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CALCIUM ACCUMULATION BY CHICK INTESTINAL MITOCHONDRIA

REGULATION BY VITAMIN D-3 AND 1,25-DIHYDROXYVITAMIN D-3

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Summary

We have evaluated the effect of vitamin D-3 and its metabolite 1,25-dihydroxyvitamin D-3 on Ca^{2+} accumulation by chick intestinal mitochondria. Ca^{2+} accumulation appears to occur in two phases: an early, transient accumulation into an Na^+-labile pool followed by an ATP-dependent accumulation into an Na^+-resistant pool. Ca^{2+} accumulation is extensive at free Ca^{2+} concentrations greater than $3 \cdot 10^{-6}$ M in the presence of ATP. Ruthenium red and dinitrophenol block Ca^{2+} accumulation, but atractyloside does not. Oligomycin blocks ATP-supported accumulation completely with a partial inhibition of ATP and malate-supported accumulation. Little difference could be found in mitochondrial preparations from vitamin D-deficient chicks compared to those from vitamin D-3 (or $1,25(OH)_2$D-3)-supplemented chicks with respect to respiratory control, oxygen consumption, efficiency of oxidative phosphorylation, affinity for Ca^{2+}, or the rate and extent of ATP-supported Ca^{2+} accumulation. Intestinal cytosol stimulated Ca^{2+} accumulation, but this was not specific with respect to vitamin D status or tissue of origin, nor was it duplicated by chick intestinal Ca^{2+}-binding protein. 30 ng/ml $1,25(OH)_2$D-3 stimulated Ca^{2+} accumulation directly, regardless of the presence of intestinal cytosol. Other vitamin D metabolites were less potent: 25-hydroxyvitamin D-3 > 24,25-dihydroxyvitamin D-3 = vitamin D-3. Since increasing the free Ca^{2+} concentration from $3 \cdot 10^{-6}$ to $1 \cdot 10^{-5}$ M increased Ca^{2+} accumulation approx. 50-fold, whereas direct stimulation by $1,25(OH)_2$D-3 in vitro increased Ca^{2+} accumulation less than 2-fold, we conclude that $1,25(OH)_2$D-3 influences

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Abbreviations: $1,25(OH)_2$D-3, 1,25-dihydroxyvitamin D-3; $24(R),25(OH)_2$D-3, 24(*R*),25-dihydroxyvitamin D-3; 25 OHD-3, 25-hydroxyvitamin D-3.

mitochondrial accumulation of Ca^{2+} in vivo primarily by altering cytosol concentrations of free Ca^{2+}.

Introduction

Little doubt remains that vitamin D-3, acting through its metabolite, 1,25-dihydroxyvitamin D-3 ($1,25(OH)_2D$-3), stimulates Ca^{2+} transport by altering directly or indirectly Ca^{2+} influx at the brush border and Ca^{2+} efflux at the basolateral membrane [1,2]. Our understanding of the mechanism by which this occurs is not complete, however. Most likely the bulk of Ca^{2+} moving through the cell is transported by intracellular organelles such as mitochondria as indicated by the transient rise in intestinal mitochondrial Ca^{2+} content following vitamin D or $1,25(OH)_2D$-3 administration to vitamin D-deficient animals [2—5]. We [5] have noted that this initial increase in mitochondrial Ca^{2+} occurs prior to the appearance of Ca^{2+}-binding protein. The subsequent decrease in mitochondrial Ca^{2+} coincides with the production of Ca^{2+}-binding protein. Cycloheximide, an agent which blocks Ca^{2+}-binding protein production, does not block Ca^{2+} transport [6] or the initial increase in Ca^{2+} accumulation by mitochondria [5]. However, cycloheximide does block the eventual decrease in mitochondrial Ca^{2+} [5].

The astonishing capacity of mitochondria from a variety of tissues to accumulate Ca^{2+} in vitro is well known [7—9]. The influence of vitamin D and its metabolites on this process has not received much attention. Vitamin D administered in vitro and in vivo [10—12] and $1,25(OH)_2D$-3 administered in vivo [13] seem to alter the ability of kidney mitochondria to retain Ca^{2+} in vitro. Vitamin D was found to expedite Ca^{2+} release, whereas $1,25(OH)_2D$-3 appeared to block Ca^{2+} release in these studies.

We explored the effects of vitamin D and $1,25(OH)_2D$-3 on intestinal mitochondrial Ca^{2+} accumulation in order to answer two questions with respect to the role of mitochondria in the stimulation of Ca^{2+} transport by $1,25(OH)_2D$-3.

1. Does $1,25(OH)_2D$-3 alter the intrinsic ability of mitochondria to accumulate Ca^{2+}?
2. Can the transient increase in Ca^{2+} accumulation by mitochondria in vivo following vitamin D or $1,25(OH)_2D$-3 be explained by other known events in the cell mediated by $1,25(OH)_2D$-3?

This report contains our observations from which we conclude that the principal effect of $1,25(OH)_2D$-3 on Ca^{2+} accumulation by intestinal mitochondria in vivo is to increase intracellular Ca^{2+} concentration to a level sufficient for extensive Ca^{2+} accumulation to occur. However, we have demonstrated a direct and specific stimulation of mitochondrial Ca^{2+} accumulation by 1,25-$(OH)_2D$-3 in vitro which may play an ancillary role in vivo.

