Stimulation of Calmodulin Binding to Skeletal Muscle Membrane Proteins by 1,25-Dihydroxy-Vitamin D_3*

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Previous work has shown that 1,25-dihydroxy-vitamin D_3 rapidly increases calmodulin levels of skeletal muscle membranes without altering the muscle cell calmodulin content. Therefore, the effects of the sterol on the binding of calmodulin to specific muscle membrane proteins were investigated. Soleus muscles from vitamin D-deficient chicks were treated *in vitro* for short intervals (5–15 min) with physiological concentrations of 1,25-dihydroxy-vitamin D_3. Proteins of mitochondria and microsomes isolated by differential centrifugation were separated on sodium dodecyl sulfate polyacrylamide gels. Calmodulin-binding proteins were identified by a [^{125}I]calmodulin gel overlay procedure followed by autoradiography. 1,25-Dihydroxy-vitamin D_3 increased the binding of labelled calmodulin to a major, calcium-independent, calmodulin-binding protein of 28 Kda localized in microsomes, and to minor calmodulin-binding proteins of 78 and 130 Kda proteins localized in mitochondria. The binding of [^{125}I]calmodulin to these proteins was abolished by flufenazine or excess non-radioactive calmodulin. 1,25-Dihydroxy-vitamin D_3 rapidly increased muscle tissue Ca uptake and cyclic AMP levels and stimulated the phosphorylation of several membrane proteins including those whose calmodulin-binding capacity potentiates. Analogously to the sterol, forskolin increased membrane calmodulin content, calmodulin binding to the 28 Kda microsomal protein and ^{45}Ca uptake by soleus muscle preparations. Forskolin also induced a similar profile of changes in muscle membrane protein phosphorylation as the hormone. These results suggest that 1,25-dihydroxy-vitamin D_3 affects calmodulin distribution in muscle cells through cyclic AMP-dependent phosphorylation of membrane calmodulin-binding proteins. These changes may play a role in the stimulation of muscle Ca uptake by the sterol.

Introduction

1,25-Dihydroxy-vitamin D_3 has been involved in the regulation of muscle intracellular Ca^{2+} levels [1–4]. The mechanism whereby the sterol regulates Ca^{2+} transport in the muscle cell remains only partially understood. There is evidence which supports the idea that 1,25-dihydroxy-vitamin D_3 acts on skeletal muscle through a nuclear mechanism as in classical target organs. Maximum responses in calcium uptake induced by the sterol in chicken soleus muscle and myoblast cultures are suppressed by protein and RNA synthesis inhibitors [5]. In addition, an intracellular receptor specific for 1,25-dihydroxy-vitamin D_3 has been detected in cultured myoblasts and muscle tissue [6, 7]. However, 1,25-dihydroxy-vitamin D_3 has been shown to cause a rapid stimulation of muscle Ca uptake, independently of *de novo* protein synthesis and which can be suppressed by calcium channel blockers [8] and calmodulin antagonists [9]. This change is paralleled, as recently shown, by an increase in calmodulin content of muscle membranes at the expense of a decrease in cytosol calmodulin concentration [9], suggesting that alterations in muscle intracellular calmodulin distribution may play a role in the rapid action of 1,25-dihydroxy-vitamin D_3 on muscle Ca influx. The present work was undertaken to obtain information about the changes underlying the effects of the sterol. Specifically, we have investigated whether 1,25-dihydroxy-vitamin D_3 affects the binding properties of muscle membrane calmodulin-binding proteins and the mechanism involved in these modifications.

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Materials and Methods

Materials

1,25-Dihydroxy-vitamin D_3 was provided by Dr. Milan Uskokovic (Hoffman-La Roche Co., Nutley, NJ). $Na^{125}I$, gamma-^{32}P-ATP and aquasol scintillation fluid were purchased from New England Nuclear (Boston, MA). Calmodulin, purified calmodulin-depleted bovine brain phosphodiesterase and forskolin, were obtained from Sigma Chemical Co. (St. Louis, MO). Cyclic AMP-assay kit was provided by Diagnostic Products Corporation (Los Angeles, CA). All other reagents were of analytical grade.

