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EFFECTS OF VITAMIN D-3 ON PHOSPHATE AND CALCIUM TRANSPORT ACROSS AND COMPOSITION OF SKELETAL MUSCLE PLASMA CELL MEMBRANES

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The effects of vitamin D-3 on calcium and phosphate transport in skeletal muscle plasma membranes were studied. Sarcolemma vesicles were isolated from vitamin D-deficient and vitamin D-treated (one week) chicks by sucrose density gradient centrifugation of a crude muscle plasma membrane fraction. Measurement of $(Na^+ + K^+)$-ATPase activity, cholesterol to phospholipid molar ratios and levels of intracellular marker enzymes showed a high degree of purification of the preparations. Administration of vitamin D-3 significantly increased active Ca^{2+} and phosphate uptake into the vesicles. The efflux of both ions from preloaded vesicles was only slightly altered by the sterol. Ca^{2+}-ATPase activity was higher in sarcolemma from treated animals. This confirms that the effects of vitamin D-3 on calcium transport are related to the Ca^{2+} pump and not to the passive permeability properties of the membrane. No changes in the protein composition of vesicles from both experimental groups were observed. However, treatment with vitamin D-3 increased sphingomyelin and phosphatidylcholine concentrations. These changes in lipid structure may play a role in the effects of vitamin D-3 on transport characteristics of sarcolemma.

Introduction

Various effects of vitamin D-3 (cholecalciferol) and derived metabolites on muscle function and structure have been described. Thus, it has been reported that 25-OH-D_3 stimulates the incorporation of leucine into protein and increases ATP levels in skeletal muscle [1]. Other studies have shown an effect of the vitamin D sterols on calcium transport and morphology of sarcoplasmic reticulum and mitochondrial membranes [2–9]. The administration of vitamin D-3 to rachitic chicks induced changes in the protein composition of mitochondria and the actomyosin contractile complex. In addition, the sterol significantly increased the phospholipid content of sarcoplasmic reticulum [10,11]. The relationships that these changes bear on each other are not known yet. Nevertheless, they may provide a biochemical basis to explain the myopathy present in nutritional vitamin D deficiency and diseases in which the metabolism of cholecalciferol is impaired [12–15].

The effects of vitamin D-3 on muscle proteins and lipids could be associated to changes in the ionic composition of the muscle cell produced by the sterol. Modifications in Ca^{2+} and phosphate ion levels may be relevant. Sarcoplasmic calcium concentrations have been implicated in the control of the synthesis of several muscle proteins [16]. Intracellular phosphate is a precursor in the synthesis of membrane phospholipids and ATP. ATP levels, in turn, affect protein synthesis.

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Abbreviations: 25-OH-D_3, 25-hydroxy-vitamin D-3; 1,25-$(OH)_2$-D_3, 1,25-dihydroxy-vitamin D-3; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Sarcolemmal membranes, in addition to sarcoplasmic reticulum and mitochondria, regulate Ca^{2+} levels in the sarcoplasm [17]. A mechanism of active extrusion of calcium and a Na^{+}-Ca^{2+} exchange system located at the plasma membrane of muscle have been described [18–21]. Transport of phosphate through sarcolemma has not been shown yet, albeit mechanisms of phosphate entry into the cell have been studied in other plasma membrane systems [22–24]. Evidence is emerging now which suggests an involvement of vitamin D-3 on calcium and phosphate fluxes across muscle plasma membranes. Bauman et al. [25] reported that vitamin D-3 or 1,25-$(OH)_2$-D_3 produce a pronounced decrease of Ca^{2+} content in muscle shortly after their injection to rachitic rats. Moreover, preliminary work has shown decreased calcium binding and uptake by sarcolemma isolated from skeletal muscle of vitamin D-deficient rats [26]. Birge and Haddad [1] have demonstrated that addition of 25-OH-D_3 to intact muscle cultures stimulates phosphate uptake [1]. Recent evidence obtained in our laboratory has shown that increased ^{32}P labelling of muscle membrane phospholipids produced by a single administration of vitamin D-3 to rachitic chicks is associated with a proportional increase in radioactivity of sarcoplasmic phosphate [10,11]. This response is compatible with an action of the sterol on phosphate fluxes through the sarcolemmal membrane.

The purpose of this work was to characterize the effects of vitamin D-3 on calcium and phosphate transport processes of muscle plasma membranes. Measurements of Ca^{2+} and phosphate uptake were performed in sarcolemma vesicles isolated from vitamin D-deficient and vitamin D-treated chicks. These data were correlated with an analysis of the phospholipid, fatty acid, cholesterol and protein composition of the membranes.

