Calcif Tissue Int (1990) 47:314–319

Calcified Tissue International

Cyclic AMP-Dependent Membrane Protein Phosphorylation and Calmodulin Binding are Involved in the Rapid Stimulation of Muscle Calcium Uptake by 1,25-Dihydroxyvitamin D_3

Luis M. Fernandez, Virginia Massheimer, and Ana R. de Boland

Departamento de Biologia, Universidad Nacional del Sur, Peru 670, 8000 Bahia Blanca, Argentina

Summary. Rapid *in vivo* effects of 1,25-dihydroxyvitamin D_3 on muscle calcium metabolism have been reported. *In vitro* studies have shown that exposure of vitamin D-deficient chick soleus muscles to the sterol for 1–10 minutes causes a significant stimulation of tissue ^{45}Ca uptake which can be suppressed by Ca channel blockers. A parallel increase in muscle membrane calmodulin content that could be mimicked by forskolin was observed. Experiments were carried out to obtain information about the mechanism underlying the fast action of 1,25-dihydroxyvitamin D_3. Like the sterol, forskolin (10 μm) rapidly increased (+48% at 5 min) soleus muscle ^{45}Ca uptake and its effect could be reversed by nifedipine (50 μm). In agreement with these observations, 1,25-dihydroxyvitamin D_3 markedly elevated tissue cAMP levels within 45 seconds to 5 minutes of treatment in a dose-dependent manner (10^{-11}–10^{-7} M). Moreover, incubation of isolated muscle microsomes with 1,25-dihydroxyvitamin D_3 increased adenylate cyclase activity and caused a similar profile of stimulation of protein phosphorylation with [γ-^{32}P]-ATP as forskolin. Major changes were detected in proteins whose calmodulin binding ability has been previously shown to be increased by 1,25-dihydroxyvitamin D_3. In addition, the calmodulin antagonists fluphenazine and compound 48/80 abolished the increase in muscle Ca uptake and membrane calmodulin content produced by the sterol. The results suggest that 1,25-dihydroxyvitamin D_3 activates muscle Ca channels through a direct membrane action which involves cAMP-dependent protein phosphorylation and calmodulin binding.

Offprint requests to: Ana R. de Boland

Key words: 1,25-dihydroxyvitamin D_3 — Skeletal muscle — Calmodulin — Cyclic AMP-calcium uptake.

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) may play a physiological role in the regulation of intracellular Ca^{2+} in skeletal muscle. Animal model studies have shown a stimulation of muscle Ca transport by the sterol [1–3]. A direct effect of $1,25(OH)_2D_3$ on muscle Ca metabolism has been demonstrated in cultured chick soleus muscle and chick embryo myoblasts [4–5]. Maximum Ca uptake responses induced by the sterol in these *in vitro* systems were suppressed by inhibitors of *de novo* RNA and protein synthesis [6]. In agreement with these observations, an intracellular receptor for $1,25(OH)_2D_3$ has been detected in cultured myoblasts and myotubes [7–9]. However, the sterol may also affect muscle Ca fluxes through a nongenomic mechanism. Exposure of chick soleus muscle preparations to the hormone causes a fast stimulation of muscle Ca uptake independently of new protein synthesis [10]. The early effect of $1,25(OH)_2D_3$ could be suppressed by Ca channel blockers [10] and was paralleled by an increase in calmodulin content of muscle membranes at the expense of a decrease in cytosol calmodulin concentration [11]. These observations may bear a physiological significance. Rapid changes in Ca content [12] and increased amounts of membrane calmodulin [11] have been observed in chick skeletal muscle in response to $1,25(OH)_2D_3$ *in vivo*. More recently, forskolin, an activator of adenylate cyclase, but not Ca ionophores, has been shown to mimic the effects of $1,25(OH)_2D_3$ on muscle calmodulin intracellular distribution. These

studies showed in addition that $1,25(OH)_2D_3$ stimulates protein phosphorylation in soleus muscles [13]. The objectives of the present work were to investigate whether changes in cyclic AMP-dependent membrane protein phosphorylation and calmodulin binding play a role in the rapid stimulation of muscle Ca uptake by the sterol.

