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Effects of Calcitriol and Its Analogues, Calcipotriol (MC 903) and 20-Epi-1α,25-dihydroxyvitamin D_3 (MC 1288), on Calcium Influx and DNA Synthesis in Cultured Muscle Cells

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ABSTRACT. The fast actions of the secosteroid hormone 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$; calcitriol] and the synthetic analogues calcipotriol (MC 903) and 20-epi-1α,25$(OH)_2D_3$ (MC 1288) on cell calcium influx were compared in rat duodenum enterocytes as well as in cells from chick embryo skeletal muscle (myoblasts) and heart (myocytes), at various concentrations (10^{-12} to 10^{-8} M) and treatment intervals (1–10 min). In enterocytes, at a concentration of 10^{-11} M, MC 1288 was significantly more active than 1,25$(OH)_2D_3$ in rapidly stimulating $^{45}Ca^{2+}$ uptake by enterocytes (80 vs 38% above controls, respectively), whereas MC 903 was devoid of activity. However, calcipotriol increased Ca^{2+} influx in myocytes and myoblasts to a greater extent than the natural hormone, whereas MC 1288 was more active only in myoblasts. Analogously to 1,25$(OH)_2D_3$, the fast MC 903- and MC 1288-induced stimulation of $^{45}Ca^{2+}$ uptake in enterocytes and muscle cells could be blocked by both verapamil and nifedipine. In addition, MC 903 and MC 1288 were more effective than 1,25$(OH)_2D_3$ in stimulating DNA synthesis in proliferating myoblasts and in inhibiting DNA synthesis in differentiating myoblasts. The results suggest, therefore, that modifications in the side-chain of the 1,25$(OH)_2D_3$ molecule increase its ability to modulate muscle cell Ca^{2+} metabolism and growth. These findings are potentially relevant for the development of analogues for the treatment of vitamin D-dependent myopathies. BIOCHEM PHARMACOL 53;12:1807–1814, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. vitamin D analogues; non-genomic effects; calcium channels; DNA synthesis; muscle; intestine

The major active metabolite of vitamin D_3, 1,25$(OH)_2D_3$† (calcitriol), is an important regulator of calcium homeostasis. 1,25$(OH)_2D_3$ exerts its effects on target tissues through a receptor-mediated nuclear mechanism [1]. In addition, the hormone induces rapid actions, independent of gene activation, in several tissues [2]. Evidence has been obtained indicating that many of these non-genomic effects are associated with activation of second messenger pathways (adenylate cyclase/cyclic AMP/protein kinase A and phospholipases C and D/diacylglycerol/protein kinase C) [3–8] and modulation of cell membrane voltage-operated calcium channels [9–12].

The vitamin D endocrine system is also involved in immunomodulation [13, 14] and regulation of cell proliferation and differentiation in both normal and malignant cells [15–17]. In addition, the vitamin D hormone plays an important role in skeletal and cardiac muscle functions, as evidenced by the development of muscle weakness and alterations in contractility in vitamin D deficiency states [18].

The major limitation of 1,25$(OH)_2D_3$ therapy is its accompanying calcemic activity. Several analogues of 1,25$(OH)_2D_3$ having side-chain modifications with low calcemic effects have been developed [19, 20]. These compounds are potentially useful as antipsoriatic agents [21–23], in cancer therapy [24, 25], and in secondary hyperparathyroidism treatment [26], with markedly decreased activity on calcium metabolism. It has been postulated that their low calcemic activity is due to a reduced affinity for vitamin D binding protein [27, 28]. Differences in the ability of various analogues to stimulate both genomic and non-genomic pathways may contribute to the potency and selectivity of their effects in target tissues [29–33].

The use of 1,25$(OH)_2D_3$ analogues as modulatory agents of muscle function has not been investigated yet. Skeletal and cardiac muscle cells contain receptors for 1,25$(OH)_2D_3$ [34, 35]. It has been demonstrated that the hormone

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† *Abbreviations:* 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3 (calcitriol); MC 903, 1,24-dihydroxy-22-ene-24-cyclopropyl-vitamin D_3 (calcipotriol); MC 1288, 20-epi-1α,25-dihydroxyvitamin D_3; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DTT, dithiothreitol; and EE, embryo extract.

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regulates muscle cellular Ca^{2+} and growth [36–38]. Characterization of the effects of $1,25(OH)_2D_3$ analogues on these processes may provide a basis for the treatment of vitamin D-dependent myopathies. In the present work, we have compared the activity of $1,25(OH)_2D_3$ and its analogues 1,24-dihydroxy-22-ene 24-cyclopropyl-vitamin D_3 (also known as MC 903 or calcipotriol) and 20-epi-1α,25-dihydroxyvitamin D_3 (MC 1288) on the influx of extracellular Ca^{2+} and DNA synthesis in cultured chick muscle cells. Primary-cultured avian myoblasts and myocytes have proven to be a useful model to characterize the action of $1,25(OH)_2D_3$ in skeletal and cardiac muscles [39]. The biological activities of both analogues and the parent molecule were also evaluated in classical target (rat intestine) cells.

MATERIALS AND METHODS

Chemicals

$1,25(OH)_2D_3$ (calcitriol), MC 903 (calcipotriol), and MC 1288 were donated by Dr. L. Binderup (Leo Pharmaceutical Products Ltd., Ballerup, Denmark). Trypsin type III from bovine pancreas, DMEM, FBS, DTT, verapamil, and nifedipine were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). $^{45}CaCl_2$ was from DuPont-New England Nuclear (Boston, MA, U.S.A.). ^{32}P was purchased from the National Atomic Energy Commission (Buenos Aires, Argentina).

Animals

Wistar rats (6 months old) were fed with standard rat food (1.2% calcium; 1.0% phosphorus), given water *ad lib.*, and maintained on a 12-hr light–12-hr dark cycle. To obtain chick embryos, fertilized eggs from white Leghorn chicks were incubated for 13 days at 38° under 90% humidity.