Materials and Methods

Animals. Chicks were raised from one day of age on a vitamin D-deficient diet with 1.6% calcium and 1.0% phosphorus [27] for 4 weeks. Treated chicks were orally dosed with 80 IU vitamin D-3 per day in 0.10 ml propyleneglycol, one week before killing. Control chicks received the vehicle alone. Both groups of animals were maintained in an environment deprived of light. At the end of the experimental period body weights were 189 ± 4.0 g and 192 ± 5.3 g in control and treated chicks, respectively. Serum Ca^{2+} and phosphorus concentrations were 10.20 ± 2.0 mg% and 2.90 ± 0.29 mg%, respectively, in chicks dosed with vitamin D-3, and 5.19 ± 1.4 mg% and 1.57 ± 0.33 mg% in the control group.

Preparation of sarcolemma vesicles. Skeletal muscles from the legs were quickly dissected out and placed in an ice bath made of distilled water. Subsequent steps were performed at 4°C. The tissue was cleaned of fat and connective tissue and minced. Sarcolemma membrane was isolated essentially by the method of Schapira et al. [28] modified according to Cheng et al. [29]. After disruption of the muscle fibers in 0.25 M sucrose/1 mM Tris-HCl (pH 7.4), actomyosin was extracted by overnight treatment with 0.5 M LiBr/0.05 mM EDTA/10 mM Tris-HCl (pH 8.5). The suspension was centrifuged at $2500 \times g$ for 10 min. This supernatant was centrifuged at $150\,000 \times g$ for 30 min. The pellet was suspended in 0.6 M KCl/10 mM Tris-HCl (pH 8.0). This suspension was allowed to rest for 10 min and centrifuged at $5000 \times g$ for 15 min. The supernatant was further centrifuged at $150\,000 \times g$ for 30 min. The pellet composed of a crude plasma membrane fraction was suspended in 8.5% sucrose and layered on the top of a discontinuous sucrose density gradient made of 50%, 45%, 40% and 30% sucrose in 1 mM Tris-HCl (pH 7.4). The gradient tubes were centrifuged at $100\,000 \times g$ for 90 min in a SW 25 rotor. The purified sarcolemma fraction was collected from the interphase between the 8.5 and 30% sucrose solutions, diluted (1 : 4, v/v) with 0.05 M Tris-HCl (pH 7.5) and centrifuged at $100\,000 \times g$ for 15 min. The pellet was suspended either on 0.25 M sucrose, 0.05 M Tris-HCl (pH 7.5) or in ion-free distilled water. Protein concentration was determined by the method of Lowry et al. [30]. Preparations were used immediately after isolation for measurements of calcium and phosphate transport. Remaining material was stored at -20°C for enzyme assays and lipid and protein compositional analysis.

Enzyme assays. $(Na^{+} + K^{+})$-ATPase (EC 3.6.1.3) activity was measured by the method of Schimmel et al. [31]. 5'-Nucleotidase (EC 3.1.3.5)

was assayed according to the procedure described by Avruch and Wallach [32]. To evaluate the degree of contamination of sarcolemma preparations the following marker enzymes were assayed: Cathepsin D [33], succinate dehydrogenase [34], azide-sensitive Mg^{2+}-ATPase [35] and NADPH-cytochrome *c* reductase [36].

Analysis of lipid and protein composition. Total lipids were isolated by extraction of sarcolemma preparations with chloroform/methanol according to the method of Folch et al. [37]. Phospholipids were separated by thin-layer chromatography on Silica gel G using chloroform/methanol/conc. NH_4OH (65 : 25 : 4, v/v) as solvent. The spots were located by exposure of the plates to iodine vapors and eluted as described previously [38]. Eluants were evaporated and analyzed for phosphate content by the method of Bartlett [39]. For determination of fatty acid composition the total lipid extract of sarcolemma was evaporated to dryness and treated with methanol/H_2SO_4 (95 : 5, v/v) under nitrogen for 90 min at 100°C [40,41]. The resulting methyl esters were extracted with *n*-hexane and concentrated. Gas chromatography was performed on a Shimadzu GC-R14 gas chromatograph equipped with a flame ionization detector using a stainless steel column (2 m × 2 mm diameter) packed with 6% EGS on Chromosorb W (100–120 mesh, acid washed and treated with dimethylchlorosilane) supplied by the Shimadzu Corporation (Japan). The carrier gas was nitrogen at a flow rate of 40 ml/min. Column temperature was maintained constant at 170°C. The methyl esters of fatty acids were identified by comparison of their retention times with those of authentic methyl esters. The composition of fatty acids was estimated from the area of the respective peaks.