Methods

White Leghorn cockerels were raised for 17—21 days on a vitamin D-deficient diet containing 0.43% calcium, 0.3% phosphorus (Teklad 75007). For certain experiments chicks were given either vitamin D-3 or $1,25(OH)_2D$-3

orally in propyleneglycol before killing. Dosage details are found in the figure legends.

Mitochondria were prepared from duodenal mucosal scrapings that had been washed in ice-cold 2 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 300 mM sucrose, 20 mM Tris-HCl adjusted to pH 6.8 at 25°C (buffer A) prior to homogenization. Homogenization of the washed mucosa was performed in 5 ml/chick of this same buffer. The supernatant from the 10 min, $800 \times g$ centrifugation was collected. The pellet was rehomogenized as above, and the $800 \times g$ supernatant collected as before. The combined $800 \times g$ supernatants were centrifuged at $10\,000 \times g$ for 15 min. The $10\,000 \times g$ supernatant was discarded. The pellet was resuspended in 2 ml/chick of buffer A, and collected again with centrifugation at $10\,000 \times g$ for 10 min. The pellet was washed once more in a similar sucrose/Tris-HCl buffer which did not contain EGTA (buffer B). The mitochondrial pellet was finally suspended in 1 ml buffer B/chick. Just prior to the Ca^{2+}-accumulation assay the mitochondria were diluted 1 : 10 in buffer B producing a suspension with approx. 1 mg protein/ml.

The preparation of intestinal cytosol for certain experiments required washing the mucosal scrapings in buffer A followed by two more washes in buffer B to remove EGTA prior to homogenization in buffer B. The mitochondrial pellet was then prepared as before. The cytosol was obtained from centrifugation at $100\,000 \times g$ for 60 min of the $10\,000 \times g$ supernatant. In these experiments cytosol rather than buffer B was used in the final 1 : 10 dilution of the mitochondria.

The Ca^{2+}-accumulation assay was performed by adding a 700 μl aliquot of the diluted mitochondrial suspension (500—800 μg protein) to 200 μl of a mixture containing in final concentration of the total 1 ml incubation mixture (see below) 10 mM sodium malate, 2 mM $MgSO_4$, 210 mM sucrose, 3 mM phosphate, 3 mM ATP, and 20 mM Tris-HCl (pH 6.8), at 25°C. After a 5 min preincubation at 25°C, we added 100 μl of a solution containing 0.5 μCi $^{45}Ca^{2+}$ (specific activity varying with final Ca^{2+} concentration), 0.5 μCi $^{36}Cl^-$ (specific activity 1 Ci/22 mol) and 50 mM EGTA/Ca^{2+} buffer [14] calculated to produce the indicated free Ca^{2+} concentrations at pH 6.8. Such calculations did not include the effect of ATP on free Ca^{2+} concentrations [15]. Therefore, the final 'free' Ca^{2+} concentration was somewhat less than that stated in incubations containing ATP. This error was minimized by the presence of Mg^{2+} and the large buffering capacity of the EGTA buffer system. 100 μl aliquots were removed at the indicated times and passed through 0.45 μm Millipore filters under vacuum. The filters were washed with 2 or 5 ml ice-cold 154 mM saline, and the $^{45}Ca^{2+}$ and $^{36}Cl^-$ radioactivity was determined by dual-label liquid scintillation techniques. The amount of Ca^{2+} accumulated into the Cl^--impermeable space was calculated.

Oxygen uptake by the mitochondria was determined with a Gilson oxygraph by the method of Dohm et al. [16].

Protein determinations were performed by the method of Lowry et al. [17].

1,25$(OH)_2$D-3 and 24(*R*),25-dihydroxyvitamin D-3 (24(*R*),25$(OH)_2$D-3) were a gift from M.R. Uskokovic, Hoffmann-La Roche. 25-hydroxyvitamin D-3 (25OHD-3) was a gift from J.C. Babcock, Upjohn. Chick intestinal Ca^{2+}-