Methods

Soleus muscles, weighing approx. 400 mg and with and average thickness of 2–3 mm, were isolated from vitamin D-deficient chicks and preincubated in Krebs-Henseleit-glucose solution for 45 min at 37 °C under 95% O_2/5% CO_2, as previously described [4]. 1,25-Dihydroxy-vitamin D_3 was added to the preparations dissolved in ethanol to give a concentration of 0.05 ng/ml. Forskolin (10 μM) was added dissolved in the same vehicle. An equal amount of ethanol alone was added to control samples. The muscles were treated for 5 to 60 min. The viability of the tissue was evidenced by the lack of significant changes in [^{3}H]leucine incorporation into protein and in ^{45}Ca and ^{32}P uptake up to 4 h of culture, in agreement with previous observations [4, 10]. After treatment, the tissue was homogenized with 5 ml 50 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 0.5 mM phenylmethylsulfonylfluoride, 5 mM EDTA, 0.2 mM EGTA, 0.25 M sucrose. EDTA and EGTA were omitted from the homogenization buffer when calmodulin activity was measured. To isolate muscle fractions, the homogenate was centrifuged for 10 min at $1200 \times g$ in a Sorvall refrigerated centrifuge. The sediment containing myofibrils was discarded and the supernatant was filtered through a triple layer of cheese-cloth. Mitochondria were collected by centrifugation for 30 min at $11{,}300 \times g$. Microsomal membranes were sedimented from this supernatant in a Beckman L 5-50 B ultracentrifuge at $120{,}000 \times g$ for 1 h. The final supernatant corresponds to the cytosolic fraction. Mitochondria and microsomes were resuspended in homogenization buffer and collected by centrifugation as described above. To assess the purity of membrane fractions, ouabain-sensitive Na + K-ATPase, Ca + Mg-ATPase and azide- and oligomycin-sensitive Ca-ATPase were measured as markers for plasma membranes, sarcoplasmic reticulum and mitochondria, respectively [9, 11]. Negligible contamination of mitochondria was detected. The microsomal fraction was shown to contain both sarcoplasmic reticulum and plasma membrane marker enzyme activities (Ca + Mg-ATPase: 0.915 μmol Pi/mg prot. min; Na + K-ATPase: 0.367 μM Pi/mg prot. min) but it was devoid of appreciable mitochondria marker enzyme. No significant differences between control and 1,25-dihydroxy-vitamin D_3-treated preparations were found.

To assay calmodulin, the homogenate and subcellular fractions were extracted with boiling 20 mM Tris-HCl, pH 8.2, 1 mM EGTA for 5 min. The calmodulin activity of extracts was measured by means of the phosphodiesterase assay [12]. 2.4 m units of purified (bovine brain) calmodulin-depleted 3′,5′-cyclic AMP phosphodiesterase were incubated in 100 μl of medium composed of 40 mM Tris-HCl, pH 8.0, 5 mM $MgSO_4$, 0.05 mM $CaCl_2$, 2 mM 3′,5′-cyclic AMP and sample (20–50 μg protein). The reaction mixture was incubated for 30 min at 30 °C. The samples were then heated for 1 min at 100 °C. Snake venom from *Crotalus atrox* (40 μg) was added and the mixture was further incubated for 10 min at 30 °C. The reaction was stopped by addition of an equal volume of 10% trichloroacetic acid. Released Pi was determined by the Fiske-Subbarow method [13].

Calmodulin-binding proteins of muscle membranes were detected using the [^{125}I]calmodulin gel overlay technique of Glenney and Weber [14], as modified by Nelson *et al.* [15]. Briefly, equal aliquots of membrane proteins (100 μg) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli [16] using 7% polyacrylamide gels. After electrophoresis the gels were fixed in 40% methanol/10% acetic acid, washed in 10% ethanol to remove SDS, then in water, and incubated in a medium composed of 20 mM imidazole, pH 7.0, 0.2 M KCl, 0.1% BSA, 0.025% NaN_3 with 5 μCi (150 μCi/nmol) [^{125}I]calmodulin/gel. The incubation medium contained either 1 mM $CaCl_2$, 1 mM

EGTA or 10^{-4} M flufenazine. [^{125}I]Calmodulin was prepared according to the procedure of Chafouleas *et al.* [17]. After 48 h incubation, the gels were washed with 20 mM imidazole, pH 7.0, 0.2 M KCl, 0.025% NaN_3, 0.02% Triton X-100, stained with Comassie Brillant Blue, dried and exposed to Alfa-Gevaert film (Eastman Kodak Co., Rochester, N.Y.) for 3 weeks at −20 °C. The radioactive bands were excised and quantitated in a gamma counter.