Duodenal Cell Isolation

Rat duodenal cells were isolated essentially as described previously [40]. Segments of the intestine (2 cm length) were placed into solution A containing 96 mM NaCl, 1.5 mM KCl, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, 27 mM sodium citrate, pH 7.3, for 10 min at 37°. After this period, solution A was discarded and replaced by isolation medium (solution B): 154 mM NaCl, 10 mM NaH_2PO_4, 1.5 mM EDTA, 0.5 mM DTT, 5.6 mM glucose, pH 7.3, for 15 min at 37° with vigorous shaking. After centrifugation at 750 *g* for 10 min, the cell pellet was washed twice with 154 mM NaCl, 10 mM NaH_2PO_4, pH 7.3, and resuspended in the incubation medium (solution D) containing 154 mM NaCl, 10 mM Na_2HPO_4, 5 mM KCl, 1 mM $MgCl_2$, 10 mM sodium MOPS, pH 7.4, 5.6 mM glucose, 0.5% BSA, 1 mM $CaCl_2$.

Muscle Cell Culture

Myoblasts were cultured as described previously [41]. Cells were isolated by gently stirring 13-day-old chick embryo pectoral muscle in Earle's balanced salt solution (SSBE) containing 0.1% trypsin and 0.05% collagenase, at 37°, under a constant flow of humidified 95% air–5% CO_2, for 30 min. Cells were collected by centrifugation, and the pellet obtained was suspended (10^6 cells/mL) in DMEM supplemented with 10% FBS, 10% chick EE, and 1% antibiotic–antimycotic solution. The medium was replaced after a 24-hr incubation, lowering the concentration of EE to 2%. Cells were seeded and cultured at 37° with 95% air–5% CO_2 until 70–78% confluence was reached.

Chick embryo myocytes were obtained as previously described [42]. Briefly, cells were isolated by gently stirring minced ventricular tissue in SSBE containing 0.1% trypsin, collected by centrifugation, and suspended in DMEM (10^6 cells/mL) with 15% FBS, 1% EE, 1 U/mL penicillin, 1 U/mL streptomycin, and 0.66 μg/mL mycostatin. Cells were plated in 12-well plates and cultured at 37° with 95% air–5% CO_2.

Measurement of Ca^{2+} Uptake

To determine duodenal cell Ca^{2+} uptake, isolated rat enterocytes were pre-equilibrated in incubation medium (D) for 20 min and then exposed for short intervals (1–10 min) to $1,25(OH)_2D_3$ or analogues, in the presence of $^{45}CaCl_2$ (1 mM $CaCl_2$; 2 μCi/mL). Immediately after treatment, aliquots of cell suspensions were transferred to ice-cold unlabeled medium containing 1 mM $LaCl_3$, centrifuged for 45 sec at 2200 *g* and solubilized in 1 N NaOH. Aliquots were taken for radioactivity and protein determination [43].

Determinations of Ca^{2+} uptake by muscle cells were performed as follows. Culture medium was replaced by Krebs–Henseleit solution containing 0.2% glucose and preincubated for 20 min at 30°. Calcium uptake was initiated by the addition of fresh Krebs–Henseleit containing $^{45}CaCl_2$ (1 mM $CaCl_2$; 2 μCi/mL), $1,25(OH)_2D_3$, analogues, or vehicle alone. The reaction was stopped by removing the incubation medium and rinsing the monolayer three times with a solution containing 140 mM NaCl, 25 mM Tris–HCl, pH 7.4, and 1 mM $LaCl_3$ [44]. Cells were dissolved with 1 N NaOH, 1% SDS. Aliquots were taken for radioactivity and protein determination. When calcium channel blockers were used in Ca^{2+} uptake experiments, they were added 5 min prior to $1,25(OH)_2D_3$ or analogue addition.

Measurement of DNA Synthesis

Myoblasts cultured in monolayers for various time intervals were washed twice with DMEM and then incubated with [*methyl*-^{3}H]thymidine (1 μCi/mL) in DMEM for 1 hr at 37° under 95% air–5% CO_2. The monolayers were then washed

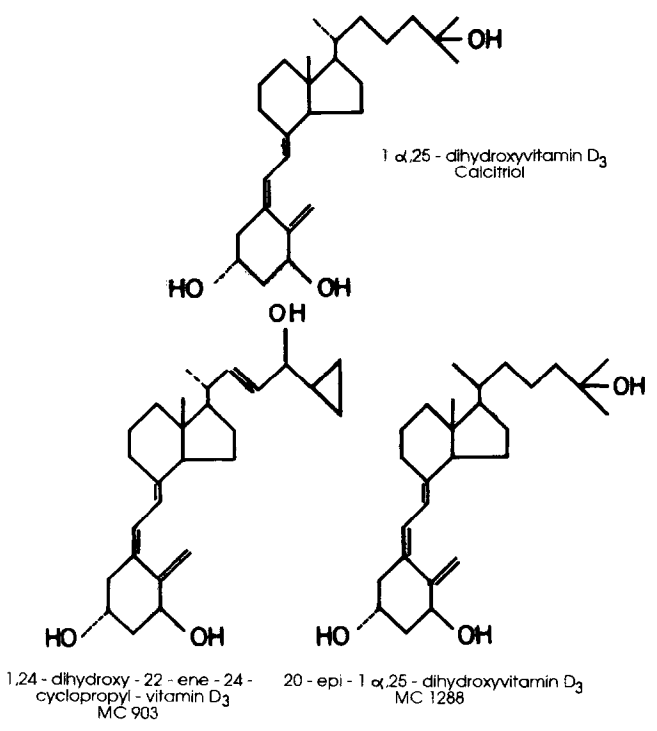

FIG. 1. Chemical structures of 1α,25-dihydroxyvitamin D_3 (calcitriol) and its analogues 1,24-dihydroxy-22-ene-24-cyclopropyl-vitamin D_3 (MC 903) and 20-epi-1α,25-dihydroxyvitamin D_3 (MC 1288).

three times with Krebs–Henseleit–0.2% glucose solution and twice with 12% trichloroacetic acid. Proteins and nucleic acids were dissolved in 1 N NaOH, and aliquots were taken for measurement of protein content by the Bradford procedure [45] and of radioactivity in a liquid scintillation counter.

