mediated system specific for the translocation of calcium [3, 6–11]. Additionally, calcium has been shown to bind to mitochondrial membranes at both high and low affinity sites [12–16] and it has been suggested that the high affinity sites are involved in the process of calcium transport [16, 17]. However, the precise relationship between binding of the divalent metal cation to the membrane and its eventual translocation has not been rigorously defined. Recently, fluorescence [18–20] and absorbance [21–23] probes specific for divalent metal cations have been utilized to monitor the binding and/or the transport of calcium and magnesium in membranous systems. In this regard the antibiotic, chlorotetracycline, was proposed by Caswell and Hutchison [18] and Caswell [19] as a probe of divalent metal cation interactions with various cellular membranes. The fluorescence of the chlorotetracycline-divalent metal cation complex was shown to increase markedly in apolar as compared to aqueous environments. Our previous studies in this area were an attempt to predict the probable submitochondrial location of magnesium and calcium in beef heart mitochondria under various metabolic conditions [20, 24, 25] using chlorotetracycline.

The present study was initiated to ascertain whether or not the chelate probe, chlorotetracycline, can be utilized as a monitor of the interaction of calcium with rat liver mitochondrial membranes under various conditions of incubation. In this study changes in the chlorotetracycline-associated fluorescence of the mitochondrial suspension were correlated with the actual translocation of radioactive calcium either into or out of isolated rat liver mitochondria.

MATERIALS AND METHODS

Rat liver mitochondria were isolated from male Sprague-Dawley rats (300–400 g) using the procedure of Chance and Mela [26]. Mitochondria were stored at 4 °C for no more than 2 h prior to use in the experiments. Mitochondrial protein was estimated using a biuret procedure [27].

The association of calcium with the mitochondrial membranes was visualized using the fluorescent chelate probe, chlorotetracycline [19, 20]. The changes in chlorotetracycline-associated fluorescence were measured in a Perkin Elmer Model MPF-2A spectrofluorimeter using an excitation wavelength of 410 nm and an emission wavelength of 560 nm. The mitochondrial incubations were performed at room temperature in a buffer containing 310 mM sucrose/20 mM Tris/chloride (pH 7.0), and other additions as indicated in the various figures. The experiments depicted in

the figures are representative of a number of experiments performed on different mitochondrial preparations. Each experiment was repeated at least 3 times.

The uptake of ^{45}Ca was measured by adding radioactive calcium chloride to the mitochondrial suspension followed by the withdrawal of samples (1.0 ml) using an Oxford Macro-Set Pipetor at various time points. The samples were centrifuged for 1 min in an Eppendorf microcentrifuge at maximum speed $8000 \times g$. In the experiments measuring ^{45}Ca uptake the concentration of radioactive calcium was 250 μM and the amount of $^{45}CaCl_2$ added to the incubation medium was 0.3 μCi/ml. Following centrifugation the supernatant was aspirated immediately and the mitochondrial pellet was resuspended in 0.5 ml of 1 % (w/v) sodium dodecyl sulfate and allowed to stand at room temperature for 2–3 h. The solubilized mitochondria were transferred to scintillation vials containing 10 ml of Aquasol (New England Nuclear) and the ^{45}Ca was counted using standard liquid scintillation procedures. Results are reported as cpm per mg mitochondrial protein.

In certain mitochondrial experiments it was necessary to pre-label the mitochondria using essentially carrier-free ^{45}Ca (1 μCi per ml of washing medium; the concentration of labeled calcium added was approximately 60 nM). The ^{45}Ca was added to the mitochondria during the first wash step of their preparation. The subsequent two wash steps and the final resuspension of the mitochondria were performed with the mannitol-sucrose solution without added ^{45}Ca. Sampling experiments with these labeled mitochondria were conducted in an identical fashion as those in which ^{45}Ca uptake was measured.

Chlorotetracycline was purchased from Nutritional Biochemical Corporation. The antibiotic, A23187, was the generous gift of the Eli Lilly Laboratories. *p*-Trifluoromethoxyphenylhydrazone of carbonyl cyanide (FCCP) was the generous gift of Dr. P. G. Heytler of the E. I. DuPont de Nemours Co. ^{45}Ca was purchased from New England Nuclear (11.6 mCi/mg). All other chemicals and reagents were of the highest quality available and were purchased from common commercial suppliers.

RESULTS

In order to investigate the movement and/or interaction of both exogenous and endogenous calcium in isolated rat liver mitochondria the fluorescent chelate probe, chlorotetracycline, was utilized. The previous studies of Caswell et al. [18, 19] and of our own laboratory [20, 24] have suggested that the fluorescence changes of chlorotetracycline may be employed as a qualitative indicator of divalent metal cation association with various membrane systems. The vast literature pertaining to the energized uptake of calcium by rat liver mitochondria renders this system appropriate for use in the present study.