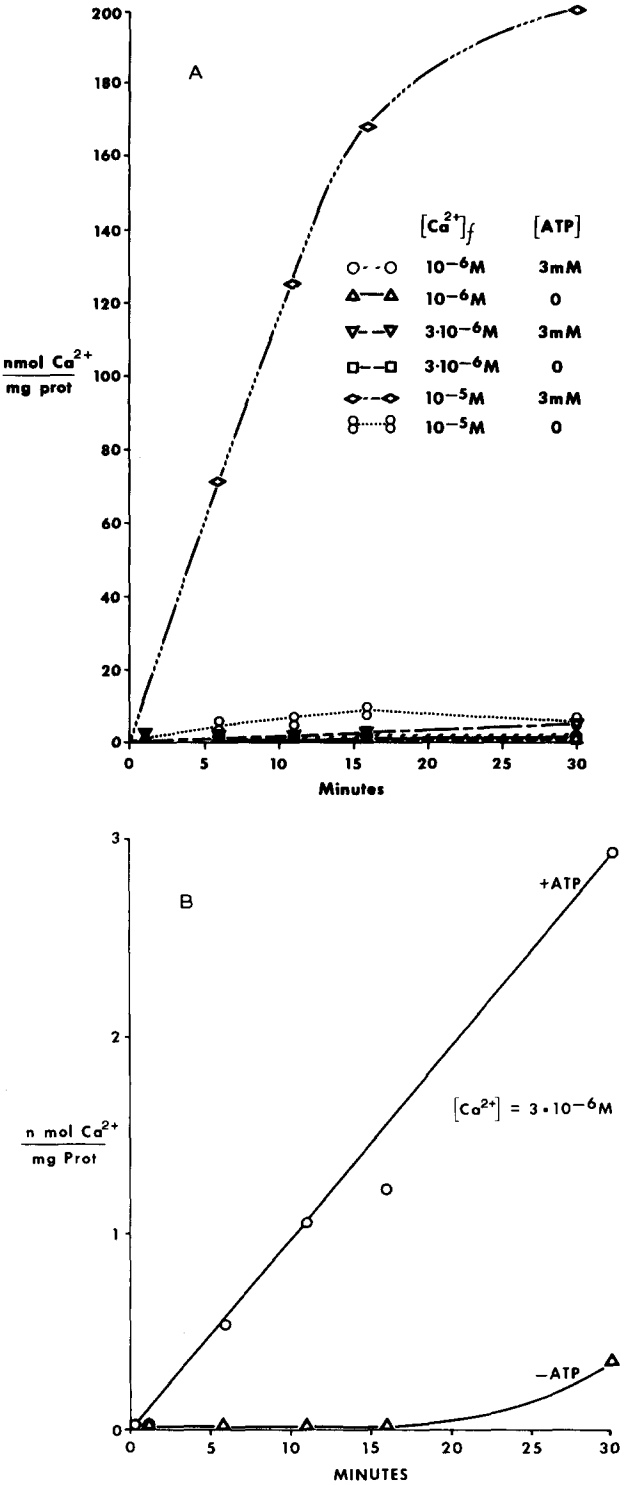

Fig. 1. Ca^{2+} accumulation by mitochondria in the presence and absence of ATP at different free Ca^{2+} concentrations. Mitochondria were separated from the incubation medium on Millipore filters and washed with 5 ml isotonic saline. The scale in (A) differs from that in (B) by 50 to permit visualization of the uptake of Ca^{2+} at $1 \cdot 10^{-5}$ M (A) and $3 \cdot 10^{-6}$ M (B).

binding protein was purified as previously described [18]. All other chemicals used were of reagent grade and obtained from commercial suppliers.

Results

Optimal Ca^{2+} accumulation by intestinal mitochondria required ATP and concentrations of Ca^{2+} greater than $3 \cdot 10^{-6}$ M (Fig. 1). At 25°C in the presence of 3 mM ATP and $1 \cdot 10^{-5}$ M Ca^{2+} using a 5 ml saline rinse at the end of the incubation, Ca^{2+} accumulation proceeded in a linear fashion with time for 10—15 min reaching a maximal accumulation of approx. 200 nmol Ca^{2+}/mg protein by 30 min (Fig. 1A). The accumulation of Ca^{2+} at $3 \cdot 10^{-6}$ M (Fig. 1B) in the presence of ATP likewise proceeded in a linear fashion for at least 30 min, but the rate and extent of accumulation was 50-fold less than at $1 \cdot 10^{-5}$ M free Ca^{2+}. In general, no Ca^{2+} release was observed under these conditions for at least 60 min unless visible clumping occurred (observed occasionally when mitochondria were incubated in cytosol). Experiments in which the mitochondrial aliquots were washed with 2 ml instead of 5 ml NaCl (Fig. 2) indicated a rapid uptake of Ca^{2+} into a pool that was not observed using 5-ml saline washes. This initial uptake was greatly increased by phosphate and ATP (Fig. 2)

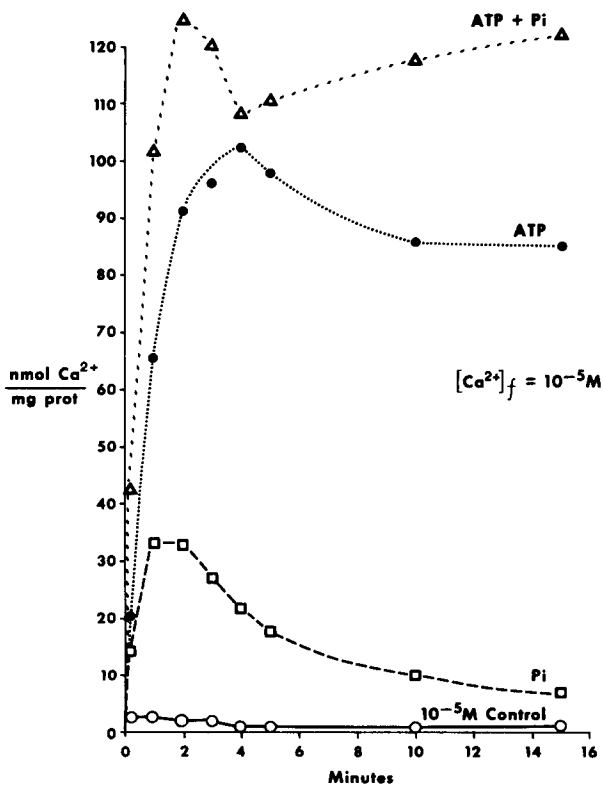

Fig. 2. Ca^{2+} accumulation by mitochondria in the presence and absence of ATP and phosphate at $1 \cdot 10^{-5}$ M free Ca^{2+}. The mitochondria were washed with 2 ml isotonic saline which permits demonstration of a more labile and transient pool of Ca^{2+} accumulation.