To perform cyclic AMP assays, muscle samples immediately frozen in liquid nitrogen after treatment, were homogenized for 15 sec in a Ultraturrax homogenizer using 5 vol of cold 10% trichloroacetic acid. The homogenate was centrifuged at 2500 × *g* for 15 min. The supernatant was pipetted off and washed five times with 6 vol of water-saturated diethyl ether. The washed extract was lyophilized and cyclic AMP measured using a competitive protein-binding technique.

A standard assay for phosphorylation of membrane proteins was used [19, 20]. Membrane samples (300 µg protein) were preincubated for 1 min at 30 °C in 100 µl medium containing 10 mM $MgCl_2$, 0.2 mM EGTA, 50 mM Tris-HCl, pH 7.0, in the absence or presence of 1 mM $CaCl_2$. Phosphorylation was started by addition of 10 µCi [γ-^{32}P]ATP to give a final concentration of 10 µM. The rate of phosphorylation of membrane proteins by intrinsic protein kinase activity is not affected at higher ATP concentrations [20]. After 1 min at 30 °C, the reaction was terminated by addition of an equal volume of electrophoresis sample buffer, and the samples were then subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis. The gels were dried and autoradiographed for 72 h at −20 °C. Phosphorylated bands were excised from the gel, dissolved in H_2O_2 at 37 °C and the radioactivity determined in a Beckman liquid scintillation spectrometer using Aquasol as scintillation fluid.

Determinations of ^{45}Ca uptake by soleus muscle were carried out using a modification of a procedure previously described [4, 21]. The muscles were incubated in Krebs-Henseleit-glucose medium containing $^{45}CaCl_2$ (2 µCi/ml) at 37 °C for 10 min. The samples were then quickly washed with cold unlabelled medium, blotted on filter paper, and dissolved in hot 1 N NaOH. Aliquots were taken for determination of protein by the procedure of Lowry *et al.* [22] and radioactivity in a scintillation counter as above.

Results

Changes in calmodulin binding by specific skeletal muscle membrane proteins which could mediate the rapid *in vitro* effects of 1,25-dihydroxy-vitamin D_3 on muscle intracellular calmodulin distribution [9] were studied. A SDS-PAGE gel overlay technique was used to identify calmodulin-binding proteins in mitochondria and microsomes isolated from vitamin D-deficient chick soleus muscle incubated 15 and 60 min in the absence and presence of physiological amounts of 1,25-dihydroxy-vitamin D_3. The proteins of these muscle membrane fractions were separated according to their relative molecular weights *via* SDS polyacrylamide gel electrophoresis. Then, the gels were incubated with ^{125}I-labelled calmodulin and subjected to autoradiography to detect individual binding proteins. Fig. 1 shows a representative autoradiogram of such experiments. The major calmodulin

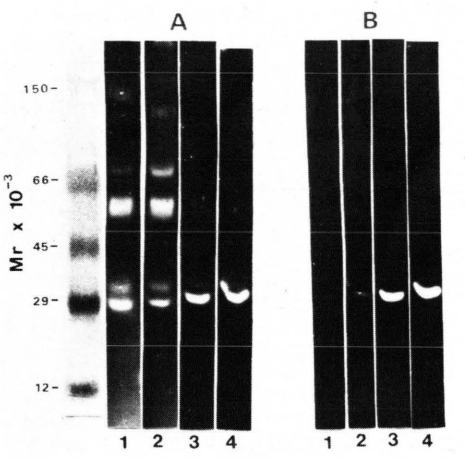

Fig. 1. Calmodulin-binding proteins of chick skeletal muscle membranes. The figure shows the autoradiogram of membrane samples (75 µg protein) isolated from vitamin D-deficient chick soleus muscles incubated 15 min in the absence (control) and presence of 1,25-dihydroxy-vitamin D_3 (10^{-10} M). The proteins were separated by slab-gel electrophoresis and then incubated with [^{125}I]calmodulin in the presence of 1 mM Ca^{2+} (A) or 1 mM EGTA (B), followed by autoradiography. Mitochondria: (1) control, (2) +1,25 $(OH)_2D_3$; microsomes: (3) control, (4) +1,25 $(OH)_2D_3$. The position of molecular weight markers are shown on the left lane.