As a starting point for our consideration of the interactions between chlorotetracycline, calcium and rat liver mitochondria the experiments described in Fig. 1 were performed. Fig. 1, panel A demonstrates that the addition of chlorotetracycline to isolated rat liver mitochondria incubated in a sucrose/Tris/chloride buffer resulted in an increase in the fluorescence of the suspension measured at the wavelength pair 410 nm → 560 nm. This increase in chlorotetracycline-associated fluorescence was likely due to an interaction between the chelate probe and endogenous divalent metal cations associated with the mitochondrial membranes. The addition of succinate as an

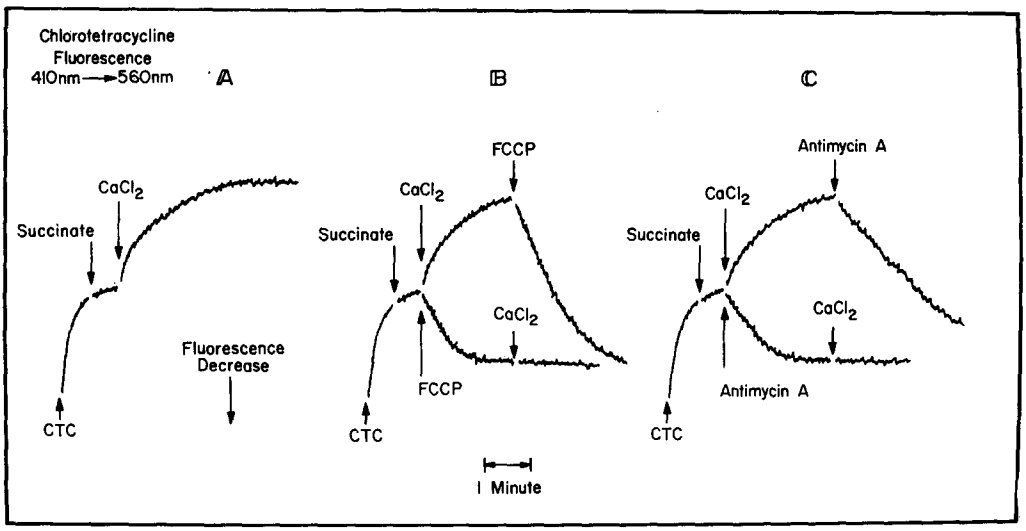

Fig. 1. Chlorotetracycline-associated fluorescence changes during energized calcium uptake by rat liver mitochondria. Rat liver mitochondria (1.0 mg protein/ml) were incubated at room temperature in a buffer containing 310 mM sucrose/20 mM Tris/chloride, (pH 7.0). 12.5 μg/mg protein chlorotetracycline (CTC), 2.5 mM succinate, 250 μM $CaCl_2$, 0.25 μM FCCP, and 2.5 μg/mg protein antimycin A, were added to the mitochondrial suspension as indicated. Fluorescence changes were monitored using an excitation wavelength of 410 nm and an emission wavelength of 560 nm.

energy source caused little alteration in the fluorescence trace. Upon the addition of exogenous calcium (250 μM) in the absence of a permeant anion, an extensive increase in chlorotetracycline-associated fluorescence was observed. That this calcium-induced change in fluorescence was energy-linked is indicated in panels B and C of Fig. 1. The addition of the uncoupler, FCCP, to the chlorotetracycline-treated mitochondria prior to calcium addition led to a rapid decrease in the baseline fluorescence and upon subsequent addition of calcium no further change in fluorescence was observed. If uncoupler was added after calcium was allowed to react with the mitochondria, an extensive fluorescence decrease was observed. Addition of the respiratory inhibitor, antimycin A, in a similar type of experiment yielded nearly identical results. This experiment is consistent with the observations of Caswell [19] and indicates that chlorotetracycline may be utilized to monitor both the endogenous divalent metal cations associated with the mitochondrial membrane as well as the interaction of exogenous calcium with the energized mitochondrial membrane. One interesting difference between the data of Caswell [19] and that shown in Fig. 1 is the fact that Caswell's experiments were performed in the presence of a rather high concentration of the permeant anion, acetate (25 mM). The effects of permeant anion addition to our mitochondrial system will be stressed in Figs. 3–7.

In order to amplify the relationship between energized calcium uptake by the mitochondrial membranes and the chlorotetracycline-associated fluorescence changes the experiment shown in Fig. 2 was performed. Sequential addition of increasing concentrations of calcium chloride up to approximately 100 μM again in the absence of permeant anion lead to a relatively constant increase in both the rate and extent of

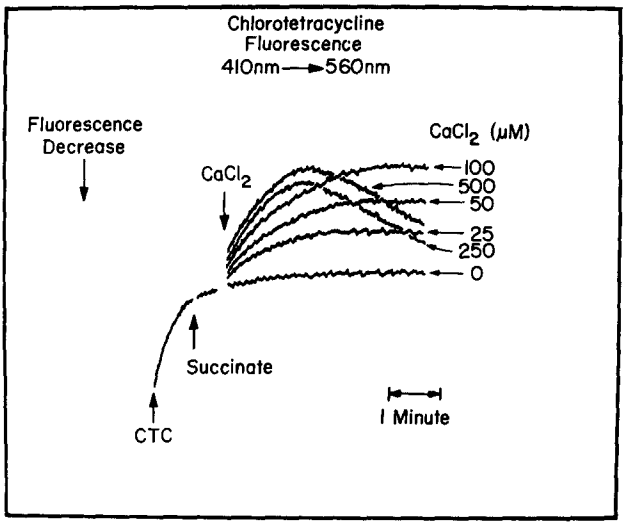

Fig. 2. Effect of increasing concentrations of $CaCl_2$ on the changes in chlorotetracycline (CTC)-associated fluorescence. Incubation conditions were the same as those described in the legend to Fig. 1 with the exception that the $CaCl_2$ concentration was varied from 0 to 500 μM.