Material and Methods

Materials

$1,25(OH)_2D_3$ was kindly donated by Dr. M. Uskokovic (Hoffman La Roche Co., NJ, USA). $^{45}CaCl_2$, [γ-^{32}P]-ATP, [α-^{32}P]-ATP, cyclic AMP assay kit, and Aquasol scintillation fluid were purchased from New England Nuclear (MA, USA). Forskolin, fluphenazine, compound 48/80, and cyclic AMP were provided by Sigma Chemical Co. (St. Louis, MO, USA).

Animals

Chicks were raised from 1 day of age on a vitamin D-deficient diet with 1.6% calcium and 1.0% phosphorus [14] for 5 weeks in an environment deprived of light.

In vitro *Treatment of Muscle Preparations*

Soleus muscles dissected from both legs of vitamin D-deficient chicks were preincubated in Krebs-Henseleit-glucose solution [15] for 30 minutes at 37°C under O_2-CO_2 (95%:5%) with constant shaking. $1,25(OH)_2D_3$ (2.4×10^{-10}M) or forskolin (10 μM) were added dissolved in ethanol. To test the effects of nifedipine, fluphenazine, and compound 48/80, these substances were added to the incubation medium dissolved in the same vehicle 5 minutes before the addition of $1,25(OH)_2D_3$ or forskolin. Ethanol alone was added to control samples. In all cases, the amount of ethanol added was lower than 0.1%.

Measurement of Ca Uptake

Determinations of ^{45}Ca uptake were carried out using a modification of a procedure previously described [4, 5]. Muscle samples prepared and treated as described above were incubated in Krebs-Henseleit-glucose medium containing $^{45}CaCl_2$ (2 μCi/ml) at 37°C for 5 minutes. The tissues were then quickly washed with cold unlabeled medium, blotted on filter paper, and dissolved in hot 1 N NaOH. Aliquots were taken for determination of protein content by the Lowry procedure [16] and radioactivity using Aquasol as scintillation fluid.

Determination of Muscle Tissue Cyclic AMP Levels

Soleus muscle samples were immediately frozen in liquid nitrogen after treatment with $1,25(OH)_2D_3$. The tissue was homogenized 15 seconds in 5 vol of cold 10% trichloroacetic acid with an Ultraturrax homogenizer (Jank and Kunkel, Staufen, FRG). The homogenate was centrifuged at $2,500 \times g$ for 15 minutes and the supernatant was washed five times with 6 vol of water-saturated diethyl ether. The washed extract was lyophilized and cyclic AMP was measured by a competitive protein binding technique [17] using a commercially available kit.

Membrane Protein Phosphorylation

Microsomes were isolated from chick soleus muscles as previously described [11]. The membranes (300 μg protein) were preincubated for 1 minute at 30°C in 100 μl of medium containing 10 mM $MgCl_2$, 0.2 mM EGTA, 1 mM $CaCl_2$, 50 mM Tris-HCl pH 7.0, and $1,25(OH)_2D_3$ (10^{-10}–10^{-8} M) or forskolin (10 μM). Phosphorylation was started by addition of 10 μCi [γ-^{32}P]-ATP to give a final concentration of 10 μM. After 1 minute at 30°C, the reaction was terminated by addition of an equal volume of 2% sodium dodecyl sulfate (SDS), 0.1% β-mercaptoethanol, 10% sucrose, and 10 mM Tris/Bicine pH 6.8. SDS-polyacrylamide gel electrophoresis of samples was performed according to Laemmli [18]. The gels were dried and autoradiographed for 72 hours at −20°C using Kodak X-Omat film. Phosphorylated bands were excised from the gel, dissolved in H_2O_2 at 37°C, and the radioactivity was determined using Aquasol as scintillation fluid.