Cholesterol and cholesterol esters were isolated by thin-layer chromatography of Folch extracts of sarcolemma on Silica gel G using petroleum ether/diethyl ether/acetic acid (90 : 10 : 1, v/v) as solvent. The corresponding spots were extracted from the plates with chloroform/methanol (4 : 1, v/v) and evaporated to dryness [42]. Quantification of the sterols in the residues was carried out by the method of Zlatkis et al. [43].

The protein composition of sarcolemma membranes purified by sucrose density gradient centrifugation was analyzed by polyacrylamide gel electrophoresis essentially as described earlier [44]. The electrophoretic gels were stained with Comassie Brillant Blue. An estimate of the relative amounts of the various proteins separated was obtained by scanning with a Varian S 634 ultraviolet-visible spectrometer equipped with a gel scanner. Trypsinogen (24 kDa), pepsin (34.7 kDa), egg albumin (45 kDa), bovine albumin (66 kDa) and γ-globulin (160 kDa) were used as molecular weight markers to calibrate gels.

Measurement of calcium uptake. Calcium uptake was measured at 22°C in a medium composed of 160 mM KCl, 22 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 2 mM Tris-ATP and 50 μM $^{45}CaCl_2$ at a protein concentration of 2–6 μg per 30 μl of medium. Samples were taken for Millipore filtration after 1, 2, 5 and 10 min of incubation previous dilution with ice-cold 200 mM KCl/5 mM Tris-HCl/0.1 mM EGTA (pH 7.4) [18]. The filters were washed with the KCl-EGTA terminating solution, placed in scintillation vials and dried at 37°C. Radioactivity was measured in a Beckman liquid scintillation counter using Liquidfluor (New England Nuclear) as scintillation fluid. The rate of calcium uptake and the calcium storing capacity of the sarcolemma vesicles were evaluated on the basis of the values obtained after 1 and 10 min incubation, respectively.

Measurement of phosphate uptake. Conditions for phosphate uptake were taken from the procedure described by Hamilton et al. [45] for mouse fibroblast vesicles. Sarcolemmal membranes (125–250 μg protein) were incubated for 15 min at 37°C in a medium composed of 200 mM sucrose, 20 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$. The reaction was started then by the simultaneous addition of $K_2H^{32}PO_4$ and NaCl (0.1 mM and 100 mM final concentrations, respectively). Samples were taken for Millipore filtration after 1, 2, 5 and 10 min of incubation previous dilution with ice-cold 0.8 M NaCl/10 mM Tris-HCl (pH 7.4). The filters were washed in this solution, dried and the ^{32}P radioactivity measured as described for Ca^{2+} uptake assays.

Measurements of calcium and phosphate efflux. Vesicles were preincubated with ^{45}Ca and ^{32}P at 37°C during 10 min in media of similar composition as for Ca^{2+} and phosphate uptake assays,

respectively. To measure calcium efflux samples preloaded with ^{45}Ca were diluted 50-fold with 160 mM KCl/20 mM Tris-HCl (pH 7.4)/0.1 mM EGTA, previously equilibrated at 37°C. Aliquots were taken at 1, 2, 5 and 10 min for Millipore filtration and the filters washed twice with the KCl-EGTA diluting medium. Radioactivity retained in the filters was determined as described before. The results were expressed as percent calcium remaining in the vesicles with respect to zero-time. For phosphate efflux measurements vesicles prelabeled with ^{32}P were diluted 10-fold with 0.2 M sucrose/20 mM Tris-HCl (pH 7.4)/ 1 mM $MgCl_2$ and processed as for Ca^{2+} efflux measurements.

ATPase assays. (Ca^{2+} + Mg^{2+})-ATPase activity was determined in a medium composed of 30 mM Tris-HCl (pH 7.6), 3 mM $MgCl_2$, 3 mM ATP (Tris salt) and 1 mM ouabain, in the presence of various amounts of $CaCl_2$ and EGTA to obtain a Ca^{2+} concentration range between 0.2 and $4 \cdot 10^{-6}$ M [46]. Mg-ATPase was determined in a medium with similar composition except that $CaCl_2$ was omitted. The reaction was initiated by adding sarcolemmal membranes (100 μg protein per ml) to the medium. After incubating 30 min at 37°C the reaction was stopped by addition of an equal volume of 10% trichloroacetic acid. Samples were centrifuged and P_i was measured in the supernatant [47].