RESULTS

We compared first the effects of calcitriol and its analogues MC 903 and MC 1288 (Fig. 1) in inducing rapid changes in calcium influx in classic vitamin D target cells. Figure 2A shows a concentration–response study performed with enterocytes isolated from adult rat duodenum. Significant differences were found at 10^{-11} M. At this concentration, MC 1288 was markedly more effective than $1,25(OH)_2D_3$ in eliciting a fast (5 min) increase in cell $^{45}Ca^{2+}$ uptake (80 vs 38% above controls, respectively), whereas MC 903 was devoid of activity. No statistically significant differences between the natural hormone and both analogues were observed at higher concentrations (10^{-10} and 10^{-9} M). The characterization of the time–course (1–10 min) of MC 1288 and $1,25(OH)_2D_3$ effects on Ca^{2+} entry into the intestinal cell at concentrations inducing maximum responses (10^{-11} M and 10^{-10} M, respectively) evidenced the greater potency of MC 1288 than $1,25(OH)_2D_3$ only after 3 min of treatment (Fig. 2B). Interestingly, a decrease in the response of Ca^{2+} influx was observed within 1 min of exposure of enterocytes to MC 1288 (control: 2.95 ± 0.3, MC 1288: 1.97 ± 0.09, $1,25(OH)_2D_3$: 3.73 ± 0.28 nmol Ca^{2+}/mg protein). An explanation for this early inhibitory effect of MC 1288, which was a consistent finding in several independent experiments, cannot be provided. A similar observation was made previously in mature cardiac muscle in response to the natural hormone, possibly reflecting increased Ca^{2+} efflux from the cell

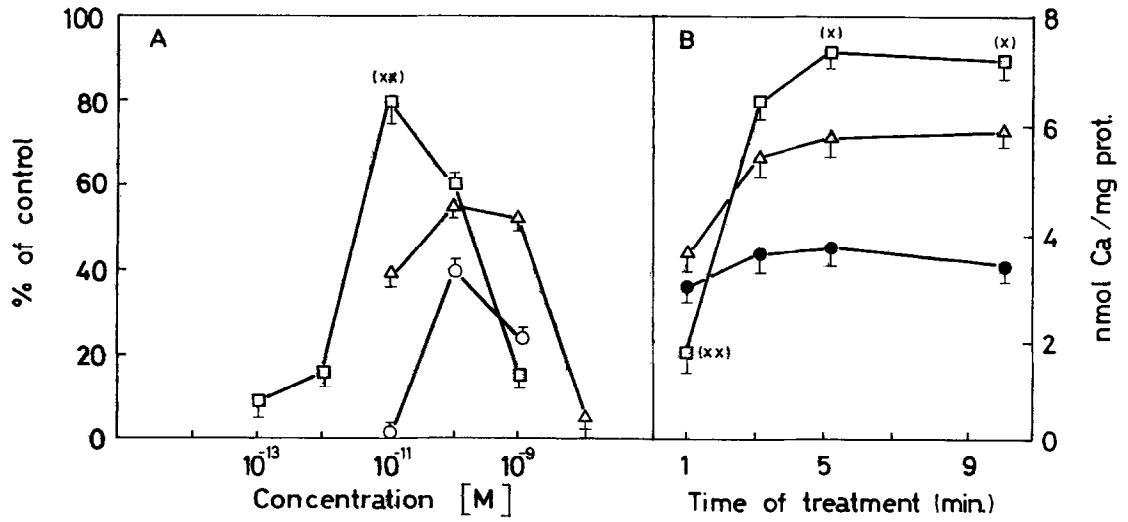

FIG. 2. Rapid effects of calcitriol and analogues MC 1288 and MC 903 on calcium uptake by rat enterocytes. Enterocytes isolated from rat duodenum were treated with ethanol, calcitriol, MC 903, or MC 1288 at the indicated concentrations and times in the presence of $^{45}CaCl_2$ (1 mM $CaCl_2$, 2 μCi/mL), followed by determination of the radioactivity incorporated into the cells as described in Materials and Methods. (A) Calcitriol (△), MC 903 (○), or MC 1288 (□) for 5 min. Control: 4.52 ± 0.67 nmol/mg protein. (B) 0.1% ethanol (●), 10^{-10} M calcitriol (△), or 10^{-11} M MC 1288 (□). Means ± SD (N = 8) are given. Key: (x) $P < 0.01$, and (xx) $P < 0.001$, MC 1288 versus $1,25(OH)_2D_3$.