Adenylate Cyclase Assays

Adenylate cyclase activity was measured in chick muscle microsomes by the method of Salomon et al. [19]. The assay mixture contained 50 mM Tris-HCl buffer pH 7.5, 0.2 mM 3-isobutyl-1-methylxanthine, 1 mM cAMP, 2.5 mM $MnCL_2$, and 1 mM (α^{32}P]-ATP (50–200 cpm/pmol). $1,25(OH)_2D_3$ was added to give a final concentration of 10^{-8} M. The reaction was then initiated by the addition of membranes (50–100 μg protein/ml).

Calmodulin Measurements

Homogenates and subcellular fractions obtained from control and $1,25(OH)_2D_3$-treated soleus muscles were extracted with boiling 20 mM Tris-HCl, pH 8.2, 1 mM EGTA for 5 minutes. The calmodulin activity of extracts was measured by means of the phosphodiesterase assay [20]. Units (2.4 m) of purified (bovine brain) calmodulin-depleted 3′,5′-cyclic AMP phosphodiesterase (Sigma Chemical Co., St. Louis, MO) 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 minutes at 30°C. The samples were then heated for 1 minute at 100°C. Snake venom from Crotalus atrox (40 μg) was added and the mixture was further incubated for 10 minutes 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 [21]. Different concentrations of homogenates and fractions were used to estimate their calmodulin activity.

The significance of the results was evaluated by Student's t test [22].

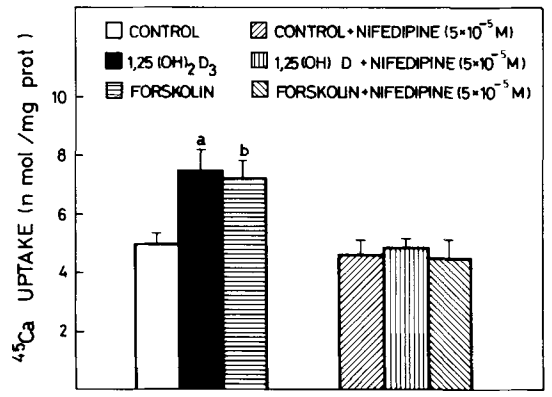

Fig. 1. Rapid stimulation of muscle calcium uptake by forskolin and $1,25(OH)_2D_3$ and its suppression by nifedipine. Soleus muscles obtained from vitamin D-deficient chicks were incubated in Krebs-Henseleit glucose solution containing 50 μM nifedipine for 5 minutes. Forskolin (10 μM) or $1,25(OH)_2D_3$ (2.4×10^{-10} M) were then added. The samples were further incubated for 10 minutes. ^{45}Ca uptake was then measured for 5 minutes indicated in Material and Methods. Each bar represents the mean value ± SE of four independent experiments. $^aP < 0.01$; $^bP < 0.025$.

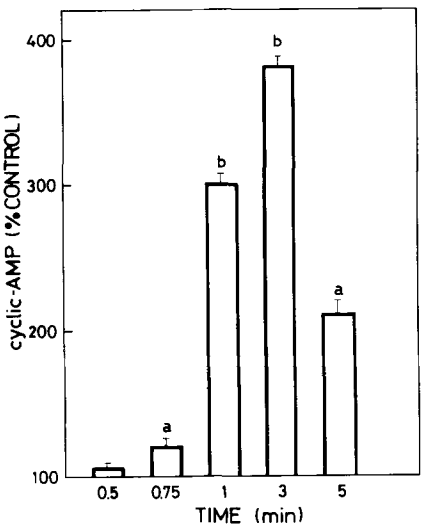

Fig. 2. Time course of effects of $1,25(OH)_2D_3$ on muscle cyclic AMP content. Vitamin D-deficient chick soleus muscles placed in Krebs-Henseleit glucose solution were treated with 10^{-8} M $1,25(OH)_2D_3$ at the times indicated. Tissue cAMP levels were measured as indicated in Material and Methods. Duplicate determinations of three experiments were done. Each bar represents the mean value ± SE. $^aP < 0.05$; $^bP < 0.001$.