Results

Purity of preparations

The purity of the membrane preparations used in this study was evaluated by measurement of marker enzymes characteristic of sarcolemma and intracellular organelles. The activity of ouabain sensitive-(Na^+ + K^+)-ATPase, an enzyme specific for plasma membrane, ranged between 25 and 30 μmol P_i per mg protein per h in vesicle preparations from both experimental groups. Levels of 5'-nucleotidase, another enzyme reported to be associated to this membrane system, were 0.129 and 0.287 μmol P_i per mg protein per h, in sarcolemma from vitamin D-deficient chicks and chicks given sterol, respectively. The activities of azide-sensitive ATPase and succinate dehydrogenase, markers for mitochondria, indicated a reduced degree of contamination with this organelle (less than 1%). In addition, negible contamination with sarcoplasmic reticulum and lysosomes was present as NADPH-cytochrome *c* reductase and cathepsin, respectively, were very low.

Calcium and phosphate transport properties of vesicles

Sarcolemmal membranes accumulated Ca^{2+} in the presence of 2 mM ATP. The active transport of the ion was significantly stimulated by administration of vitamin D-3, as shown in Fig. 1. The initial rate of uptake was 16.8 nmol/mg protein per min in sarcolemma from treated chicks as compared to 11.7 nmol/mg protein per min in membranes from vitamin D-deficient animals ($P < 0.05$). Calcium storing capacity of the vesicles, measured after 10 min of incubation, was increased from 16.4 nmol/mg protein to 28.5 nmol/mg protein by treatment with the sterol ($P < 0.02$). In the absence of ATP in the incubation medium, calcium uptake by the vesicles was consistently reduced and no differences were observed between preparations from vitamin D-deficient and vitamin D-3-treated chicks.

In addition, vitamin D-3 markedly stimulated phosphate transport of sarcolemma mediated by an externally imposed Na^+ gradient (Fig. 1). The

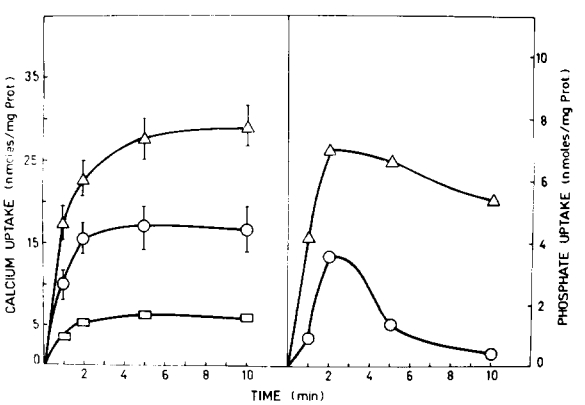

Fig. 1. Time course of calcium and phosphate transport into sarcolemmal vesicles isolated from vitamin D-deficient (○) and vitamin D-3-treated chicks (△). Isolation of vesicles and uptake assays are described under Material and Methods. Ca^{2+} transport was measured in the presence (○, △) and absence (□) of 2 mM ATP in the incubation medium. Values are the mean of determinations on samples from five chicks. Phosphate transport was performed in the presence of a 100 mM NaCl gradient. Values from a typical experiment are shown.

rate of phosphate accumulation was 0.9 nmol/mg protein per min and 4.2 nmol/mg protein per min in depleted chicks and chicks given the sterol, respectively ($P < 0.01$). The transport reaction was lineal up to 2 min of incubation and then rapidly declined in the case of vesicles from vitamin D-deficient animals. The absence of a pronounced overshoot in phosphate uptake of vesicles from the treated group, observed in various experiments, remains unexplained. In a few instances, however, a more rapid loss of phosphate was seen in this preparation (for example 50% loss after 10 min incubation). In the absence of NaCl gradient a low phosphate uptake into the vesicles was measured and no differences between the two types of preparations were observed.

In Fig. 2 it is shown the time course of calcium and phosphate efflux from preloaded vesicles. The exit of both ions is relatively fast and after 10 min approx. 60% of the initial ion content had left the membrane. Previous administration of vitamin D-3 to the animals decreased only slightly the release of calcium and phosphate by sarcolemma.