the chlorotetracycline-associated fluorescence. A further increase in the calcium concentration resulted in an initial rapid increase in fluorescence followed by a transient phase in which the fluorescence level usually returned to the baseline within 5 to 8 min following calcium addition. This cyclic behavior of the chlorotetracycline-associated fluorescence trace occurring at higher calcium concentrations was consistent from preparation to preparation, the only variable being the exact concentration needed to initiate the cyclic behavior. It can be seen in Fig. 1, Panel A that the addition of 250 μM calcium chloride did not lead to the fluorescence cycle whereas with the mitochondrial preparation used in the experiment described in Fig. 2, the cyclic response began at approximately 250 μM calcium chloride.

A correlation of changes in chlorotetracycline-associated fluorescence and the uptake of ^{45}Ca by the mitochondria in the presence and absence of phosphate is shown in the experiment described in Fig. 3. The addition of $^{45}CaCl_2$ to the succinate-energized mitochondria resulted in an expected increase in the fluorescence trace (Panel A) and a rapid uptake of ^{45}Ca reaching a constant level approximately 2 min following addition of the radioactive metal cation (Panel B). After the fluorescence trace had attained its maximum level, inclusion of the permeant anion, phosphate resulted in an extensive decrease in chlorotetracycline fluorescence but led to an increase in the amount of ^{45}Ca retained by the mitochondria. In results not presented, addition of other permeant anions including acetate affected the fluorescence trace in nearly an identical fashion as phosphate. If phosphate was included in the incubation medium prior to ^{45}Ca addition, instead of causing a fluorescence increase, calcium addition resulted in a rather extensive decrease in the fluorescence of the probe. Under these latter conditions (e.g. addition of phosphate prior to ^{45}Ca) the ^{45}Ca retained by the mitochondria was greater in the initial sample but rapidly decreased within a minute after calcium addition. It is apparent that there was correspondence between

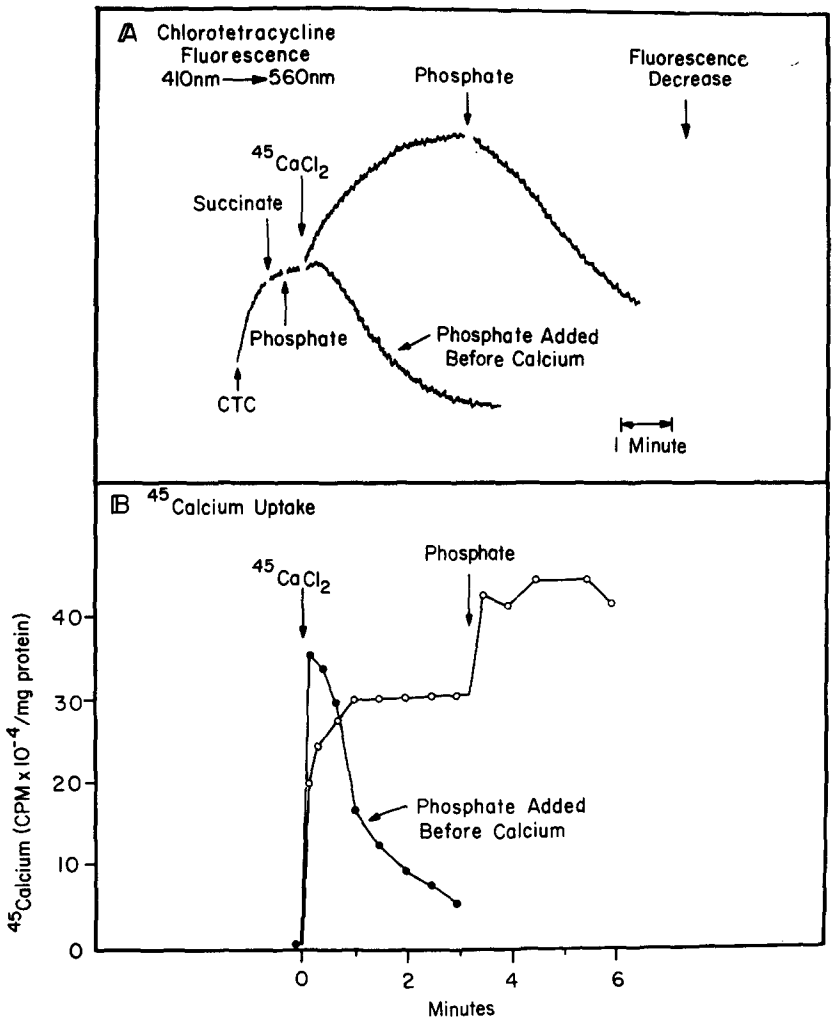

Fig. 3. The effect of the permeant anion, phosphate, on the chlorotetracycline (CTC) fluorescence and ^{45}Ca accumulation by energized rat liver mitochondria. Reaction conditions were identical to those described in the legend for Fig. 1, except that 1 mM potassium phosphate, was added at the points indicated in the figure. ^{45}Ca uptake was measured by the procedure described in Materials and Methods; the $CaCl_2$ concentration in this experiment was 250 μM.

the directional change in the fluorescence trace and the amount of ^{45}Ca retained by the mitochondria. This situation was not observed in the experiment to which ^{45}Ca was added before phosphate addition.