Results and Discussion

The operation of a nongenomic mechanism of action of $1,25(OH)_2D_3$ at the membrane level, possibly related to the activation of Ca^{2+} channels in skeletal muscle, has been recently suggested [10]. We studied effects of several inhibitors to gain inferential insights into the mechanism of nongenomic $1,25(OH)_2D_3$-dependent Ca^{2+} uptake in skeletal muscle. In agreement with previous observations [10], exposure of soleus muscle preparations from vitamin D-deficient chicks to physiological concentrations of $1,25(OH)_2D_3$ for only 10 minutes caused a significant increase (53%, $P < 0.01$) of tissue ^{45}Ca uptake (Fig. 1). Forskolin (10 μM), an activator of adenylate cyclase, mimicked the effects of the sterol. The calcium channel blocker nifedipine, at a concentration of 50 μM, effectively blocked the rapid stimulation of muscle Ca uptake produced either by $1,25(OH)_2D_3$ or forskolin.

In addition, treatment *in vitro* of chick soleus muscles with $1,25(OH)_2D_3$ rapidly increased cyclic AMP muscle tissue content. The increment could be already detected at 30 seconds and occurred in a time-dependent fashion up to 3 minutes (280% increase with respect to control preparations) and then declined (+110%) after a 5 minute treatment interval (Fig. 2). Forskolin (10 μM) increased muscle cAMP levels approximately to the same extent as $1,25(OH)_2D_3$ (215% and 126% at 1 minute and 5 minutes of treatment, respectively). A dose-response study of the fast effects of $1,25(OH)_2D_3$ on

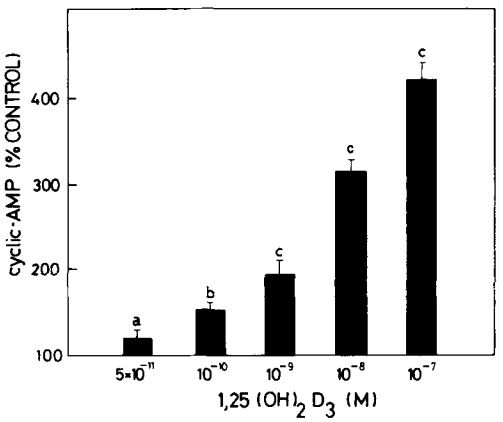

Fig. 3. Dose-response profile for the effects of $1,25(OH)_2D_3$ on muscle cyclic AMP content. Chick soleus muscles were incubated with various concentrations of $1,25(OH)_2D_3$ for 3 minutes followed by determination of their cAMP content as in Figure 2. Means of three experiments ± SE are given. $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.0025$.

cAMP concentration of soleus muscle was performed using a sterol concentration range of 5×10^{-11} M–10^{-7} M. Figure 3 shows a 20% elevation of muscle cAMP levels at the lowest dose used. The response to the hormone further increased up to a concentration of 10^{-7} M (+315%).

Recent studies have shown that treatment of chick soleus muscle with $1,25(OH)_2D_3$ for short