Ca^{2+}-ATPase activity of vesicles

Ca^{2+}-ATPase activity was measured in skeletal muscle plasma membranes from vitamin D-deficient and repleted chicks which differed in calcium transport. Incubation of the sarcolemmal fraction in a medium containing 3 mM MgATP in the absence and in the presence of various physiological concentrations of Ca^{2+} gave the profiles shown in Fig. 3. An increase in ATP hydrolysis in the presence of Ca^{2+} was observed. Optimum activation was at 1.5–2.0 μM Ca^{2+}. The activity of the enzyme was higher in preparations from vitamin D-3-treated animals at all calcium concentrations tested. Apparent K_m values derived from Lineweaver-Burk analysis were 0.45 and 0.32 μM for

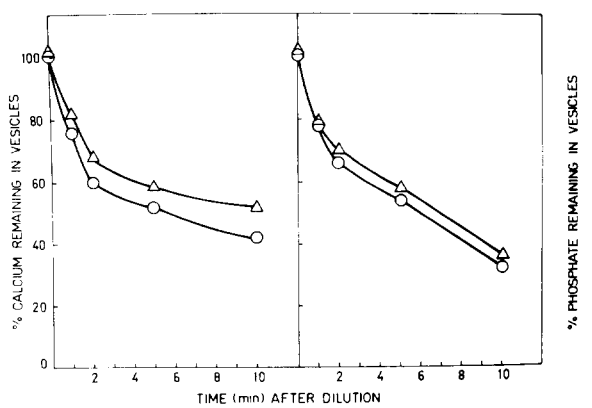

Fig. 2. Time course of calcium and phosphate efflux from sarcolemmal vesicles isolated from vitamin D-deficient (○) and vitamin D-3-treated (△) chicks. Membranes were preloaded with ^{45}Ca and ^{32}P as described under Material and Methods. At zero-time control samples were assayed and the remaining samples were rapidly diluted with uptake buffer. The diluted samples were filtered through Millipore filters at the time points indicated and radioactivity in the filters was measured. Values are the mean of determinations on preparations from three chicks.

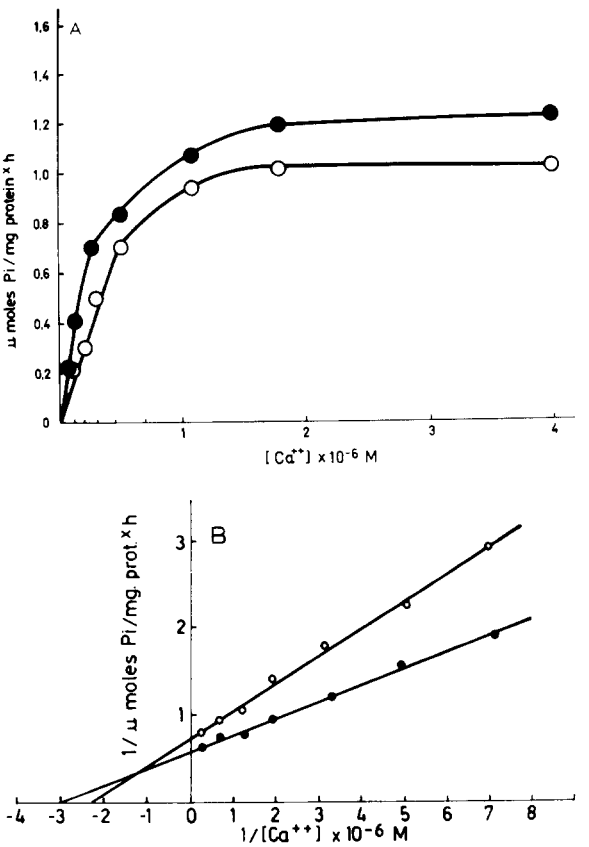

Fig. 3. (A) Effect of Ca^{2+} concentration on Ca^{2+}-ATPase activity of sarcolemmal vesicles isolated from vitamin D-deficient (○) and vitamin D-3-treated (●) chicks. ATPase assays were performed as described under Material and Methods. Values correspond to the difference between (Ca^{2+} and Mg^{2+})- and Mg^{2+}-ATPase activities and are the mean of determinations on samples from three chicks. (B) Lineweaver-Burk plots of the data.

deficient and treated chicks, respectively. V_{max} was increased by the sterol from 1.02 to 1.31 μmol P_i per mg protein per h. The effects of vitamin D-3 on kinetics parameters could be reproduced consistently in several experiments.