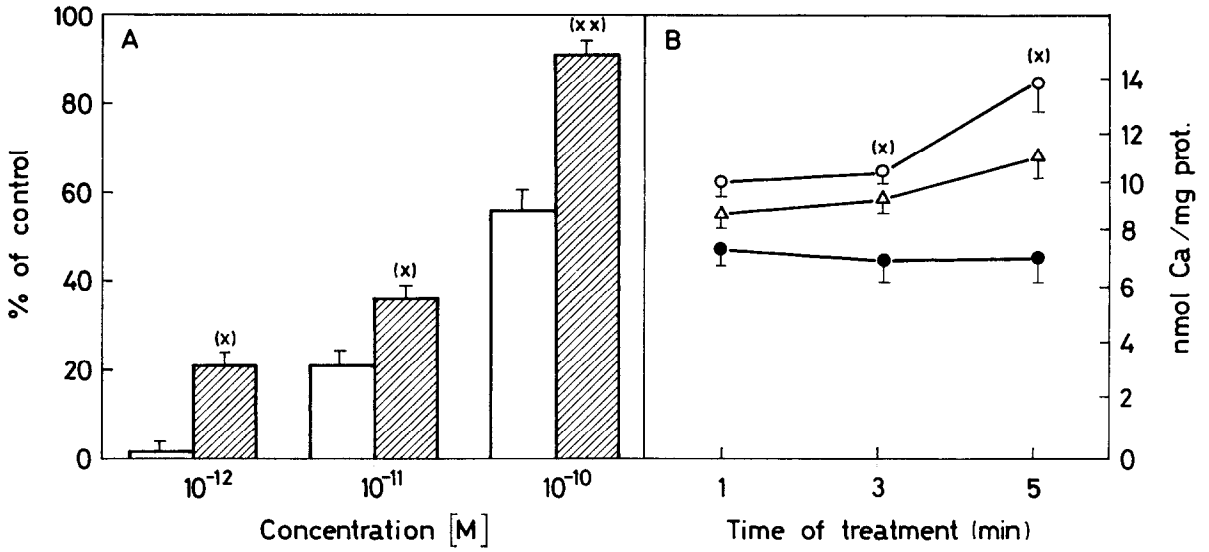

FIG. 3. Rapid effects of calcitriol and analogue MC 903 on myocyte calcium uptake. Cultured chick embryo myocytes at 75–80% confluence were treated with ethanol (0.1%), $1,25(OH)_2D_3$, or MC 903 at the indicated concentrations and times in the presence of $^{45}CaCl_2$ (1 mM $CaCl_2$; 2 μCi/mL), followed by determination of radioactivity incorporated into the cells as described in Materials and Methods. (A) Calcitriol (□) and calcipotriol (▨) for 5 min. Control: 6.6 ± 0.5 nmol/mg protein. (B) Ethanol (●); 10^{-10} M calcitriol (△) or 10^{-10} M MC 903 (○). Means ± SD (N = 4) are given. Key: (x) $P < 0.05$, and (xx) $P < 0.005$, MC 903 versus calcitriol.

caused by an initial release of Ca^{2+} from intracellular stores [12].

In chick embryo cardiac myocytes the analogue MC 903 showed a higher potency than $1,25(OH)_2D_3$ in stimulating cell $^{45}Ca^{2+}$ uptake when tested in the concentration range of 10^{-12} M to 10^{-10} M for 5 min (Fig. 3A). MC 903 increased Ca^{2+} influx 23, 36 and 93% above controls at 10^{-12}, 10^{-11}, and 10^{-10} M, respectively. $1,25(OH)_2D_3$ was devoid of activity at 10^{-12} M, whereas at 10^{-11} and 10^{-10} M it stimulated myocyte Ca^{2+} uptake 21 and 57%, respectively. The analogue MC 1288 failed to induce changes in myocyte Ca^{2+} entry (4.66 ± 0.5 nmol Ca^{2+}/mg protein for control and 5.10 ± 0.63, 4.49 ± 0.30, 4.76 ± 0.35, 4.66 ± 0.30, and 4.89 ± 0.42 for cells treated with 10^{-13} to 10^{-9} M MC 1288, respectively). As shown in Fig. 3B, MC 903 was more effective than $1,25(OH)_2D_3$ in stimulating $^{45}Ca^{2+}$ uptake by cardiac cells at different treatment intervals (1–5 min).

In cultured myoblasts isolated from chick embryo skeletal muscle, both MC 903 and MC 1288 were more potent than calcitriol in eliciting a fast stimulation of cell $^{45}Ca^{2+}$ uptake within the concentration range of 10^{-11} to 10^{-8} M (Fig. 4A). At 10^{-11} M, both synthetic analogues elicited a significant stimulation of radioactive Ca^{2+} influx (MC 1288: 39%; MC 903: 21% above controls), whereas the effects of calcitriol were not significant (+7%). Maximum responses, obtained at 10^{-9} M, were 86, 64, and 46% for MC 1288, MC 903, and $1,25(OH)_2D_3$, respectively. Characterization of the time–course (1–5 min) of changes in myoblast Ca^{2+} influx further evidenced the higher activity of both analogues compared with $1,25(OH)_2D_3$ (Fig. 4B). It is known from previous studies that the effects of the hormone on Ca^{2+} uptake by myocytes and myoblasts at 10 min are lower than or equal to those at 5 min [42, 46].

Considering that in intestinal and muscle cells it has been demonstrated previously that calcitriol exerts its stimulatory effects on Ca^{2+} influx through activation of voltage-operated calcium channels [2], experiments were carried out to evaluate whether the effects of the $1,25(OH)_2D_3$ analogues on these vitamin D target cells could be altered by Ca^{2+} channel blockers. As shown in Table 1, both verapamil (5 μM) and nifedipine (5 μM) effectively blocked the fast increase in $^{45}Ca^{2+}$ uptake induced by compounds MC 903 and MC 1288 in rat enterocytes and chick embryo myoblasts and myocytes.

Membrane protein phosphorylation is a key metabolic event participating in agonist regulation of calcium channel activity [47]. However, no significant differences in myoblast membrane phosphorylation patterns could be detected in preliminary experiments in which myoblasts prelabeled with ^{32}P were treated with calcitriol, calcipotriol, or MC 1288 for 5 min followed by SDS–PAGE analysis of the distribution of radioactivity incorporated into microsomal proteins (data not shown).

The effects of calcitriol and the analogue MC 903 on DNA synthesis in cultured myoblasts were compared. As shown in Fig. 5, during the proliferative phase of myoblasts $1,25(OH)_2D_3$ and MC 903 increased the incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material by 15 and 53% at 10 hr, and 43 and 67% at 18 hr of treatment, respectively. On the contrary, at the stage where myoblasts fuse and differentiate into myotubes, $1,25(OH)_2D_3$ and MC 903 decreased DNA synthesis by 15 and 40% at 24 hr, and 12 and 11% at 48 hr, respectively.