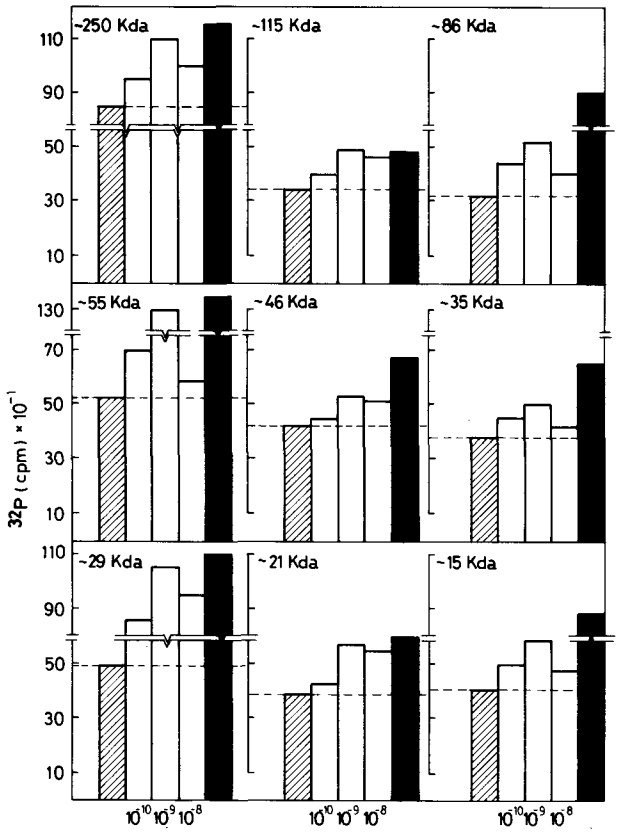

Fig. 4. Direct stimulation of muscle membrane protein phosphorylation by $1,25(OH)_2D_3$ and forskolin. Microsomes isolated from vitamin D-deficient chick soleus muscles were incubated with 10^{-10}–10^{-8} M $1,25(OH)_2D_3$ or 10 μM forskolin for 1 minute. The membranes were phosphorylated with [γ-^{32}P]-ATP (1 minute) and subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. Proteins from radioactive bands were extracted from the gel and the radioactivity was measured in a liquid scintillation counter as described in Material and Methods. Results are the average of four separate experiments. Control (▨), $1,25(OH)_2D_3$ (□), forskolin (■).

Table 1. Effects of $1,25(OH)_2D_3$ on adenylate cyclase activity of muscle membranes

Time (min)	Adenylate cyclase (pmol cAMP/mg prot) Control	$1,25(OH)_2D_3$	% increase
5	16.20 ± 0.90	19.80 ± 0.85	22
10	19.90 ± 1.10	23.30 ± 1.70	17
20	30.00 ± 1.27	31.90 ± 2.05	6

Adenylate cyclase was measured in microsomes from vitamin D-deficient chick soleus muscles incubated in assay medium containing [α-^{32}P]-ATP [19] in the presence of 10^{-8} M $1,25(OH)_2D_3$ for 5–20 minutes

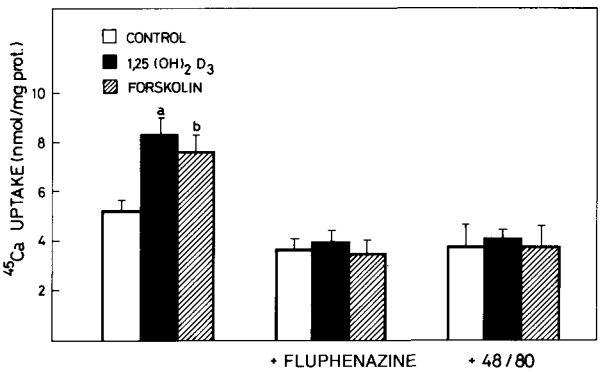

Fig. 5. Suppression by calmodulin antagonists of the rapid stimulation of muscle calcium uptake caused by $1,25(OH)_2D_3$ or forskolin. Vitamin D-deficient chick soleus muscles were treated for 10 minutes with $1,25(OH)_2D_3$ (2.4×10^{-10}M) or forskolin (10 μM) in the absence and presence of fluphenazine (100 μM) or compound 48/80 (10 μg/ml). ^{45}Ca uptake was then measured for 5 minutes as indicated in Materials and Methods. Each bar represents the mean value ± SE of four independent experiments. $^aP < 0.001$; $^bP < 0.01$.