which also appeared necessary to convert this initial uptake into the NaCl-resistant form of accumulation depicted in Fig. 1. The accumulation of Ca^{2+} by mitochondria was blocked completely by $2.5 \cdot 10^{-5}$ M ruthenium red and $5 \cdot 10^{-4}$ M dinitrophenol (Fig. 3). $1 \cdot 10^{-5}$ M atractyloside (Fig. 3) did not block Ca^{2+} accumulation. 10 μg/ml oligomycin was only partly effective in blocking Ca^{2+} accumulation (Fig. 3) unless malate was omitted from the incubation medium. In the absence of malate, oligomycin completely blocked ATP-supported Ca^{2+} accumulation (data not shown).

Intestinal mitochondria from chicks given 625 pmol $1,25(OH)_2D$-3 19 h before killing could not be distinguished from mitochondria obtained from their vitamin D-deficient hatchmates in terms of their respiratory control index, state 3 or state 4 oxygen consumption, or phosphorylation efficiency (ADP/O ratio) (Table I). These mitochondria demonstrated a respiratory control index greater than 6, state 3 oxygen uptake approx. 170 ngatoms O/min per mg protein, and a phosphorylation ratio of 2.5. Clearly, no fundamental abnormality in oxidative phosphorylation was observed in vitamin D-deficient mitochondria. Likewise, in vivo administration of $1,25(OH)_2D$-3 failed to alter the ability of subsequently isolated mitochondria to accumulate Ca^{2+} at suboptimal Ca^{2+} concentrations ($3 \cdot 10^{-6}$ M) or at higher concentrations ($1 \cdot 10^{-5}$ M) (Fig. 4).

Intestinal cytosol enhanced Ca^{2+} accumulation by mitochondria (Table II). This effect did not depend on the vitamin D status of the animal from which

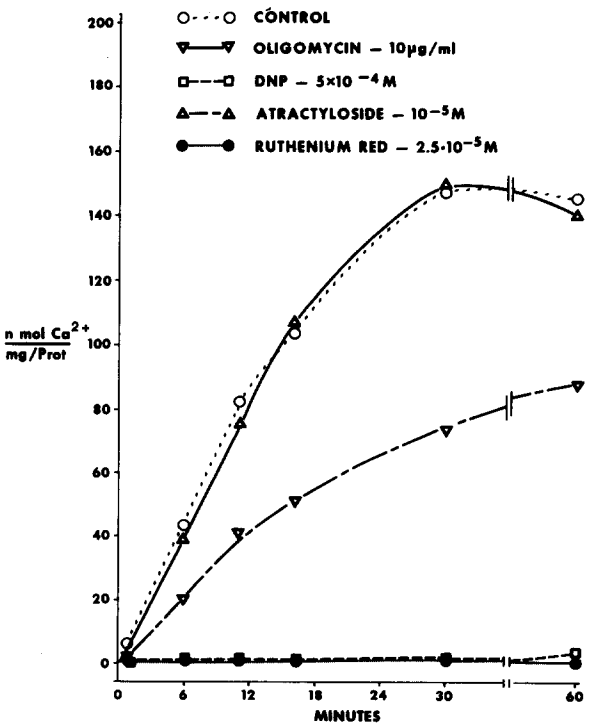

Fig. 3. Ca^{2+} accumulation by mitochondria in the presence of various inhibitors. The incubation contained ATP and $1 \cdot 10^{-5}$ M free Ca^{2+}. The mitochondria were washed with 5 ml saline.

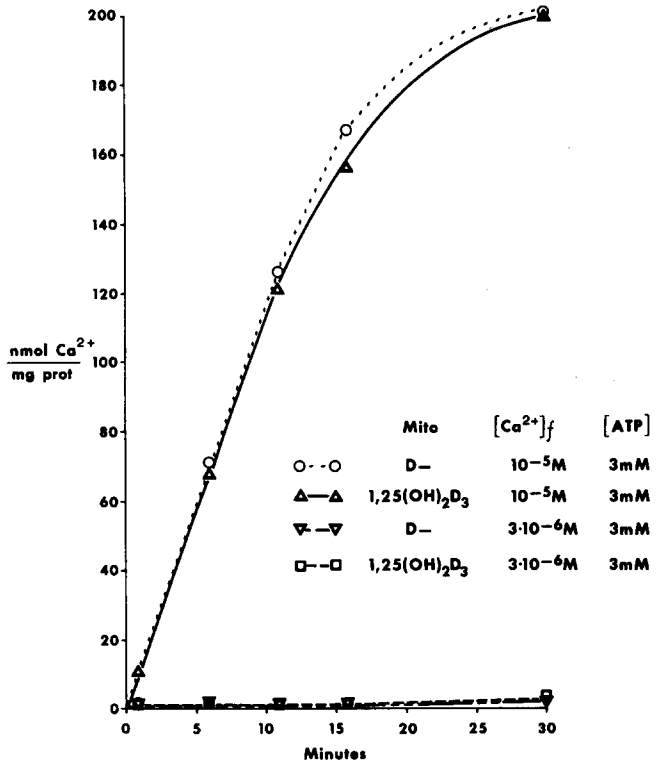

Fig. 4. Ca^{2+} accumulation by mitochondria from vitamin D-deficient chicks or from their hatchmates given 625 pmol $1,25(OH)_2D$-3 16 h before killing. The incubation medium contained 3 mM ATP. The mitochondria were washed with 5 ml isotonic saline at the end of the incubation.

either the cytosol or the mitochondria were prepared (Table II). Furthermore, liver cytosol was just as effective (data not shown).