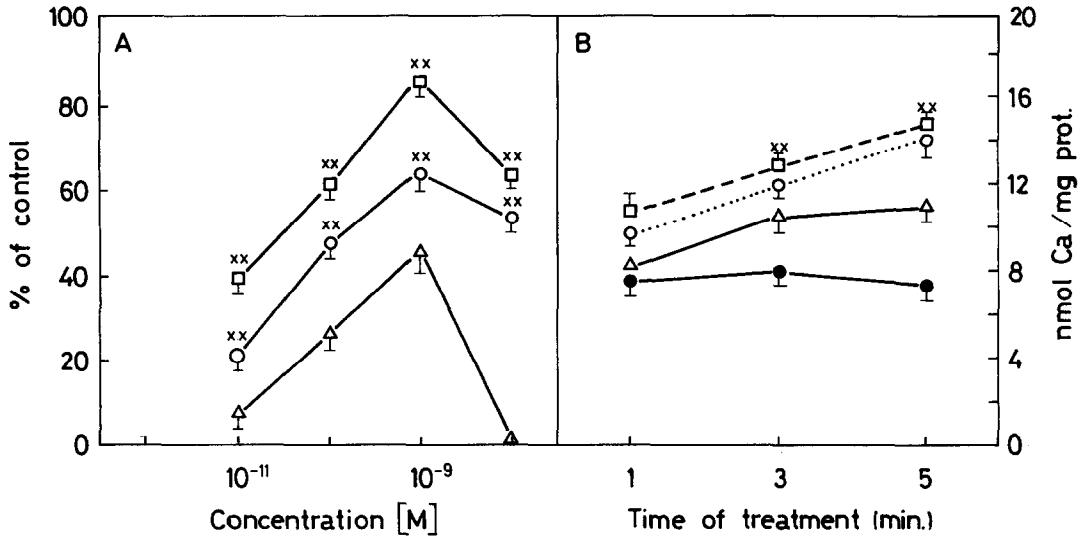

FIG. 4. Rapid stimulation of calcium uptake induced by calcitriol and analogues MC 1288 and MC 903 in cultured myoblasts. Chick embryo myoblast cultures (75–80% confluence) were treated with ethanol (●), calcitriol (△), MC 1288 (□), or MC 903 (○) at the indicated concentrations and times in the presence of $^{45}CaCl_2$ (1 mM $CaCl_2$; 2 μCi/mL), followed by determination of radioactivity incorporated into the cells as described in Materials and Methods. (A) Calcitriol, MC 1288, or MC 903 for 5 min. Control: 7.8 ± 0.6 nmol/mg protein. (B) Ethanol (0.1%), calcitriol, MC 1288, or MC 903 (10^{-9} M). Means ± SD (N = 6) are given. Key: (xx) $P < 0.001$, MC 1288 and MC 903 versus calcitriol.

At this stage, MC 1288 also inhibited the incorporation of [^{3}H]thymidine into DNA (36 and 23% at 24 and 48 hr, respectively; data not shown).

DISCUSSION

The present work provides for the first time information on the effects of synthetic analogues of $1,25(OH)_2D_3$ on Ca^{2+} influx and DNA synthesis by muscle cells. The hormone has been shown to modulate cytoplasmic Ca^{2+} and cell proliferation in cultured chick embryo skeletal muscle myoblasts and cardiac myocytes both through receptor-mediated regulation of gene transcription and via non-genomic pathways that involve opening of voltage-gated Ca^{2+} channels and intracellular Ca^{2+} mobilization [18, 34, 36, 39]. These observations, as well as various other lines of evidence, have lent support to the concept that $1,25(OH)_2D_3$ is a regulator of muscle calcium metabolism and growth.

Numerous vitamin D_3 analogues have been developed in an attempt to dissociate non-classical actions from effects on intestinal calcium absorption and bone resorption. Most analogues synthesized have modifications in the side-chain of the $1,25(OH)_2D_3$ molecule. One of the most representative compounds of this class is the analogue MC 903, which inhibits cell proliferation and DNA synthesis and

TABLE 1. Effect of calcium channel blockers on rapid calcitriol-, MC 903-, and MC 1288-induced $^{45}Ca^{2+}$ uptake stimulation in enterocytes, myocytes, and myoblasts

	Ca^{2+} (nmol/mg protein)			
	Control	Calcitriol	MC 903	MC 1288
Enterocytes				
− Verapamil	4.7 ± 0.6	7.2 ± 1.2		9.26 ± 1.8*
+ Verapamil	4.9 ± 0.1	4.7 ± 0.1		4.90 ± 0.2
Myocytes				
− Nifedipine	9.0 ± 1.5	15.0 ± 1.3	18.5 ± 1.6*	
+ Nifedipine	10.5 ± 0.9	10.3 ± 0.9	10.8 ± 1.2	
Myoblasts				
− Nifedipine	12.3 ± 0.8	14.3 ± 0.6	16.9 ± 0.7†	18.1 ± 0.7‡
+ Nifedipine	12.4 ± 0.3	12.9 ± 1.0	12.8 ± 0.7	12.9 ± 1.0

Cells were treated for 5 min with $1,25(OH)_2D_3$ or analogues in the presence of $^{45}CaCl_2$ to measure calcium uptake as described in Materials and Methods. In enterocytes, 10^{-10} M calcitriol or 10^{-11} M MC 1288 were used. Myocytes were exposed to 10^{-10} M calcitriol or MC 903 and myoblasts to 10^{-9} M calcitriol, MC 903, or MC 1288. Controls were treated with ethanol (0.1%). Nifedipine and verapamil (5 μM) were added 5 min prior to treatments. Means ± SD (N = 5) are given.

*–‡ Significantly different from calcitriol: * $P < 0.01$, † $P < 0.05$, and ‡ $P < 0.025$.