treatment intervals (5–15 minutes) stimulates the phosphorylation of microsomal proteins [13]. To investigate whether the effects of the hormone are exerted at the membrane level and require the participation of cAMP, microsomes isolated from soleus muscle preparations were directly exposed to $1,25(OH)_2D_3$ (10^{-10}–10^{-8} M) or forskolin (10 μM) for 1 minute and then phosphorylated with (γ^{32}P)-ATP, followed by SDS-PAGE fractionation of proteins and autoradiography. A quantitative evaluation of the changes in protein phosphorylation caused by both treatments is shown in Figure 4. The phosphorylation of proteins of relative molecular masses of 250, 55, and 29 Kdalton were clearly affected by $1,25(OH)_2D_3$. Lesser changes were also observed in proteins of 115, 86, 46, 35, 21, and 15 KDa. The extent of the effects of $1,25(OH)_2D_3$ on the incorporation of ^{32}P into muscle membrane proteins varied with the dose used, maximum responses being detected at a sterol concentration of 10^{-9} M (twofold increase for the proteins of 55 and 28 KDa). The effects of the sterol on microsomal protein phosphorylation were mimicked by the addition of forskolin (Fig. 4). The protein species affected by $1,25(OH)_2D_3$ and forskolin may be components of either sarcoplasmic reticulum and/or plasma membranes as significant activity levels of both Ca + Mg-ATPase and Na + K-ATPase are found in chick muscle microsome preparations [11]. In agreement with the preceding observations, chick soleus muscle microsomes treated with $1,25(OH)_2D_3$ (10^{-8} M) for 5–10 minutes exhibited increased adenylate cyclase activity (Table 1). No changes were detected with incubation of the mem-

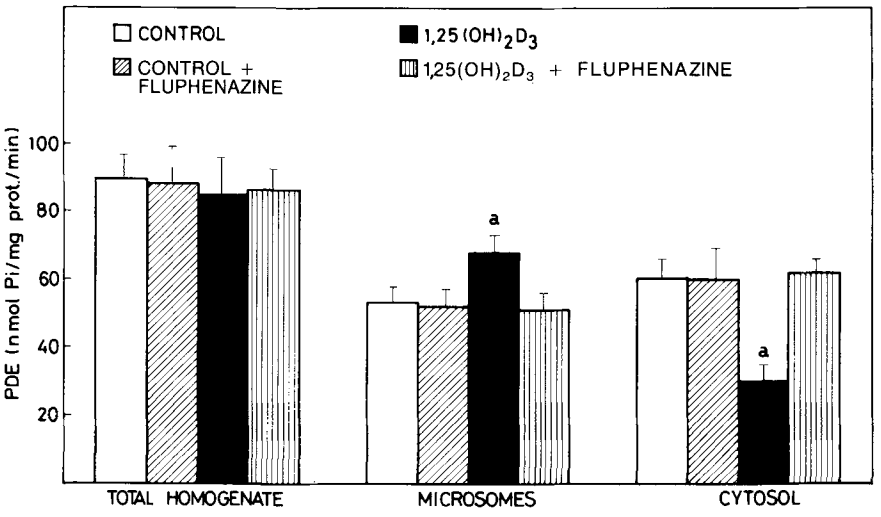

Fig. 6. Suppression by calmodulin antagonists of the effects of $1,25(OH)_2D_3$ on muscle calmodulin subcellular distribution. Chick soleus muscles were treated with $1,25(OH)_2D_3$ (2.4×10^{-10} M) for 10 minutes in the absence and presence of 100 μM fluphenazine. The muscles were homogenized followed by isolation of cytosol and microsomes as previously described [11]. Calmodulin activity was determined using the phosphodiesterase assay [20]. Bars represent calmodulin specific activity in homogenates and fractions expressed as phosphodiesterase activity. Values are the means ± SE of three experiments. $^aP < 0.025$.

branes for 5 minutes with 10^{-8} M $25(OH)_2D_3$ (data not shown). Effects of $1,25(OH)_2D_3$ on cyclic nucleotide metabolism have been previously shown. In contrast to the present results, it has been reported that $1,25(OH)_2D_3$ inhibits adenylate cyclase stimulation by parathyroid hormone in isolated kidney plasma membranes [23]. More recently, Barsony and Marx [24] have demonstrated an increase in cyclicGMP content of cultured human skin fibroblasts after short treatment intervals with the sterol [24]. However, the observation that $1,25(OH)_2D_3$ and forskolin caused similar patterns of stimulation of protein phosphorylation suggests that changes in cAMP play a more significant role in the effects of $1,25(OH)_2D_3$ on muscle.