The experiment described in Fig. 4 provides further evidence relative to the relationship between the chlorotetracycline-associated fluorescence trace and the uptake of ^{45}Ca by the mitochondria. In this experiment the mitochondria were pretreated with the respiratory chain inhibitor, antimycin A (see panel A, Fig. 4). Following an initial decrease in chlorotetracycline fluorescence lasting approximately 2 min $^{45}CaCl_2$ was added either in the presence or absence of the permeant anion, phos-

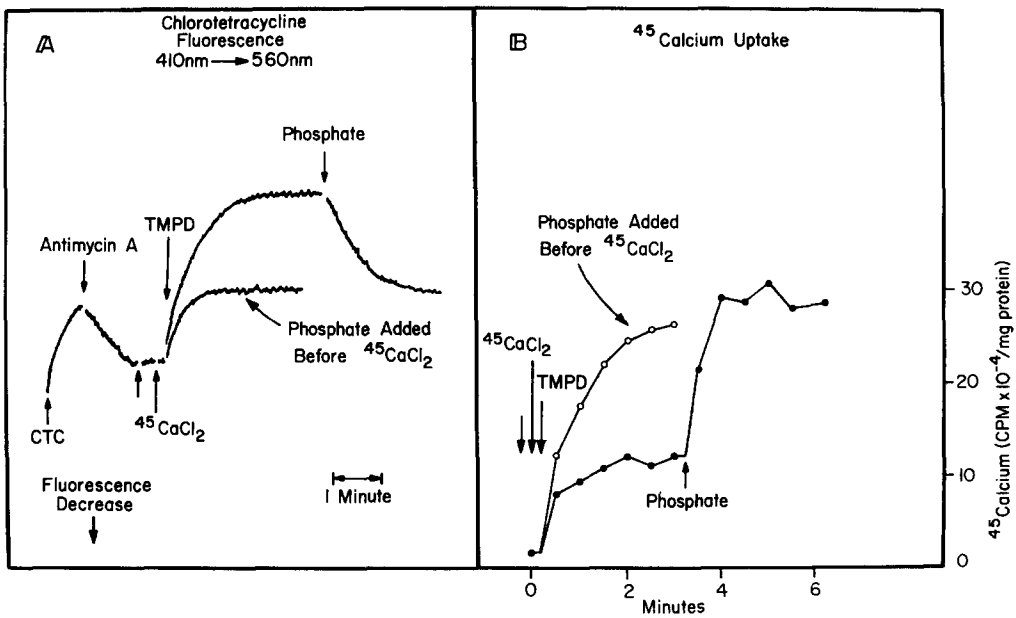

Fig. 4. Effect of potassium phosphate addition on chlorotetracycline (CTC) fluorescence and ^{45}Ca uptake by rat liver mitochondria following a de-energization-energization cycle. Incubation conditions were identical to those described in the legend to Fig. 1. The mitochondria were pretreated with antimycin A, 2.5 μg/mg protein, prior to energization with 100 μM TMPD. Ascorbate (2.5 mM) was present in the incubation prior to addition of mitochondria. The concentration of $^{45}CaCl_2$ and potassium phosphate were 250 μM and 1 mM respectively. ^{45}Ca uptake was measured as described in Materials and Methods.

phate. Energy was then resupplied to the system by the addition of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD); ascorbate was present in the reaction medium from the beginning of the incubation period. Similar to the experiment described in Fig. 3, in the absence of phosphate there was a rapid and extensive fluorescence increase upon energization of the mitochondria (Panel A), concomitant with an increase in the ^{45}Ca taken up by the mitochondria. Inclusion of phosphate in the incubation following the attainment of the maximum fluorescence resulted in a rapid decrease in fluorescence but lead to a much greater uptake of ^{45}Ca. Addition of phosphate prior to ^{45}Ca resulted in a diminished fluorescence increase upon energization with TMPD and, again, a more rapid and extensive uptake of ^{45}Ca. The maximum level of ^{45}Ca attained in the mitochondria was nearly equivalent to the amount of ^{45}Ca retained by the mitochondria in the experiment in which ^{45}Ca addition preceded the phosphate addition. There exists an interesting difference between the mitochondrial ^{45}Ca uptake experiments shown in Figs. 3 and 4. In Fig. 3, the mitochondria pretreated with phosphate showed an initial increase in ^{45}Ca uptake followed by a nearly complete loss of ^{45}Ca within 2 min. The fluorescent probe apparently did not see the initial uptake of ^{45}Ca and merely indicated an extensive discharge of metal cation from the mitochondria. This observation is contrasted in the experiment shown in Fig. 4 in which the antimycin A-treated mitochondria to which phosphate was added exhibited a return to the original base fluorescence level upon ^{45}Ca addition and

subsequent re-energization. In this case an extensive uptake of the ^{45}Ca occurred and this level of ^{45}Ca was maintained for at least 2.5 min following ^{45}Ca addition. The ^{45}Ca uptake experiments were performed both in the presence and absence of chlorotetracycline with nearly identical results.