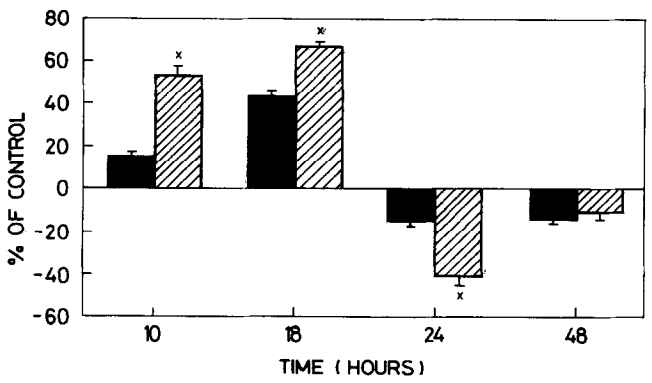

FIG. 5. Biphasic effects of $1,25(OH)_2D_3$ and analogue MC 903 on DNA synthesis in cultured myoblasts. Chick embryo myoblasts, cultured for 24 hr as described in Materials and Methods, were treated with 10^{-9} M $1,25(OH)_2D_3$ or MC 903 for 10–48 hr. The cells were then incubated with [^{3}H]thymidine in DMEM for 1 hr, and trichloroacetic acid-insoluble radioactivity was determined in a liquid scintillation counter. Means ± SD (N = 3) are given. Control values: 3,630 ± 930, 11,490 ± 140, 8,130 ± 530, and 4,310 ± 200 cpm/mg protein at 10, 18, 24, and 48 hr, respectively. Key: $1,25(OH)_2D_3$ (■); MC 903 (▨); and (x) $P < 0.01$, MC 903 versus $1,25(OH)_2D_3$.

induces cell differentiation with the same potency as $1,25(OH)_2D_3$, but has 100–200 times less calcemic activity than the natural hormone due to a higher rate of metabolic clearance [48]. Of particular interest are the novel 20-epi-vitamin D_3 analogues, among them MC 1288, characterized by an altered stereochemistry at carbon 20 in the side-chain. These compounds are considerably more potent regulators of cellular growth, differentiation, and immune responses than $1,25(OH)_2D_3$. They have a longer half-life than other analogues of this group, which makes them more suitable for systemic use [49].

In this study, MC 903 exhibited a considerably greater capacity than $1,25(OH)_2D_3$ to rapidly (1–5 min) stimulate the influx of $^{45}Ca^{2+}$ in both myoblasts and myocytes. The effects of the analogue were already evident at 10^{-12} M, a concentration at which the hormone was devoid of activity. In myoblasts, the analogue MC 1288 was even more potent than MC 903, while it lacked activity in myocytes. Target tissue differences in the non-genomic responses elicited by the analogues MC 903 and 22-oxacalcitriol have been observed by other investigators [29, 30, 50]. At low levels (10^{-13} to 10^{-11} M) and after treatment intervals longer than 3 min, MC 1288 was also more effective *in vitro* than $1,25(OH)_2D_3$ at stimulating Ca^{2+} uptake in classic target intestinal cells (rat enterocytes), an observation that may be related to the greater activity of the 20-epi-vitamin D_3 analogue on calcium metabolism *in vivo* [49]. The cell-specific responses displayed by compound MC 1288 remain unexplained.

The rapid (5 min) stimulation of Ca^{2+} uptake by MC 903 and MC 1288 in myoblasts and myocytes was abolished completely by nifedipine and verapamil, suggesting that modulation of voltage-operated Ca^{2+} channels is involved in the non-genomic action of both analogues in muscle cells as previously shown for $1,25(OH)_2D_3$ in these as well as in other target cells [2, 7, 9, 12, 36, 39]. In agreement with our observations, it has been reported that MC 903 induces a rapid increase in intracellular Ca^{2+} in Caco-2 cells more effectively than does $1,25(OH)_2D_3$ [30].

Various lines of evidence have suggested that $1,25(OH)_2D_3$ regulation of Ca^{2+} channel activity in muscle and intestinal cells involves cyclic AMP-mediated phosphorylation of membrane proteins [3–5, 12]. However, in spite of the greater effectiveness of MC 903 and MC 1288 than $1,25(OH)_2D_3$ in promoting the influx of extracellular Ca^{2+} in myoblasts, preliminary experiments showed that both analogues induced changes in myoblast membrane protein phosphorylation similar to those of the hormone (data not shown). The contribution of this mechanism to analogue potency is being further investigated in our laboratory. In addition, factors other than protein phosphorylation may explain the differences in Ca^{2+} influx regulation between the synthetic derivatives of $1,25(OH)_2D_3$ and the parent molecule. There is evidence that polyphosphoinositide turnover controls Ca^{2+} influx from outside the cell [51, 52]. Fast (less than 1 min) increases in inositol 1,4,5-trisphosphate (IP_3) induced by $1,25(OH)_2D_3$ activation of phosphoinositide-phospholipase C have been observed in myoblasts [7, 8]. MC 903 has been shown to be more effective than $1,25(OH)_2D_3$ in increasing IP_3 in Caco-2 cells [30]. Therefore, it might be possible that differences in the extent of inositol phosphate liberation account for the greater stimulation of extracellular Ca^{2+} influx through calcium channels by analogues MC 903 and MC 1288 in muscle cells. This hypothesis is also currently under investigation.

The analogues MC 903 and MC 1288 were also more potent than $1,25(OH)_2D_3$ in modulating DNA synthesis in cultured myoblasts. Previous studies showed that $1,25(OH)_2D_3$ stimulates DNA synthesis in proliferating myoblasts, e.g. at early stages of culture prior to cell fusion, whereas it inhibits DNA synthesis during the subsequent phase of myoblast differentiation. Changes in other biochemical parameters were also consistent with a role of the hormone in the regulation of muscle cell proliferation and differentiation [37]. We have now observed that the biphasic action of MC 903 on myoblast DNA synthesis is more pronounced than that of $1,25(OH)_2D_3$. MC 1288 also exerts a greater inhibitory effect on DNA synthesis than the hormone. It is conceivable that these properties are related, at least in part, to the greater potency of the analogues in activating calcium channels, in view of the role of Ca^{2+} in $1,25(OH)_2D_3$ regulation of myoblast proliferation [38].