The early action of $1,25(OH)_2D_3$ on skeletal muscle Ca uptake *in vitro* is paralleled by a transfer of calmodulin from cytosol to membranes [11]. Increased amounts of muscle membrane-bound calmodulin are also seen in response to forskolin. The effects of $1,25(OH)_2D_3$ have been associated with increased ability of a microsomal protein of 28–29 KDa to bind calmodulin [13]. In addition, similar effects of forskolin on the calmodulin binding properties of this protein were also shown (Massheimer, Fernandez and de Boland, unpublished data), suggesting that the sterol stimulates the binding of calmodulin to specific skeletal muscle proteins through cAMP-dependent phosphorylation. The observation that both $1,25(OH)_2D_3$ and forskolin significantly increased the phosphorylation of a 29 KDa protein in isolated microsomes (Fig. 4) supports this interpretation.

The possibility that modifications in calmodulin intracellular distribution might be involved in the rapid stimulation of muscle Ca uptake induced by $1,25(OH)_2D_3$ and forskolin was investigated. We observed that the increase in ^{45}Ca labeling of vitamin D-deficient chick soleus muscles caused by treatment with either substance for 10 minutes was inhibited by the calmodulin antagonists fluphenazine (100 μM) and compound 48/80 (10 μg/ml) (Fig. 5). In tissues other than skeletal muscle, it has been proposed that calmodulin inhibitors may interact with Ca^{2+} channels directly by binding to a calmodulin-like site on the channel [25]. As shown in Figure 6, reversal of the effects of $1,25(OH)_2D_3$ and forskolin on muscle Ca uptake by fluphenazine was accompanied by a suppression of the changes in relative amounts of calmodulin associated with microsomes and cytosol induced by these agents. However, the increase in muscle tissue cAMP concentration induced by $1,25(OH)_2D_3$ was not blocked by fluphenazine (data not shown).

In conclusion, the results presented in this report suggest that $1,25(OH)_2D_3$ stimulates muscle calcium uptake through a direct membrane action which involves both cAMP-dependent protein phosphorylation and calmodulin binding. It is possible that the sterol activates Ca channels as its effects can be suppressed by nifedipine and verapamil [10]. However, direct measurement of Ca channel activity is required to firmly establish this concept. In addition, future studies will be necessary to identify the cellular binding site(s) for $1,25(OH)_2D_3$ which is coupled to the initiation of rapid Ca^{2+} uptake by skeletal muscle.

Acknowledgments. This work was supported by grants from the Consejo Nacional de Investigaciones Cientificas y Técnicas (CONICET) and the Comision de Investigaciones Cientificas de la Provincia de Buenos Aires, Argentina. We thank Dr. Mirta Flawia (Instituto de Genética y Biologia Molecular, CONICET, Buenos Aires) for advice and assistance in adenylate cyclase

assays. We thank Ms. Silvia Benassati for technical help in electrophoretic analysis.