These experimental results are consistent with our previous studies of magnesium and calcium uptake by isolated beef heart mitochondria [25]. In these studies chlorotetracycline-associated fluorescence was utilized to follow energized membrane loading of divalent metal cations in the absence of permeant anions and the subsequent transport of metal cation toward the matrix space (i.e. increased uptake, but decreased fluorescence of the probe) upon addition of anion.

The increase in the fluorescence of the mitochondrial suspension upon the addition of chlorotetracycline in the absence of exogenous divalent metal cation was interpreted as an interaction between the fluorescent probe and endogenous, membrane-associated divalent metal. Experiments were performed to define the nature of this basal fluorescence and the results are shown in Figs. 5, 6 and 7. For these experiments rat liver mitochondria were pre-labeled with essentially carrier-free ^{45}Ca using the procedure described in Materials and Methods. Following the initial interaction between chlorotetracycline and the mitochondria, it was found that addition of the uncoupler, FCCP, or the respiratory chain inhibitor, antimycin A, caused a significant

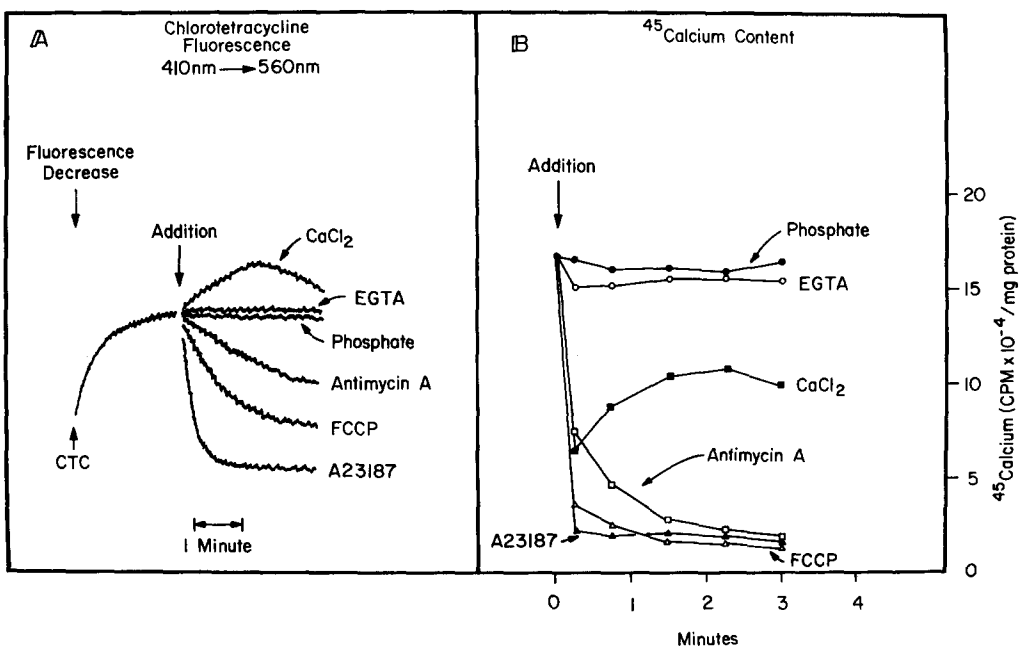

Fig. 5. Effect of various agents on the chlorotetracycline (CTC) fluorescence and ^{45}Ca content of rat liver mitochondria. Mitochondria were pre-labeled with ^{45}Ca during preparation as described in Materials and Methods, and were incubated under the conditions described in the legend to Fig. 1, except that no oxidizable substrate was added during the experiment. 2 μg/mg protein A23187, 0.25 μM FCCP, 2.5 μg/mg protein Antimycin A, 1 mM EGTA, 1 mM potassium phosphate, and 250 μM $CaCl_2$ were added at the points indicated. The ^{45}Ca content of the mitochondria was measured using the procedure described in Materials and Methods.

decrease in fluorescence (Fig. 5, Panel A) and nearly a complete loss of ^{45}Ca from the mitochondria (Fig. 5, Panel B). Similarly, the divalent ionophorous antibiotic, A23187, resulted in an even more rapid fluorescence decrease and loss of ^{45}Ca from the mitochondria. Addition of the permeant anion, phosphate, and the polar chelator, EGTA, had no measurable effect on either the fluorescence level or on the ^{45}Ca content of the mitochondria. Inclusion of unlabeled calcium chloride (250 μM) to