In conclusion, the results of these investigations suggest that modifications in the side-chain of the $1,25(OH)_2D_3$ molecule increase its ability to modulate muscle cell Ca^{2+} metabolism and growth. These findings are potentially relevant for the development of analogues for the treatment of vitamin-dependent myopathies.

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References

1. Minghetti PP and Norman AW, Vitamin D receptors: Gene regulation and genetic circuitry. *FASEB J* **2:** 3043–3053, 1988.
2. de Boland AR and Nemere I, Rapid actions of vitamin D compounds. *J Cell Biochem* **49:** 32–36, 1992.
3. Caffrey JM and Farach-Carson MC, Vitamin D_3 metabolites modulate dihydropyridine calcium currents in clonal rat osteosarcoma cells. *J Biol Chem* **264:** 20265–20274, 1989.
4. de Boland AR and Norman A, Evidence for involvement of protein kinase C and cyclic adenosine 3′,5′ monophosphate-dependent protein kinase in the 1,25-dihydroxy-vitamin D_3-mediated rapid stimulation of intestinal calcium transport, (transcaltachia). *Endocrinology* **127:** 39–45, 1990.
5. Selles J and Boland R, Evidence on the participation of the 3′,5′-cyclic AMP pathway in the non-genomic action of 1,25-dihydroxy-vitamin D_3 in cardiac muscle. *Mol Cell Endocrinol* **82:** 229–235, 1991.
6. Lieberherr M, Grosse B, Duchambon P and Druecke T, A functional cell surface type receptor is required for the early action of 1,25-dihydroxyvitamin D_3 on the phosphoinositide metabolism in rat enterocytes. *J Biol Chem* **264:** 20403–20406, 1989.
7. Morelli S, de Boland AR and Boland RL, Generation of inositol phosphates, diacylglycerol and calcium fluxes in myoblasts treated with 1,25-dihydroxyvitamin D_3. *Biochem J* **289:** 675–679, 1993.
8. Nemere I and Norman AW, At the cutting edge. Steroid hormone actions at the plasma membrane: Induced calcium uptake and exocytosis. *Mol Cell Endocrinol* **80:** c165–c169, 1991.
9. de Boland AR and Boland RL, Rapid changes in skeletal muscle calcium uptake induced *in vitro* by 1,25-dihydroxyvitamin D_3 are suppressed by calcium channel blockers. *Endocrinology* **120:** 1858–1864, 1987.
10. Tornquist K and Tashjian AH Jr, Dual actions of 1,25-dihydroxycholecalciferol on intracellular Ca^{2+} in GH_4C_1 cells: Evidence for effects on voltage-operated Ca^{2+} channels and Na^+/Ca^{2+} exchange. *Endocrinology* **124:** 2765–2775, 1989.
11. de Boland AR, Nemere I and Norman AW, Ca^{2+}-channel agonist BAY K8644 mimics $1,25(OH)_2$-vitamin D_3 rapid enhancement of Ca^{2+} transport in chick perfused duodenum. *Biochem Biophys Res Commun* **166:** 217–222, 1990.
12. Selles J and Boland R, Rapid stimulation of calcium uptake and protein phosphorylation in isolated cardiac muscle by 1,25-dihydroxyvitamin D_3. *Mol Cell Endocrinol* **77:** 67–73, 1991.
13. Manolagas SC, Hustmyer FG and Yu X-P, Immunomodulating properties of 1,25-dihydroxyvitamin D_3. *Kidney Int* **38:** S9–S16, 1990.
14. Binderup L, Immunological properties of vitamin D analogues and metabolites. *Biochem Pharmacol* **43:** 1885–1892, 1992.
15. Suda T, The role of $1\alpha,25$-dihydroxyvitamin D_3 in the myeloid cell differentiation. *Proc Soc Exp Biol Med* **191:** 214–220, 1989.
16. Bikkle DD and Pillai S, Vitamin D, calcium, and epidermal differentiation. *Endocr Rev* **14:** 3–19, 1993.
17. Koeffler HP, Amatruda T, Ikekawa N and Kobayashi Y, Induction of macrophage differentiation of human normal and leukemic myeloid stem-cells by 1,25-dihydroxyvitamin D_3 and its fluorinated analogs. *Cancer Res* **44:** 5624–5628, 1984.
18. Boland R, The role of vitamin D in skeletal muscle function. *Endocr Rev* **7:** 434–448, 1986.
19. Brown A, Finch J, Lopez-Hilker S, Dusso A, Ritter C, Pernalete N and Slatopolsky E, New active analogues of vitamin D with low calcemic activity. *Kidney Int* **38:** 22–27, 1990.
20. Jones G and Calverley MJ, A dialogue on analogues: Newer vitamin D-drugs for use in bone disease, psoriasis and cancer. *Trends Endocrinol Metab* **4:** 297–303, 1993.
21. Holick MF, 1,25-Dihydroxyvitamin D_3 and the skin: A unique application for the treatment of psoriasis. *Proc Soc Exp Biol Med* **191:** 246–262, 1989.
22. Kragballe K, Gjertsen BT, de Hoop D, Karlsmark T, van de Kerkhof PCM, Larkö O, Nieboer C, Roed-Petersen J, Strand A and Tikjøb G, Double-blind, right/left comparison of calcipotriol and betamethasone valerate in treatment of psoriasis vulgaris. *Lancet* **337:** 193–196, 1991.
23. Van de Kerkhof PCM, Biological activity of vitamin D analogues in the skin, with special reference to antipsoriatic mechanisms. *Br J Dermatol* **132:** 675–682, 1995.
24. Coombes RC, New targets for cancer therapy. *Mol Aspects Med* **14:** 373–449, 1993.
25. Hamada K and Shinomiya H, Novel vitamin D_3 analogs and their potential for inhibiting cell growth. *Drugs Future* **18:** 1057–1061, 1993.
26. Slatopolsky E, Berkoben M, Kellber J, Brown A and Delmez J, Effects of calcitriol and non-calcemic vitamin D analogs on secondary hyperparathyroidism. *Kidney Int* **42:** S43–S49, 1992.
27. Bouillion R, Allewaert K, Xiang D, Tan B and Van Baelen H, Vitamin D analogs with low affinity for the vitamin D binding protein: Enhanced *in vitro* and decreased *in vivo* activity. *J Bone Miner Res* **5:** 1051–1057, 1991.
28. Pols HA, Birkenhäger JC and van Leeuwen JPTM, Vitamin D analogues: From molecule to clinical application. *Clin Endocrinol* **40:** 285–291, 1994.
29. Farach-Carson MC, Sergeev I and Norman AW, Non-genomic actions of 1,25-dihydroxyvitamin D_3 in rat osteosarcoma cells: Structure–function studies using ligand analogs. *Endocrinology* **129:** 1876–1884, 1991.
30. Tien X-Y, Brasitus TA, Qasawa BM, Norman AW and Sitrin MD, Effect of $1,25(OH)_2D_3$ and its analogues on membrane phosphoinositide turnover and $[Ca^{2+}]_i$ in Caco-2 cells. *Am J Physiol* **265:** G143–G148, 1993.
31. Norman AW, Bouillon R, Farach-Carson MC, Bishop JE, Zhou LX, Nemere I, Zhao J, Muralidharan KR and Okamura WH, Demonstration that $1\beta,25$-dihydroxyvitamin D_3 is an antagonist of the nongenomic but not genomic biological responses and biological profile of three A-ring diastereomers of $1\alpha,25$-dihydroxyvitamin D_3. *J Biol Chem* **268:** 20022–20030, 1993.
32. Khoury R, Ridall AL, Norman AW and Farach-Carson MC, Target gene activation by 1,25-dihydroxyvitamin D_3 in osteosarcoma cells is independent of calcium influx. *Endocrinology* **135:** 2446–2453, 1994.
33. Bouillon R, Okamura WH and Norman AW, Structure–function relationships in the vitamin D endocrine system. *Endocr Rev* **16:** 200–257, 1995.
34. Boland R, Norman AW, Ritz E and Hasselbach W, Presence of a 1,25-dihydroxyvitamin D_3 receptor in chick skeletal