Purified chick intestinal Ca^{2+}-binding protein at a concentration (10 μg/ml) equivalent to that found in the intestinal cytosol preparation from chicks given $1,25(OH)_2D$-3 16 h before killing did not result in a comparable stimulation of Ca^{2+} accimulation (Fig. 5). At this concentration, Ca^{2+}-binding protein had no apparent effect on Ca^{2+} accumulation with or without ATP at $3 \cdot 10^{-6}$ or $1 \cdot 10^{-5}$ M free Ca^{2+}. No consistent effect of Ca^{2+}-binding protein on Ca^{2+}

TABLE I

THE RESPIRATORY CONTROL INDEX (RCI), STATE 3 OXYGEN UPTAKE, AND PHOSPHORYLATION RATIO (ADP/O) OF MITOCHONDRIA FROM VITAMIN D-DEFICIENT CHICKS OR FROM CHICKS RECEIVING 625 PMOL $1,25(OH)_2$ D-3 19 h BEFORE KILLING

The substrate was pyruvate malate.

	RCI	Oxygen uptake (ngatoms/min per mg)	ADP/O
$1,25(OH)_2$ D-3	6.39	178	2.52
Vitamin D-deficient	6.87	165	2.47

TABLE II

Ca^{2+} ACCUMULATION BY INTESTINAL MITOCHONDRIA FROM VITAMIN D-DFFICIENT CHICKS (D—) OR FROM CHICKS GIVEN 625 pmol $1,25(OH)_2$ D-3 16 h BEFORE KILLING (D+) IN THE PRESENCE AND ABSENCE OF INTESTINAL CYTOSOL FROM THE SAME ANIMALS

The free Ca^{2+} concentration in the incubation medium was $1 \cdot 10^{-5}$ M. Each value is the mean of duplicate determinations which varied within 5% of the mean.

	Buffer	Cytosol	
		D+	D—
Mitochondria			
D+	188 *	302	315
D—	190	285	264

* nmol Ca^{2+}/mg protein per 30 min.

accumulation could be observed even if the Ca^{2+}-binding protein concentration was raised to 160 μg/ml (data not shown).

$1,25(OH)_2$D-3 added to the incubation medium stimulated mitochondrial Ca^{2+} accumulation in the presence of $1 \cdot 10^{-5}$ M free Ca^{2+} at doses as low as 30 ng/ml (Fig. 6A). A similar stimulation was exerted by vitamin D-3 but at

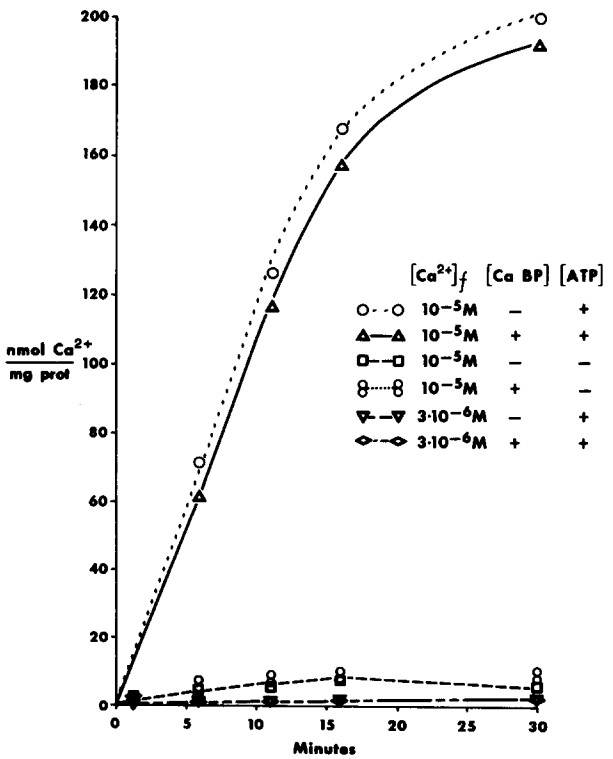

Fig. 5. Ca^{2+} accumulation by mitochondria incubated in the presence or absence of 10 μg/ml Ca^{2+}-binding protein (CaBP), 3 mM ATP, and $1 \cdot 10^{-5}$ or $3 \cdot 10^{-6}$ M free Ca^{2+}. The mitochondria were washed with 5 ml saline at the end of incubation.