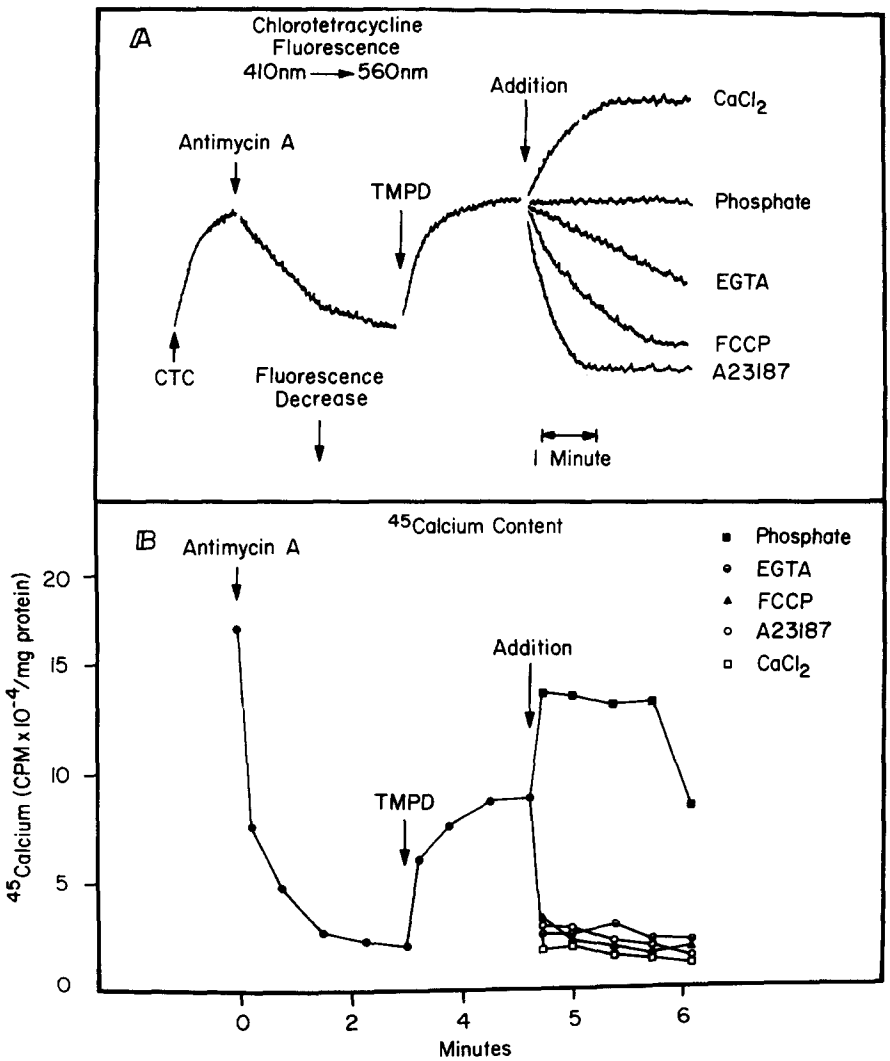

Fig. 6. Effects of various agents on the chlorotetracycline (CTC)-fluorescence and ^{45}Ca content of rat liver mitochondria following a de-energization-energization cycle. Rat liver mitochondria were pre-labeled using the procedure described in Materials and Methods and were incubated under the conditions described in the legends to Figs. 1 and 4. Potassium phosphate, EGTA, FCCP, A23187, and $CaCl_2$ were added to the incubation at the points indicated at the concentrations defined in the legend to Fig. 5.

these unenergized mitochondria resulted in a slight increase in the chlorotetracycline fluorescence level and a rapid initial decrease in ^{45}Ca content followed by a slower re-accumulation of a portion of the ^{45}Ca lost by the mitochondria. This effect of unlabeled calcium addition is likely due to a rapid initial exchange of the endogenous ^{45}Ca with the exogenous calcium followed by an uptake or reassociation of the ^{45}Ca with the mitochondria.

The re-energizatisn of antimycin A-treated mitochondria with TMPD resulted in a nearly complete restoration of the basal fluorescence level of the suspension (Fig. 6, Panel A). If the ^{45}Ca content of the mitochondria in this type of experiment was followed using the pre-labeling technique only about 50 % of the ^{45}Ca released from the mitochondria upon antimycin A treatment was re-accumulated upon energization with TMPD.

In the experiment described in Fig. 5, A23187, FCCP and antimycin A caused qualitatively similar effects on both the chlorotetracycline fluorescence trace and the ^{45}Ca content while phosphate and EGTA were without any noticeable effect. In contrast after antimycin A treatment and subsequent re-energization, A23187, FCCP and EGTA each caused both a decrease in the fluorescence trace and nearly a complete release of the re-accumulated ^{45}Ca. Phosphate, added at this point in the incubation caused no change in the fluorescence trace but resulted in nearly a complete restoration to the mitochondria of the ^{45}Ca which had been released previously to the incubation medium. Unlabeled calcium chloride addition caused a further increase in the fluorescence trace but resulted in apparent complete loss of ^{45}Ca from the mitochondria.

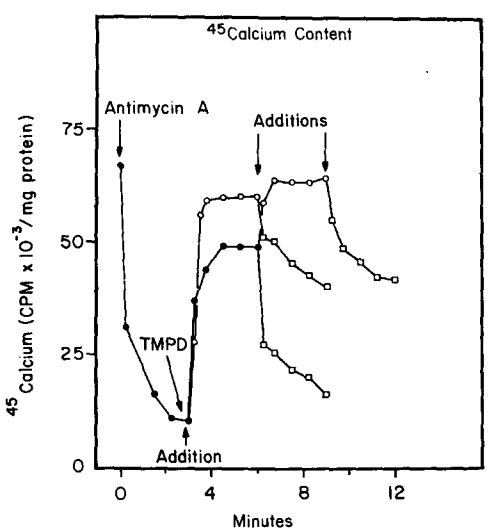

Fig. 7. The effect of potassium phosphate and EGTA on the ^{45}Ca content of pre-labeled rat liver mitochondria following a de-energization-energization cycle. Rat liver mitochondria were pre-labeled with ^{45}Ca using the procedure described in Materials and Methods and the incubations were performed as described in the legends to Figs. 1 and 4. 1 mM Potassium phosphate and 1 mM EGTA were added at the points indicated. The symbol used in the figure following the addition of phosphate to the mitochondria is an open circle and the symbol used following EGTA addition is an open square.