Chemical and electrophoretic analysis

As the effects of vitamin D-3 on transport properties of sarcolemma could be related to changes in its chemical composition, the protein and lipid profiles of membrane preparations from vitamin D-deficient chicks and chicks dosed with the sterol were compared. Administration of vitamin D-3 to depleted animals increased the relative phospholipid content of sarcolemma, as seen in Table I. Cholesterol levels were not modified by treatment with the sterol. Administration of vitamin D-3, however, altered the relative proportions of phospholipid classes (Table II). Sphingomyelin and phosphatidylcholine concentrations were increased significantly, while phosphatidylethanolamine concentration decreased. Only minor variations in the levels of phosphatidylserine and phosphatidylinositol were observed.

The fatty acid composition of total sarcolemma lipids was not modified appreciably by treatment of depleted chicks with vitamin D-3 (Table III).

Analysis of the protein profiles of skeletal muscle plasma membranes from both experimental groups by polyacrylamide gel electrophoresis did not reveal differences.

TABLE I

PHOSPHOLIPID AND CHOLESTEROL CONTENT OF SKELETAL MUSCLE SARCOLEMMA FROM VITAMIN D-DEFICIENT AND VITAMIN D-3-TREATED CHICKS

The phospholipid content of sarcolemma was measured after Folch extraction as described in Material and Methods. Cholesterol isolated from the lipid extracts by thin-layer chromatography was quantitated by the method of Zlatkis et al. [43]. Each value represents the mean ± S.D. of five different preparations.

	− Vitamin D	+ Vitamin D-3
Phospholipd (μmol/mg protein)	0.78 ± 0.15	0.95 ± 0.12
Cholesterol (mg/mg protein)	0.20 ± 0.01	0.20 ± 0.02
Cholesterol/Phospholipid (molar ratio)	0.66	0.60

TABLE III

FATTY ACID COMPOSITION OF SKELETAL MUSCLE SARCOLEMMA FROM VITAMIN D-DEFICIENT AND VITAMIN D-3-TREATED CHICKS

Methyl esters of fatty acids isolated from total membrane lipids were separated and quantitated by gas-liquid chromatography. Results are presented as percentage of total peak area. Each value represents the mean ± S.D. of four different preparations.

Fatty acid	Percentage	
	− Vitamin D	+ Vitamin D-3
14 : 0	0.7 ± 0.77	0.4 ± 0.95
14 : 1	3.6 ± 1.16	4.3 ± 0.54
16 : 0	17.6 ± 1.78	17.4 ± 2.23
16 : 1	2.1 ± 0.39	2.1 ± 0.46
18 : 0	26.5 ± 2.59	27.6 ± 1.80
18 : 1 − 9	15.1 ± 2.59	17.3 ± 0.60
18 : 2 − 6	29.6 ± 2.97	25.6 ± 3.10
20 : 4 − 6	2.3 ± 0.58	1.7 ± 0.53
22 : 5 − 3	0.5 ± 0.24	0.2 ± 0.36
22 : 6 − 3	1.4 ± 1.79	2.6 ± 1.05

TABLE II

PHOSPHOLIPID COMPOSITION OF SKELETAL MUSCLE SARCOLEMMA FROM VITAMIN D-DEFICIENT AND VITAMIN D-3-TREATED CHICKS

Phospholipid extracts prepared according to Folch et al. [37] were fractionated by thin-layer chromatography. Phosphate content in each spot was determined by the Bartlett's procedure [39]. Values represent percentage of total lipid P and add to 100% with P found at origin of TLC plates. * $P < 0.001$, ** $P < 0.05$ compared to vitamin D-deficient chicks.

	PI	PS	SM	PC	PE
− vit. D	3.40 ± 0.60	5.90 ± 1.52	10.62 ± 0.56	44.54 ± 0.71	30.68 ± 4.28
+ vit. D-3	2.95 ± 0.57	5.69 ± 1.69	17.11 ± 0.42 *	47.20 ± 1.58 **	26.55 ± 2.86

Discussion

Direct proof that vitamin D-3 affects calcium and phosphate transport through sarcolemmal membranes was obtained. Previous investigations had suggested effects of the sterol on skeletal muscle plasma membranes. Neville and DeLuca observed that administration of ^{3}H-labeled vitamin D-3 to intact animals results in appreciable accumulation of radioactivity in sarcolemma [48]. More recently, indirect evidence obtained by other authors implied involvement of cholecalciferol in calcium and phosphate fluxes across this membrane system [1,10,11,25].