targets in mitochondrial membranes have apparent molecular masses of 28 and 56 Kda; proteins of 30, 70, 130 and 145 Kda bound labelled calmodulin to a lesser extent (Fig. 1 A). Binding of calmodulin to these mitochondrial proteins was shown to be Ca-dependent by the absence of bands in autoradiograms of gels incubated with a solution of [^{125}I]calmodulin containing 1 mM EGTA instead of 1 mM Ca (Fig. 1 B). In microsomes, a major calmodulin-binding protein of 28 Kda which bound [^{125}I]calmodulin either in the presence or absence of Ca was detected (Fig. 1 A and B). In addition, minor microsomal calmodulin-binding proteins of 60 and 170 Kda were barely detected in the gels. The presence of flufenazine (0.1 mM), a calmodulin antagonist, or an excess of non-radioactive calmodulin in the gel labelling medium prevented the binding of [^{125}I]calmodulin to proteins of both mitochondria and microsomes (data not shown). These results are in general agreement with studies which have shown the presence of various calmodulin-binding proteins in mitochondria and microsomes of different tissues [23, 24]. As shown in Fig. 1, 1,25-dihydroxy-vitamin D_3 increases in mitochondria the amount of radioactivity associated to the [^{125}I]calmodulin-labelled 78 and 130 Kda proteins. A decrease in labelling of the protein of 28 Kda was also evidenced. In addition, the metabolite increased the levels of [^{125}I]calmodulin bound to the 28 Kda macromolecule of microsomes. To quantitate the effects of the sterol, the calmodulin-binding proteins localized by the SDS-PAGE overlay procedure were removed from the portion of the dried gel identified by autoradiography and their ^{125}I content was determined using gamma scintillation spectroscopy. The results of three separate experiments are summarized in Table I. After 15 min of treatment with the sterol, there is a marked stimulation in calmodulin binding to the 28 Kda microsomal proteins (+300%) and to the 78 Kda (+220%) and 130 Kda (+150%) mitochondrial proteins (Table I). The 3-fold 1,25-dihydroxy-vitamin D_3-dependent increase in [^{125}I]calmodulin binding by the 28 Kda protein of microsomes was less evident in the autoradiogram (Fig. 1) probably due to the narrow range of linearity of the photographic film. The effects of the sterol on the minor 170 and 60 Kda proteins localized in the microsomal fraction were less apparent. After 60 min of treatment with 1,25-dihydroxy-vitamin D_3, the stimulation of calmodulin binding to muscle membrane proteins sharply decreases (Table I).

The possibility that 1,25-dihydroxy-vitamin D_3 would affect calmodulin binding to skeletal muscle membranes by a stimulation of cAMP-dependent phosphorylation of muscle membrane proteins was investigated. Modulation of calmodulin binding after phosphorylation has been described for a series of proteins [25–27]. Changes in cAMP levels

Table I. Stimulation by 1,25-dihydroxy-vitamin D_3 of calmodulin binding to skeletal muscle membrane proteins. Proteins from membrane fractions isolated from vitamin D-deficient chick soleus muscle treated *in vitro* with 10^{-10} M 1,25 $(OH)_2D_3$ (15 and 60 min) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gels were incubated with [^{125}I]calmodulin followed by autoradiography. Proteins from radioactive bands were then extracted from the gel and the radioactivity measured in a gamma counter as described in Methods. Results are the average of three separate experiments.

Membrane fraction	Relative molecular mass [Kda]	Calmodulin-binding stimulation (% of control)	
		15 min	60 min
Mitochondria	130	150 ± 37	21 ± 6
	78	220 ± 55	38 ± 11
Microsomes	170	40 ± 10	25 ± 8
	60	13 ± 6	15 ± 7
	28	300 ± 60	20 ± 6

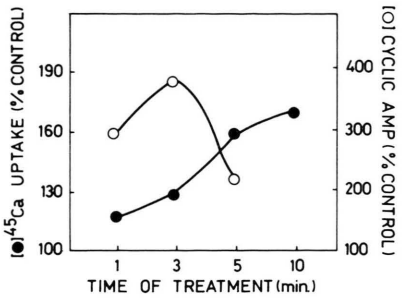

Fig. 2. Increase in cyclic AMP content and $^{45}Ca^{2+}$ uptake by chick muscle in response to short *in vitro* treatment intervals with 1,25 $(OH)_2D_3$. Samples of soleus muscles obtained from vitamin D-deficient chicks were incubated in Krebs-Henseleit-glucose solution in the absence or presence of 1,25 $(OH)_2D_3$ (10^{-10} M) at 37 °C for various times. Cyclic AMP and ^{45}Ca uptake were then measured as described in Methods. Values are the average of three independent experiments.