muscle myoblasts. *Biochem Biophys Res Commun* **128:** 305–309, 1985.
35. Walters MR, Wicker DC and Riggle PC, 1,25-Dihydroxyvitamin D_3 receptors identified in the rat heart. *J Mol Cell Cardiol* **18:** 67–72, 1986.
36. de Boland AR and Boland R, Non-genomic signal transduction pathway of vitamin D in muscle. *Cell Signal* **6:** 717–724, 1994.
37. Drittanti L, de Boland AR and Boland R, Modulation of DNA synthesis in cultured muscle cells by 1,25-didroxyvitamin D_3. *Biochim Biophys Acta* **1014:** 112–119, 1989.
38. Bellido T, Morelli S, Fernandez LM and Boland R, Evidence for the participation of protein kinase C and 3′,5′-cyclic AMP-dependent protein kinase in the stimulation of muscle cell proliferation by 1,25-dihydroxyvitamin D_3. *Mol Cell Endocrinol* **90:** 231–238, 1993.
39. Boland R, de Boland AR, Marinissen MJ, Santillan G, Vazquez G and Zanello S, Avian muscle cells as targets for the secosteroid hormone 1,25-dihydroxyvitamin D_3. *Mol Cell Endocrinol* **114:** 1–8, 1995.
40. Massheimer V, Boland R and de Boland AR, Rapid 1,25-dihydroxy-vitamin D_3 stimulation of calcium uptake by rat intestinal cells involves a dihydropyridine-sensitive cAMP-dependent pathway. *Cell Signal* **6:** 299–304, 1994.
41. Giuliani D and Boland R, 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, 1984.
42. Selles J, Bellido T and Boland R, Modulation of calcium uptake in cultured cardiac muscle cells by 1,25-dihydroxyvitamin D_3. *J Mol Cell Cardiol* **26:** 1593–1599, 1994.
43. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
44. Renaud JF, Meaux JP, Romey G, Schmid A and Lazdunski M, Activation of the voltage-dependent Ca^{2+} channel in rat heart cells by dihydropyridine derivatives. *Biochem Biophys Res Commun* **126:** 405–412, 1984.
45. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
46. Vazquez G and de Boland AR, Stimulation of dihydropyridine-sensitive Ca^{2+} influx in cultured myoblasts by $1,25(OH)_2D_3$. *Biochem Mol Biol Int* **31:** 677–684, 1993.
47. Reuter H, Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature* **301:** 569–574, 1983.
48. Binderup L and Kragballe K, Origin of the use of calcipotriol in psoriasis treatment. *Rev Contemp Pharmacother* **3:** 357–365, 1992.
49. Binderup L, Latini S, Binderup E, Bretting C, Calverley M and Hansen K, 20-epi-Vitamin D_3 analogues: A novel class of potent regulators of cell growth and immune responses. *Biochem Pharmacol* **42:** 1569–1575, 1991.
50. Farach-Carson MC, Abe J, Nishii Y, Khoury R, Wright GC and Norman AW, 22-Oxacalcitriol: Dissection of $1,25(OH)_2D_3$ receptor-mediated and Ca^{2+} entry-stimulating pathways. *Am J Physiol* **265:** F705–F711, 1993.
51. Irvine RF, Inositol phosphates and Ca^{2+} entry: Toward a proliferation or a simplification. *FASEB J* **6:** 3085–3091, 1992.
52. Berridge MJ, Inositol trisphosphate and calcium signalling. *Nature* **361:** 315–325, 1989.