The effect of EGTA on the ^{45}Ca content of liver mitochondria in the presence and absence of phosphate is illustrated more completely in the experiment described in Fig. 7. The mitochondria were pre-labeled with ^{45}Ca as described in the methods, were treated with antimycin A to deplete the endogenous calcium and were re-energized in the presence or absence of phosphate. As seen in Fig. 6, TMPD addition in the absence of phosphate resulted in an uptake of the previously released ^{45}Ca to between 50–60 % of the basal level. Following this ^{45}Ca uptake the addition of EGTA led to virtually a complete release of the ^{45}Ca content; alternatively, addition of phosphate alone at the same point caused an increased uptake of ^{45}Ca as seen in Fig. 6. EGTA addition following phosphate caused a partial loss of ^{45}Ca, the level of ^{45}Ca retained by the mitochondria was slightly below that observed prior to the addition of phosphate. If phosphate was added to the incubation prior to TMPD-mediated energization, the uptake phase was more rapid and extensive. Subsequent addition of EGTA resulted in a partial loss of ^{45}Ca and the amount of ^{45}Ca remaining in the mitochondria under these conditions equaled the ^{45}Ca content of the mitochondrial incubation to which phosphate and EGTA were added after the uptake of ^{45}Ca. Thus, it appears that the location and/or the properties of the endogenous ^{45}Ca in the experiments described in Figs. 5, 6 and 7 were considerably altered by energetic manipulations of the mitochondrial system, and by the presence of permeant anions.

DISCUSSION

The energized uptake of calcium by mitochondrial membranes is one of the most extensively studied cellular transport processes. In an attempt to characterize the kinetics of this process techniques have been developed for continuous monitoring of calcium ion movements in membranous systems which employ compounds whose absorption or fluorescence characteristics differ depending upon whether or not the compound is complexed with calcium. Examples of this type of monitor are murexide [21], aequorin [22], and chlorotetracycline [18–20, 24]. Also, sensitive calcium specific electrodes have been used to monitor changes in the concentration of calcium in solutions bathing various membrane preparations [28]. Finally, measurement of the consequences of calcium uptake such as proton extrusion [3, 29], calcium-stimulated respiration [3], or changes in the oxidation-reduction state of various respiratory chain components [30] have been used as indirect indicators of calcium accumulation in mitochondrial systems.

The present study was initiated to ascertain whether the measurement of fluorescence changes in the chelate probe, chlorotetracycline, could be employed to define the properties of the calcium translocation system and the nature of the endogenous membrane-associated divalent metal cations in isolated rat liver mitochondria. In this regard certain important considerations must be addressed. First, the specificity of chlorotetracycline for various divalent metal cations is of interest in the interpretation of the results using the probe in complex membranous systems. Based upon slight fluorescence spectral differences between the calcium and magnesium complexes of chlorotetracycline in methanolic solution, Caswell [19] suggested that the probe could be used to distinguish between movements of the individual metal cations in isolated mitochondria. However, our previous studies in which calcium and magnesium uptake were monitored in beef heart mitochondrial systems indicated that

the suggestion of Caswell was at best optimistic [20, 24]. In the present study we have chosen to correlate movements of a single divalent metal cation, calcium, with fluorescent changes of the probe. To our advantage in the present study is the observation that rat liver mitochondria do not actively accumulate magnesium under usual conditions of incubation.

We have chosen not to use the probe as a quantitative monitor of calcium. Instead, we have attempted merely to correlate directional changes in the fluorescence of the probe with movements of calcium on and off the mitochondrial membranes using conventional sampling and assay techniques under a variety of experimental conditions. Another important question is whether chlorotetracycline is taken up or bound by mitochondrial membranes in the absence of divalent metal cations. Our previous studies [20, 24] indicated that chlorotetracycline will migrate into the beef heart mitochondrial membranes only if there is a divalent metal ion either co-migrating with the chlorotetracycline or already present in the membrane. If membranes are specifically depleted of divalent metal cations, the probe usually does not enter the lipophilic portion of the system where there would be a considerable fluorescence enhancement of the probe-metal cation complex. Also we have observed that at the concentration used in the present study chlorotetracycline does not have a significant ionophoretic effect on calcium accumulation, but at higher concentrations of chlorotetracycline ionophoretic effects could be a significant consideration (unpublished observations).

The data presented in Fig. 1 indicate that a maximum increase in the fluorescence of chlorotetracycline occurred upon the addition of calcium in the presence of energy and in the absence of a permeant anion. Energy dependent uptake of calcium by mitochondria in the absence of permeant anions has previously been reported by Lehninger [31] and Reynafarje et al. [32] and has been referred to as membrane loading. With these studies in mind and knowing the reported properties of chlorotetracycline, i.e., chlorotetracycline is a very lipophilic substance which may be taken up by various cellular membranes, and whose fluorescence properties change markedly when complexed with divalent metal cations, it is our conclusion that the chelate probe was likely monitoring the membrane-associated calcium in the present experiments.