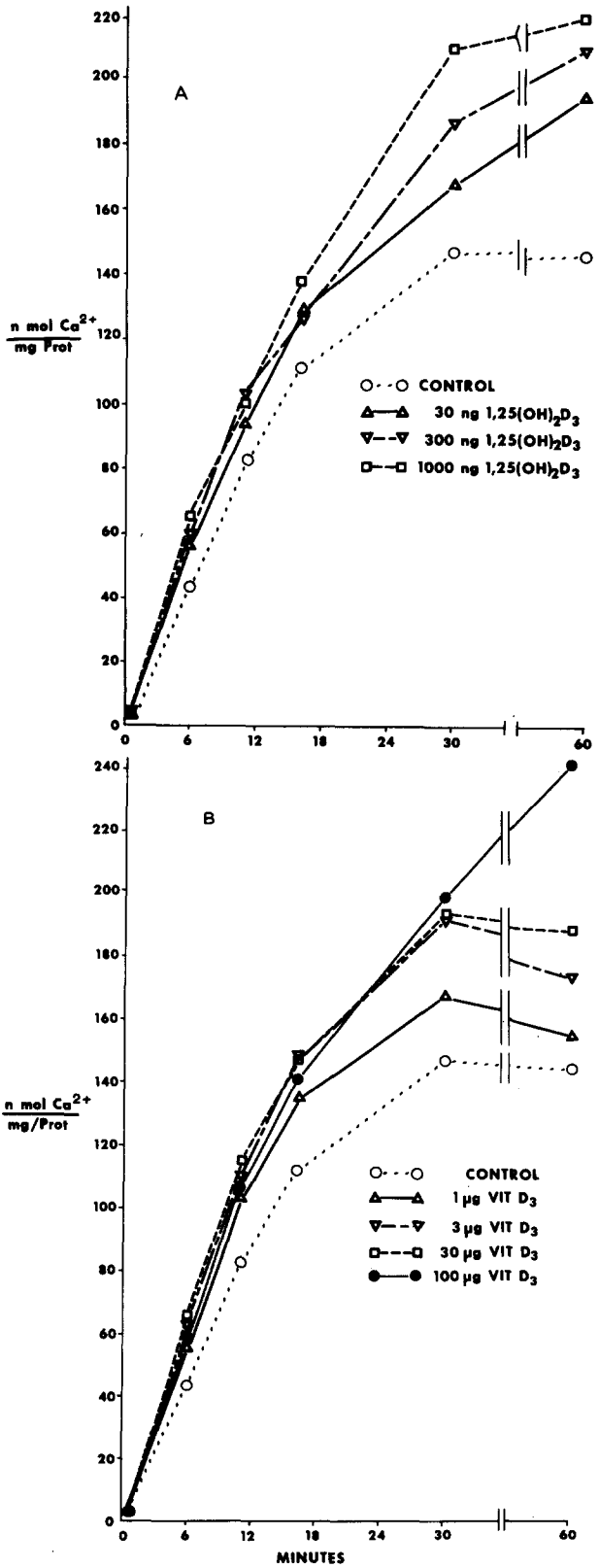

Fig. 6. Ca^{2+} accumulation by mitochondria incubated in the presence of variable concentrations of $1,25(OH)_2D$-3 (A) or vitamin D-3 (B) added in 10 µl ethanol. The incubation contained 3 mM ATP, $1 \cdot 10^{-5}$ M free Ca^{2+}, and was ended with a 5 ml saline wash.

TABLE III

Ca^{2+} ACCUMULATION BY MITOCHONDRIA INCUBATED FOR 30 MIN IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF $1,25(OH)_2$ D-3 AT TWO DIFFERENT FREE Ca^{2+} CONCENTRATIONS

Each value is the mean of duplicate determinations, each of which was within 5% of the mean or within 0.5 ng Ca^{2+}/mg of the mean.

Ca^{2+} (M)	Concn. $1,25(OH)_2$ D-3 (ng/ml)						
	0	1	3	10	30	100	300
$1 \cdot 10^{-5}$	151 *	153	153	156	158	202	240
$3 \cdot 10^{-6}$	5.4	4.2	4.8	4.8	3.9	4.9	4.0

* nmol Ca^{2+}/mg protein.

much higher concentrations (1 μg/ml) (Fig. 6B). In the presence of 1 μg/ml $1,25(OH)_2$D-3, Ca^{2+} accumulation at 30 min was 44% above control in this experiment. The ability of $1,25(OH)_2$D-3 to stimulate Ca^{2+} accumulation at $1 \cdot 10^{-5}$ M free Ca^{2+} was not due to an increase in affinity for Ca^{2+}, apparently,