References

1. Curry OB, Basten JF, Francis MJO, Smith R (1974) Calcium uptake by sarcoplasmic reticulum of muscle from vitamin D-deficient rabbits. Nature 249:83–87
2. Matthews C, Heimberg KW, Ritz E, Agostini B, Fritzche J, Hasselbach W (1977) Effect of 1,25-dihydroxycholecalciferol on impaired calcium transport by the sarcoplasmic reticulum in experimental uremia. Kidney Int 11:227–235
3. de Boland AR, Gallego S, Boland R (1983) Effects of vitamin D_3 on phosphate and calcium transport across and composition of skeletal muscle plasma cell membranes. Biochim Biophys Acta 733:264–270
4. Giuliani D, Boland R (1984) Effects of vitamin D_3 metabolites on calcium fluxes in intact chicken skeletal muscle and myoblasts cultured in vitro. Calcif Tissue Int 36:200–205
5. de Boland AR, Boland R (1985) In vitro cellular muscle calcium metabolism. Characterization of effects of 1,25-dihydroxy-vitamin D_3 and 25-hydroxyvitamin D_3. Z Naturforsch [C] 40:102–108
6. de Boland AR, Boland R (1985) Suppression of 1,25-dihydroxy-vitamin D_3-dependent calcium transport by protein synthesis inhibitors and changes in phospholipids in skeletal muscle. Biochim Biophys Acta 845:237–241
7. Simpson RU, Thomas GA, Arnold AJ (1985) Identification of 1,25-dihydroxyvitamin D_3 receptors and activities in muscle. J Biol Chem 260:8882–8891
8. Boland R, Norman A, Ritz E, Hasselbach W (1985) Presence of a 1,25-dihydroxy-vitamin D_3 receptor in chick skeletal muscle myoblasts. Biochem Biophys Res Commun 128:305–311
9. Costa EM, Blau HM, Feldman D (1986) 1,25-Dihydroxyvitamin D_3 receptors and hormonal responses in cloned human skeletal muscle cells. Endocrinology 119: 2214–2220
10. de Boland AR, Boland R (1987) Rapid changes in skeletal muscle calcium uptake induced in vitro by 1,25-dihydroxy-vitamin D_3 are suppressed by calcium channel blockers. Endocrinology 120:1858–1864
11. de Boland AR, Massheimer V, Fernandez LM (1988) 1,25-Dihydroxyvitamin D_3 affects calmodulin distribution among subcellular fractions of skeletal muscle. Calcif Tissue Int 43:370–375
12. Bauman VK, Valinietse MY, Babarykin DA (1984) Vitamin D_3 and 1,25-dihydroxyvitamin D_3 stimulate the skeletal muscle-calcium mobilization in rachitic chicks. Arch Biochem Biophys 231:211–216
13. Fernandez L, Massheimer V, de Boland AR (1988) Dihydroxy-vitamin D_3 increases calmodulin binding to skeletal muscle membranes. In: Norman AW, Schaefer K, Grigoleit HG, Herrath DV (eds) Vitamin D, molecular, cellular and clinical endocrinology. Walter de Gruyter & Co, Berlin, New York pp 454–455
14. Wasserman RH, Taylor AN (1973) Intestinal absorption of phosphate in the chick: effect of vitamin D_3 and other parameters. J Nutr 103:586–599
15. Paul J (1975) Cell and tissue culture. Churchill Livingstone, Great Britain, p 90
16. Lowry OH, Rosebrought NJ, Farr AL, Randal PJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
17. Tovey KC, Oldham KG, Whelan JAM (1974) A simple direct assay for cyclic AMP in plasma and other biological samples using an improved competitive protein binding technique. Clin Chim Acta 56:221–234
18. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277:680–685
19. Salomon Y, Londos C, Rodbell M (1974) A highly sensitive adenylate cyclase assay. Anal Biochem 58:541–548
20. Wallace RW, Tallant EA, Cheung WY (1983) Assay of calmodulin by Ca^{2+}-dependent phosphodiesterase. Methods Enzymol 102:39–47
21. Fiske CH, Subbarow Y (1925) The colorimetric determination of phosphorus. J Biol Chem 66:375–400
22. Snedecor GW, Cochran WG (1967) Statistical methods. The Iowa State University Press, Ames, Iowa
23. Cloix JF, d'Herbigny E, Ulmann A (1980) Renal parathyroid hormone-dependent adenylate cyclase in vitamin D-deficient rats. J Biol Chem 255:11280–11283
24. Barsony J, Marx SJ (1988) Receptor-mediated rapid action of 1α,25-dihydroxycholecalciferol: increase of intracellular cGMP in human skin fibroblasts. Proc Natl Acad Sci USA 85:1223–1226
25. Luchowski EM, Jousif F, Triggle DJ, Maurer SC, Sarmiento JG, Janis RA (1984) Effects of metal cations and calmodulin antagonists on [^{3}H]-nitrendipine binding in smooth and cardiac muscle. J Pharmacol Exp Ther 230:607–613

Received June 5, 1989, revised September 25, 1989.