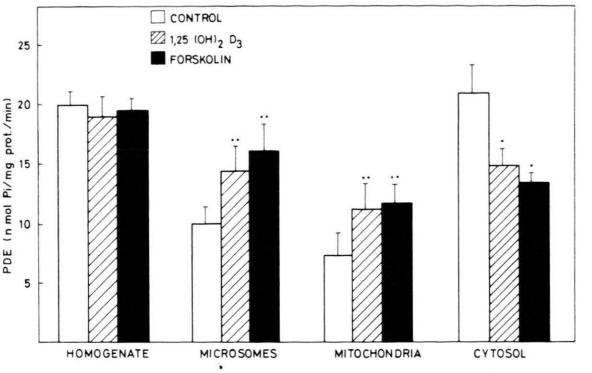

Fig. 3. Subcellular distribution of calmodulin in chick skeletal muscle treated *in vitro* with 1,25 $(OH)_2D_3$ and forskolin. Vitamin D-deficient chick soleus muscles were treated 15 min with either 1,25-dihydroxy-vitamin D_3 (10^{-10} M) or forskolin (10^{-5} M). Subcellular fractions were isolated by differential centrifugation. Calmodulin activity was determined using the phosphodiesterase assay, as described in Methods. Different concentrations of homogenates and fractions were employed. The stimulation of phosphodiesterase to a great extent was Ca^{2+}-dependent (basal activity in the presence of 10 mM EGTA was 2.5 nmol Pi/mg prot./min) and could be abolished (90%) by the calmodulin antagonists flufenazine and compound 48/80. Bars represent calmodulin-specific activity in each fraction expressed as phosphodiesterase activity (mean ± S.D., $n = 3$). * $p < 0.005$, ** $p < 0.01$, with respect to the corresponding control.

and/or adenylate cyclase activity in response to the sterol have been reported for other tissues [28, 29]. Treatment of soleus muscle from vitamin D-deficient chicks for 1 to 5 min with 1,25-dihydroxy-vitamin D_3 (10^{-10} M) resulted in a 3-, 4- and 2-fold increase in the content of muscle tissue cAMP at 1, 3 and 5 min of treatment, respectively (Fig. 2). This rapid increase in cAMP paralleled the initial stimulation of muscle ^{45}Ca uptake elicited in response to the sterol. It was then determined whether forskolin, an activator of adenylate cyclase, would mimic the effects of 1,25-dihydroxy-vitamin D_3 on calmodulin subcellular distribution in skeletal muscle. To this end, the calmodulin levels of membranes and cytosol isolated from chick soleus muscles treated 15 min *in vitro* with forskolin or the sterol were estimated by measuring their capacity to activate bovine brain calmodulin-depleted cAMP phosphodiesterase. Analogously to 1,25-dihydroxy-vitamin D_3, forskolin increased the calmodulin content of muscle mitochondria and microsomes whereas it decreased that of the cytosolic fraction (Fig. 3). In agreement with these observations, it was observed by gel overlay that forskolin causes a similar pattern of stimulation of [^{125}I]calmodulin binding to muscle microsomal membrane proteins as 1,25-dihydroxy-vitamin D_3 (Fig. 4). These results suggest a role of cAMP in the effects of 1,25-dihydroxy-vitamin D_3 on the intracellular distribution of calmodulin in muscle. Experiments were carried out to correlate the me-

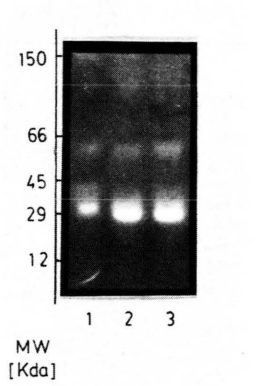

Fig. 4. Effects of forskolin on calmodulin binding to muscle microsomal proteins. Membrane samples (45 µg protein) were isolated from vitamin D-deficient chick soleus muscles and incubated 15 min in the absence (control) and presence of forskolin (10 µM) or 1,25-dihydroxy-vitamin D_3 (10^{-10} M). The proteins were separated by slab-gel electrophoresis and then incubated with [^{125}I]calmodulin followed by autoradiography. (1) Control; (2) +1,25 $(OH)_2D_3$; (3) + forskolin.