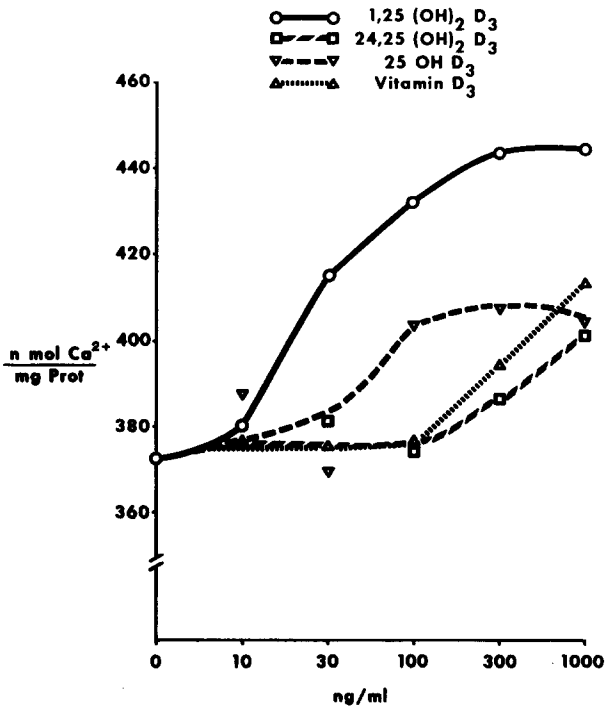

Fig. 7. Ca^{2+} accumulation by mitochondria incubated for 30 min in cytosol. Both mitochondria and cytosol were obtained from the duodenal mucosa of vitamin D-deficient chicks. The original homogenization of the mucosa was performed in 2 ml buffer B/g wet wt. of mucosa to produce a cytosol concentration with 8.4 mg protein/ml. Ca^{2+} accumulation is expressed on the basis of mitochondrial protein. The vitamin D metabolites were added in 10 μl ethanol. The incubation medium contained 3 mM ATP and $1 \cdot 10^{-5}$ M free Ca^{2+}. Each point is the mean of duplicate determinations, all of which varied less than 5% and generally less than 2% of the mean for each point.

since no effect on Ca^{2+} accumulation at $3 \cdot 10^{-6}$ M free Ca^{2+} could be demonstrated (Table III). In this experiment 300 ng/ml $1,25(OH)_2D$-3 stimulated Ca^{2+} accumulation 60% at $1 \cdot 10^{-5}$ M free Ca^{2+} with no stimulation at $3 \cdot 10^{-6}$ M Ca^{2+}. The ability of $1,25(OH)_2D$-3 to stimulate Ca^{2+} accumulation was not increased by the presence of vitamin D-deficient intestinal cytosol (Fig. 7). As depicted in Fig. 7, 25 OHD-3 stimulated Ca^{2+} accumulation with a potency less than that of $1,25(OH)_2D$-3 but greater than that of vitamin D-3. $24(R),25$-$(OH)_2D$-3 did not stimulate Ca^{2+} accumulation more effectively than vitamin D-3. The magnitude of the stimulation by these agents (up to 71 nmol Ca^{2+}/mg protein) was comparable to that seen in the absence of cytosol, although the percent stimulation above control (less than 20%) was smaller because of the marked stimulation by cytosol itself.

Discussion

By studying Ca^{2+} accumulation under conditions in which the free Ca^{2+} concentration is buffered with EGTA, we can observe accumulation for at least 30 min and evaluate the dependence of this accumulation on Ca^{2+} concentration (Fig. 1). Similar results were obtained with kidney mitochondria [19] using the same Ca^{2+} buffer system and liver mitochondria using a different Ca^{2+} buffer system [20]. ATP substantially increased Ca^{2+} accumulation especially into a pool resistant to the NaCl wash procedure (Figs. 1 and 2). Na^+ promotes the extrusion of Ca^{2+} from mitochondria [21]. We employed a 5 ml NaCl wash in these experiments to remove this transient, labile pool of Ca^{2+}, which we believe is Ca^{2+} binding, in order to evaluate the slower, but more stable phase of Ca^{2+} accumulation. ATP is required to stabilize the accumulated Ca^{2+} into an amorphous calcium phosphate deposit [7,22] which permits massive loading of Ca^{2+} into mitochondria [8] and, presumably, reduces its susceptibility to release by Na^+.

Ruthenium red, a specific inhibitor of mitochondrial Ca^{2+} accumulation [23], and dinitrophenol, an uncoupler of oxidative phosphorylation known to block Ca^{2+} accumulation by mitochondria from other tissues [24], completely blocked Ca^{2+} accumulation by intestinal mitochondria (Fig. 3). Oligomycin, an inhibitor of mitochondrial ATPase [25], partially blocked Ca^{2+} accumulation (Fig. 3) in the presence of malate indicating that in this system exogenous ATP provides at least part of the energy required for Ca^{2+} accumulation in addition to the oligomycin-insensitive ability of ATP to enhance Ca^{2+} retention [26,27]. This conclusion was confirmed by noting that Ca^{2+} accumulation supported by ATP in the absence of malate was blocked completely by oligomycin. Atractyloside, an inhibitor of the adenine nucleotide translocase [28], did not block the ability of ATP to stimulate Ca^{2+} accumulation (Fig. 3) despite blocking the ability of ADP to stimulate oxygen consumption (data not shown). This suggests either that ATP must facilitate the accumulation of Ca^{2+} by a process that does not require entry of ATP into the mitochondrion via the adenine nucleotide translocase or that during Ca^{2+} accumulation the adenine nucleotide translocase is insensitive to atractyloside [29]. Similar findings have been observed with cardiac mitochondria [29].