In this study it was shown that administration of vitamin D-3 to vitamin D-deficient chicks significantly increases in vitro ATP-dependent calcium accumulation by sarcolemma vesicles. The data obtained suggest that the effects of cholecalciferol on Ca^{2+} transport are related to changes in activity of the Ca^{2+} pump rather than to modifications in the passive permeability properties of the membrane. Differences in Ca^{2+} efflux from preloaded vesicles between vitamin D-depleted and vitamin D-3-treated chicks were smaller than the variations observed in active calcium uptake. The sterol, however, produced an stimulation of sarcolemmal Ca^{2+}-ATPase activity. The study of its kinetic properties varying Ca^{2+} concentrations in the medium revealed that cholecalciferol induces a decrease of K_m and an increase of V_{max}. These changes are compatible with an increase in the enzyme affinity for calcium and concentration of active sites in the membrane by the sterol. The high affinity of the transport ATPase for Ca^{2+} ($K_m < 1\ \mu M$) is consistent with its participation in the mechanism of regulation of intracellular calcium concentration in the muscle cell [17].

It is highly unlikely that the variations observed in Ca^{2+} transport and Ca^{2+} sensitive-ATPase activities of sarcolemmal membranes from vitamin D-depleted and vitamin D-3-treated chicks are due to contaminations with mitochondria, sarcoplasmic reticulum or actomyosin. The levels of marker enzymes were very low and comparable in both preparations. Moreover, calcium uptake by vesicles was not affected by addition of NaN_3 and ruthenium red, compounds which inhibit mitochondrial calcium uptake activity. In agreement with previous reports [18], sarcolemmal Ca^{2+} transport, in contrast to sarcoplasmic reticulum, was not stimulated by 5 mM oxalate. In addition, myofibrils are extracted during the isolation procedure by exhaustive treatment with 0.6 M LiBr and KCl. Comparison of ($Na^+ + K^+$)-ATPase specific activities and cholesterol/phospholipid molar ratios with values reported in the literature [28,29, 31,49–51] suggests a high degree of purification of the sarcolemmal fraction used in this study. The activity of 5′-nucleotidase, however, was approx. 10 times lower than the values obtained by Schapira et al. [28]. As a possible explanation for this apparent discrepancy it should be mentioned that other authors were able to identify ($Na^+ + K^+$)-ATPase and 5′-nucleotidase activities in different sucrose density gradient fractions [31].

In agreement with our observations similar effects of vitamin D-3 on calcium uptake by rat skeletal muscle sarcolemma have been recently reported in abstract form [26].

Calcium accumulation by sarcolemma preparations probably reflect the activity of Ca^{2+}-ATPase in a vesicle population with inverted membranes or 'inside-out' since the physiological function of the pump is to extrude Ca^{2+} from the cells [18]. Therefore, the differences observed in vitro between vesicles from depleted and vitamin D-3-treated chicks would imply that the in vivo calcium content of the muscle cell is increased in vitamin D deficiency. Recent work by Bauman et al. [25] supports this contention. These authors found augmented calcium levels in skeletal muscle from rachitic rats. Acute treatment of the animals with vitamin D-3 or 1,25-$(OH)_2$-D_3 decreased the calcium content of the tissue.

It is not likely that the degree of sidedness and resealing of the vesicles in the sarcolemmal preparations would contribute to their differences in Ca^{2+} transport. It could be verified that the total Ca^{2+}-ATPase activity obtained after detergent treatment or osmotic shock was still higher in vesicles from vitamin D-3-treated chicks than in vesicles from vitamin D-deficient animals. The increase in Ca^{2+}-ATPase activity allowed to estimate that in the preparations from both groups about the same proportion of inside-out plus leaky vesicles were present.

Moreover, the differences in Ca^{2+} pump activ-

ity between vesicles from vitamin D-deficient and vitamin D-3-treated animals are not probably due to differences in the amounts of calmodulin present in both preparations. First, an effect of vitamin D-3 on calmodulin content of sarcolemmal membranes is not likely in view of recent reports. Halloran et al. [52] have shown a lack of dependence on vitamin D of calmodulin activity of erythrocytes. In addition, Thomasset et al. [53] demonstrated that 1,25-$(OH)_2$-D_3 does not influence calmodulin concentration in duodenal cells. Second, sufficient amounts of calmodulin to fully activate the Ca^{2+}-ATPase should be present in sarcolemma from both experimental groups taking into consideration the conditions of the isolation procedure employed. It has been reported that plasma membranes from smooth muscle still have abundant calmodulin after osmotic shock and EDTA-treatment [54]. In heart sarcolemma hypotonic and hypertonic shock in the presence of EGTA is required to remove calmodulin [55].