Another observation of some interest is illustrated in Fig. 2. When the calcium concentration added to the energized mitochondria in the absence of permeant anions exceeded 100 μM a cycling of the fluorescence trace was observed. There occurred an initial uptake of calcium followed by a release of the accumulated calcium by the membrane. In experiments not presented here, it was observed that this cycling behavior of the fluorescence trace was completely prevented by including magnesium in the incubation. Whether this cycling effect observed at increased calcium concentrations was due to an alteration of the nature of the membrane such that the affinity of the membrane for calcium was altered or to an uncoupling of the energy transducing system of the membrane by calcium must await additional experiments.

The effect of permeant anions on the fluorescence of chlorotetracycline in this system was assessed (see Fig. 3). When phosphate was added after the mitochondria were loaded with ^{45}Ca, the fluorescence trace decreased to nearly the baseline level while the uptake of ^{45}Ca actually increased. On the other hand, when the mitochondria were pre-treated with phosphate prior to ^{45}Ca addition, the fluorescence trace decreased far below the baseline level while the uptake of ^{45}Ca showed a rapid spike

followed by an extensive loss of ^{45}Ca from the mitochondria. Hence, it is likely that permeant anion addition to chlorotetracycline-treated mitochondria either before or after addition of exogenous calcium led to a discharge of the calcium from the membrane toward the matrix side of the inner membrane. These observations were further illustrated in Fig. 4. From this experiment it is apparent that there exists a pool of calcium which is associated with the mitochondrial membrane which can be visualized with the chelate probe and which is dependent upon the presence or absence of permeant anions.

The occurrence of a time-dependent interaction of chlorotetracycline with the mitochondria implies that an endogenous divalent metal component is associated with the membrane. It was our experimental rationale that if this proposed pool of divalent metal ions could be labeled with ^{45}Ca during the isolation procedure, this endogenous calcium pool could be studied using a correlation of the chlorotetracycline fluorescence and the movement of ^{45}Ca from the mitochondria. It was shown that the endogenous ^{45}Ca was released from the membrane by the addition of uncoupler, a respiratory chain inhibitor and the divalent metal specific ionophore. In light of the previous experiments it was interesting that permeant anions did not affect either the fluorescence trace or the ^{45}Ca content of the mitochondria. This observation suggests that endogenous calcium was dissimilar in its properties and/or its intramitochondrial location to the calcium which was taken up by the membrane during exogenous calcium uptake in the absence of phosphate. Polar chelators also apparently did not have access to the endogenous calcium. It is likely that endogenous magnesium may also be lost during treatment with uncouplers, respiratory chain inhibitors and A23187. The experiments of Binet and Volfin [33] have indicated a relationship between the loss of calcium and magnesium from the mitochondria following treatment with A23187.

When the location of the endogenous calcium was manipulated by alternately de-energizing the mitochondria with antimycin A and re-energizing with TMPD, the properties of this calcium were altered. The fact that only about 50 % of the ^{45}Ca which was originally present in the mitochondria was re-accumulated upon energization while the fluorescence trace returned completely to the original level, implied that the ^{45}Ca which was re-accumulated was that portion of the original ^{45}Ca which was associated with the membrane, the remainder being derived from the matrix space. However, we cannot exclude the possibility that a portion of the endogenous calcium was associated with the membrane but was inaccessible to chlorotetracycline. An important difference in the properties of the original component of endogenous ^{45}Ca and the ^{45}Ca which was re-accumulated was the accessibility of the latter to the polar chelator EGTA. This observation may indicate that the re-accumulated ^{45}Ca was oriented on the outer aspect of the inner mitochondrial membrane whereas the endogenous ^{45}Ca was oriented on the inner aspect of the inner mitochondrial membrane. However, in both cases the calcium was accessible to the chelate probe and was maintained in its location in an energy dependent fashion.

The fact that the permeant anion, phosphate, caused a further accumulation of ^{45}Ca with no apparent change in the fluorescence level indicates that our previous proposal that the TMPD-mediated reaccumulation of ^{45}Ca represented a pool of calcium associated primarily with the mitochondrial membrane and that the remainder of the ^{45}Ca lost upon deenergization originated in the matrix was likely a reasonable suggestion.

It is our conclusion that the combined utilization of chlorotetracycline fluorescence and conventional sampling and assay procedures can be employed effectively to study the movements of calcium in isolated rat liver mitochondria. Further, these techniques can be used to distinguish between divalent cations associated with the membrane and those merely translocated into the matrix.

The present studies raise various questions regarding the importance and utility of endogenous divalent metal cations. Recently we have suggested that mitochondrial membrane-associated divalent metal cations may serve as important regulatory species which can be alternately released and resequestered depending upon the energy state of the membrane. Further, various enzymes located in the different subcompartments of the mitochondrion may be activated or inhibited by the energy and counter ion dependent uptake and egress of metal cations from the membrane.

Another interesting facet of the nature of the endogenous divalent cations is their possible involvement in the structural integrity of the membrane. Binet and Volfin [33] have proposed recently the existence of a pool of magnesium involved in the structural integrity and permeability properties of isolated rat liver mitochondria. Also in this regard Kun et al. [34] suggested that the loss of intramitochondrial magnesium may be symptomatic of the structural disintegration of the mitochondrial membranes. Precedent for the involvement of divalent metal cations in the structural and functional integrity of other membranous systems may be taken from a variety of studies of the erythrocyte membrane [35, 36].

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