In general, our results indicate that Ca^{2+} accumulation by vitamin D-defi-

cient chick intestinal mitochondria is an active process operating at Ca^{2+} concentrations somewhat higher than expected for the presumed intracellular levels of Ca^{2+} in the resting cell [30]. We then evaluated the impact of vitamin D-3 and its active metabolites on this process. After reassuring ourselves that no obvious difference existed in the mitochondria from vitamin D-deficient and vitamin D-treated chicks with respect to the general parameters of oxidative phosphorylation (Table I), we examined the ability of these mitochondrial preparations to accumulate Ca^{2+}. Such studies indicated that treatment with vitamin D-3 or $1,25(OH)_2$D-3 in vivo did not change the ability of intestinal mitochondria to accumulate Ca^{2+} with respect to rate, extent, or affinity (Fig. 4). Krawitt et al. [31] noted a similar lack of effect in mitochondrial function when animals were adapted to different dietary Ca^{2+} concentrations, a manipulation that changes $1,25(OH)_2$D-3 concentrations in the gut [32,33].

Intestinal cytosol stimulated mitochondrial Ca^{2+} accumulation (Table II). However, this stimulation is not specific for intestinal cytosol. Little consistent difference between cytosol from the tissue of vitamin D-deficient compared to $1,25(OH)_2$D-3-treated chicks could be demonstrated (Table II). We excluded the possibility that Ca^{2+}-binding protein accounted for this stimulation (Fig. 5), but have not distinguished among the multitude of other possibilities because of the lack of specificity of this effect.

Since vitamin D and $1,25(OH)_2$D-3 administered in vivo did not appear to enhance the intrinsic ability of intestinal mitochondria to accumulate Ca^{2+} in vitro, we evaluated whether these agents could act directly on mitochondria in vitro with respect to Ca^{2+} accumulation. The results depicted in Figs. 6 and 7 and Table III demonstrate that $1,25(OH)_2$D-3 does exert a direct stimulatory effect on Ca^{2+} accumulation at concentrations as low as 30 ng/ml (Fig. 6A). Vitamin D-3 itself also stimulates Ca^{2+} accumulation but at concentrations nearly two orders of magnitude higher (Fig. 6B). $1,25(OH)_2$D-3 does not appear to act by increasing the affinity of the mitochondrial pump for Ca^{2+} (Table III). Comparable stimulation was observed when the mitochondria were prepared in intestinal cytosol from vitamin D-deficient chicks (Fig. 7). 25 OHD-3 seemed to be more potent than vitamin D-3 in this experiment (Fig. 7) but less potent than $1,25(OH)_2$D-3. $24(R),25(OH)_2$D-3 was no more effective than vitamin D-3. These results indicate a specific and direct effect of $1,25(OH)_2$D-3 on mitochondrial Ca^{2+} accumulation demonstrated in vitro which operates in the absence of the nucleus. The cytosol receptors for $1,25(OH)_2$D-3 do not appear to be necessary for this effect of $1,25(OH)_2$D-3 since the presence of cytosol (Figs. 6 and 7, and Table II) did not alter the amount of stimulation or improve the specificity. The concentration of $1,25(OH)_2$D-3 required (30 ng/ml or $7.2 \cdot 10^{-8}$ M) for stimulation seems somewhat high compared to intestinal mucosal $1,25(OH)_2$D-3 concentrations calculated to exist in vivo [35]. However, this level is consistent with the concentration ($4.7 \cdot 10^{-8}$ M) required to stimulate Ca^{2+} uptake by isolated intestinal epithelial cells in vitro [36].

Our studies examined only Ca^{2+} accumulation. Guilland et al. [13] reported that $1,25(OH)_2$D-3 administered in vivo increased the retention of Ca^{2+} by kidney mitochondria assayed in vitro. No study of a direct in vitro effect of $1,25(OH)_2$D-3 was reported. DeLuca and colleagues [10—12,27] in a number

of reports demonstrated that vitamin D-3 in vivo or in vitro stimulated the release of Ca^{2+} from kidney mitochondria. They did not find an increase in Ca^{2+} accumulation. The vitamin D-3 concentrations used by these investigators were comparable to those required by us to demonstrate increased Ca^{2+} accumulation (Fig. 6B). Such large concentrations of vitamin D-3 ($2.6 \cdot 10^{-6}$ M) exceed the physiological range. Whether vitamin D-3 stimulates Ca^{2+} release as well as accumulation by intestinal mitochondria has not yet been demonstrated. Hamilton and Holdsworth [37,38] report that Ca^{2+}-binding protein stimulates Ca^{2+} release from intestinal mitochondria. The concentration of Ca^{2+}-binding protein required (20 μg/ml) for this effect in their hands had no effect on Ca^{2+} accumulation in our system.

Although $1,25(OH)_2D$-3 stimulates mitochondrial Ca^{2+} accumulation directly, the extent of this stimulation over a 100-fold range of $1,25(OH)_2D$-3 concentrations is less than 2-fold. In contrast, increasing the Ca^{2+} concentration from $3 \cdot 10^{-6}$ to $1 \cdot 10^{-5}$ M increases Ca^{2+} accumulation approx. 50-fold in the absence of $1,25(OH)_2D$-3. Therefore, it seems more likely to us that the increase in mitochondrial Ca^{2+} following vitamin D or $1,25(OH)_2D$-3 stimulated Ca^{2+} transport across the gut [3—5] is secondary to the increase in intracellular Ca^{2+} concentration [18] rather than to the increase in $1,25(OH)_2D$-3 concentration. The mitochondria protect the cell from Ca^{2+} toxicity under such circumstances, and probably help transport the Ca^{2+} through the cell.

Acknowledgements

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