Vitamin D-3 affected, in addition, phosphate accumulation by sarcolemma. Previous treatment of vitamin D-deficient chicks with cholecalciferol produced a 4-fold increase in the active transport of the anion measured in vitro. The effects of the sterol on phosphate uptake into the vesicles were dependent on the presence of an external Na^+ gradient. In the absence of 100 mM NaCl in the uptake medium, the vesicles did not accumulate phosphate. These observations are similar to previous reports on phosphate transport of vesicles isolated from other cells and indicate the operation of a sodium-phosphate symport mechanism [45,56–58]. In contrast to the Ca^{2+} pumps, detailed knowledge on phosphate carriers of cell membranes is lacking, except for recent advances with the mitochondrial PO_4 transport system [59,60].

The modifications produced by vitamin D-3 on phosphate transport properties of sarcolemma vesicles support previous observations. Birge and Haddad [1] reported that 25-OH-D_3 stimulates phosphate uptake by intact muscle in vitro. In vivo studies have recently shown that prior administration of vitamin D-3 to chicks partially depleted of vitamin D stimulates incorporation of [^{32}P]phosphate to skeletal muscle sarcoplasm. Since the specific activity of serum P_i did not differ between control and treated animals, the experiments suggests an involvement of the sterol on phosphate fluxes though the muscle plasma membrane [11].

The possibility that the effects observed in isolated sarcolemmal membranes are due to contamination with mitochondria, which possess a rapid transport process, should be discarded since a 100 mM NaCl gradient has little effect on phosphate accumulation by the organelle [45,61,62].

The lack of differences in cholesterol levels and fatty acid composition of sarcolemma from vitamin D-deficient and vitamin D-3-treated chicks agree with the fact that the calcium and phosphate passive permeability properties of the vesicles were not significantly altered by the sterol. On the other hand, administration of cholecalciferol increased the total amounts of phospholipids and the concentration of sphingomyelin and phosphatidylcholine in the membrane. The absence of changes in protein composition suggest that the modifications in the content and distribution of phospholipids may play a role in the mechanism by which vitamin D affects calcium and phosphate transport in skeletal muscle plasma membranes. These changes in membrane lipids may result in modifications of the environment of transport proteins, therefore influencing their activity. This would be in line with evidence obtained by other authors which suggests that the effects of vitamin D sterols on intestinal plasma membrane calcium transport are mediated by changes in the lipid structure of this membrane. Thus, a close correlation between the time course of de novo synthesis of phosphatidylcholine and Ca^{2+} transport has been shown in brush border membrane vesicles after 1,25-$(OH)_2$-D_3 administration to vitamin D-deficient chicks. Similar to sarcolemma, intestinal membranes from depleted animals contain less phosphatidylcholine. Moreover, neither the changes in lipid structure nor those in Ca^{2+} transport were blocked by previous treatment of deficient chicks with cycloheximide indicating that the action of the sterol does not require protein synthesis [63,64]. Contrary to our findings other authors have demonstrated that the administration of 1,25-$(OH)_2$-D_3 also induces changes in the composition and metabolism of the membrane fatty acids [64,65].

These studies have clearly demonstrated that

vitamin D affects in muscle, similarly as in intestine and kidney, calcium and phosphate fluxes across plasma membranes. 1,25-Dihydroxycholecalciferol has been identified as the vitamin D derivative which stimulates the transport of both ions in intestine and kidney [66]. Only fragmentary evidence is available respect to the active metabolite in muscle tissue. Birge and Haddad [1] have shown that 25-OH-D_3 affects phosphate absorption by muscle. Other work suggests, however, that 1,25-$(OH)_2$-D_3 is implicated in calcium fluxes through muscle plasma membranes [25]. These aspects deserve clarification in further studies.

Finally, the action of vitamin D-3 on sarcolemmal calcium and phosphate transport may result in modifications of the levels of both ions in muscle cytosol. An attractive possibility is to postulate that these changes may in turn be responsible for several effects on muscle membranes and contractile proteins described for the vitamin [1–11]. This hypothesis should, however, be tested experimentally.

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