Table II. The effects of 1,25-dihydroxy-vitamin D_3 and forskolin on the phosphorylation of skeletal muscle proteins. Mitochondria and microsomes were isolated from vitamin D-deficient chick soleus muscle treated *in vitro* (15 min) with either 1,25-dihydroxy-vitamin D_3 (10^{-10} M) or forskolin (10^{-5} M). The membranes were phosphorylated with [γ-^{32}P]ATP and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by autoradiography. Proteins from radioactive bands were extracted from the gel and the radioactivity measured in a liquid scintillation counter as described in Methods. Results are the average of 4 separate experiments.

Relative molecular mass (Kda)	Mitochondria		Microsomes	
	1,25 $(OH)_2$-vit. D_3	Forskolin	1,25 $(OH)_2$-vit. D_3	Forskolin
	Protein phosphorylation (% of control)			
180	264 ± 30	354 ± 48	–	–
170	–	–	66 ± 8	102 ± 10
141	–	–	152 ± 19	206 ± 25
130	240 ± 28	900 ± 100	–	–
90	68 ± 7	93 ± 12	–	–
78	580 ± 75	670 ± 60	74 ± 10	103 ± 13
60	35 ± 5	50 ± 6	124 ± 18	190 ± 22
45	–	–	40 ± 5	60 ± 5
35	–	–	42 ± 6	65 ± 6
31	62 ± 10	105 ± 10	–	–
28	–	–	102 ± 11	170 ± 19
21	–	–	51 ± 4	71 ± 8
15–16	–	–	37 ± 5	55 ± 4

diation by cAMP of the action of the sterol with modifications in muscle membrane protein phosphorylation. Membranes isolated from soleus treated with 1,25-dihydroxy-vitamin D_3 were phosphorylated with [γ-^{32}P]ATP followed by analysis of labelled phosphoproteins by SDS-PAGE and autoradiography. 1,25-Dihydroxy-vitamin D_3 stimulated the phosphorylation of several membrane proteins including those whose calmodulin binding capacity was potentiated by the sterol. Table II shows the percentage increase with respect to control values. In mitochondria, the most pronounced changes were seen in proteins of molecular masses of 180, 130 and 78 Kda; proteins of 90, 60 and 31 Kda were phosphorylated to a lesser extent in response to 1,25-dihydroxy-vitamin D_3. In microsomes, the sterol mainly affected the phosphorylation of proteins of 170, 141, 78, 60 and 28 Kda; in addition, a minor stimulation was observed in proteins of 45, 31, 21 and 15 Kda. The effects of 1,25-dihydroxy-vitamin D_3 on muscle membrane protein phosphorylation could be mimicked by forskolin (Table II).

Similarly to 1,25-dihydroxy-vitamin D_3, forskolin (10^{-5} M) produced a significant increase in ^{45}Ca uptake by chick soleus muscles after 5–15 min treatment (Table III).

Table III. Rapid stimulation of skeletal muscle calcium uptake by forskolin. Soleus muscles from vitamin D-deficient chicks were incubated in Krebs-Henseleit-glucose solution in the absence and presence of forskolin (10^{-5} M) at 37 °C for 5–15 min. The uptake of $^{45}CaCl_2$ was then measured during a 10 min interval as indicated in Methods. The effects of 1,25 $(OH)_2D_3$ (10^{-10} M) were compared to those of forskolin under similar treatment conditions. Results are the average of three separate experiments.

Time [min]	^{45}Ca uptake (% of control)	
	Forskolin	1,25 $(OH)_2D_3$
5	135 ± 2.5[b]	140 ± 3.5[a]
10	130 ± 2.0[a]	145 ± 3.7[b]
15	127 ± 1.9[a]	150 ± 2.5[a]

[a] $p < 0.005$; [b] $p < 0.01$, with respect to controls.

Discussion

Previous investigations have shown that 1,25-dihydroxy-vitamin D_3 affects the intracellular distribution of calmodulin in chick skeletal muscle, namely, it increases membrane calmodulin levels at the expense of a reduction in cytosol calmodulin

concentration [9]. In the present work, it was shown by means of a [^{125}I]calmodulin gel-binding technique that the sterol exerts its effects through a stimulation of calmodulin binding to a protein of 28 Kda localized in microsomes and to proteins of 78 and 130 Kda associated to mitochondria. The binding of labelled calmodulin to these proteins appeared to be specific as it was inhibited by the calmodulin antagonist flufenazine or non-radioactive calmodulin. The 28 Kda microsomal protein was a major, Ca-independent, calmodulin-binding protein whereas those of 78 and 130 Kda detected in mitochondria-bound calmodulin to a lesser extent, suggesting that the former may play a more important role in the modulation of muscle cell calmodulin distribution by 1,25-dihydroxy-vitamin D_3. The specific subcellular localization of this protein remains to be established. The microsomal fraction isolated from chick soleus muscle in this study contained high Ca + Mg-ATPase and Na^+ + K^+-ATPase activities, indicating that it is composed of sarcoplasmic reticulum and plasma membranes. The presence of calmodulin target proteins in these skeletal muscle membrane components has been only partially studied [30, 31] as to make adequate extrapolations.

Higher calmodulin levels of chick intestine brush border membranes observed in response to treatment with 1,25-dihydroxy-vitamin D_3 *in vivo* have been related to increased calmodulin-binding ability of a 102–105 Kda, Ca-independent, membrane protein [32, 33]. No information is available on the specific mechanism by which the sterol affects binding of calmodulin to this protein.

The results of the present investigation suggest that 1,25-dihydroxy-vitamin D_3 stimulates the binding of calmodulin to specific skeletal muscle membrane proteins through cyclic AMP-dependent phosphorylation of these proteins. The sterol significantly increased the cAMP levels of soleus muscles within 1–5 min of incubation, in accord with the short *in vitro* treatment intervals (5–15 min) at which the increase in both muscle membrane calmodulin content [9] and calmodulin binding to muscle membrane proteins (Table I) were observed. Similarly as 1,25-dihydroxy-vitamin D_3, forskolin which specifically activates cAMP production, rapidly increased the concentration of calmodulin in soleus muscle membranes without altering total calmodulin levels in the tissue (Fig. 3). Moreover, these effects of forskolin were also related to an increased calmodulin-binding capacity of the protein of 28 Kda located in microsomes (Fig. 4). In addition, similar patterns of stimulation of protein phosphorylation with [γ-^{32}P]ATP were obtained in isolated mitochondria and microsomal membranes after treatment of muscle with 1,25-dihydroxy-vitamin D_3 and forskolin (Table II). The most pronounced changes in phosphorylation were observed in protein fractions with electrophoretic mobilities similar to those whose calmodulin-binding ability was affected by the sterol and forskolin to the greatest extent, further supporting a relationship between 1,25-dihydroxy-vitamin D_3-dependent changes in protein phosphorylation and calmodulin binding. These observation are in general agreement with other studies which have shown that the affinity for calmodulin of various calmodulin-binding proteins is modulated by cyclic AMP-dependent phosphorylation [25–27].

The modifications in muscle membrane protein phosphorylation and in turn of calmodulin binding may be involved in the fast stimulation of muscle Ca transport induced by 1,25-dihydroxy-vitamin D_3. The early increase in soleus muscle ^{45}Ca uptake was accompanied by a significant elevation of tissue cAMP levels (Fig. 2). The fact that treatment with forskolin rapidly increased ^{45}Ca uptake by skeletal muscle (Table III) also supports this contention. Moreover, calmodulin antagonists inhibit muscle Ca fluxes regulated by 1,25-dihydroxy-vitamin D_3 [9]. It has been shown that the early action of the sterol on muscle Ca uptake can be suppressed by Ca channel blockers [8] which is in agreement with the participation of protein phosphorylation and calmodulin in the modulation of cell membrane calcium channels [34, 35]. Future work should investigate whether any of the proteins whose phosphorylation and calmodulin-binding activity is affected by 1,25-dihydroxy-vitamin D_3 takes part in muscle membrane Ca channel function.

The possibility that the stimulation of calmodulin binding to muscle membranes caused by 1,25-dihydroxy-vitamin D_3 is the consequence of an increase in the concentration of cytosolic Ca^{2+} is not likely. The ability of the microsomal 28 Kda protein to bind calmodulin is affected by the sterol independently of Ca (Fig. 1). In addition, treatment

with Ca ionophore does not alter muscle calmodulin subcellular distribution (V. Massheimer, L. M. Fernandez, and A. R. de Boland, unpublished observations).

Finally, the present observations may be physiologically significant as 1,25-dihydroxy-vitamin D_3 also affects *in vivo* muscle cell calmodulin distribution [9] and calcium fluxes [36].

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