British Journal of Pharmacology (1999) 126, 1815–1823
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Rapid actions of calcitriol and its side chain analogues CB1093 and GS1500 on intracellular calcium levels in skeletal muscle cells: a comparative study

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1 The ability of synthetic analogues of the secosteroid hormone 1α,25-dihydroxy-vitamin-D_3 [calcitriol, CT; 1,25$(OH)_2D_3$] to exert non-genomic (rapid) effects on target cells has been scarcely studied. To evaluate the pharmacological potential of the CT side-chain analogues CB1093 and GS1500, we compared their fast effects on intracellular calcium concentration ($[Ca^{2+}]_i$) in chick skeletal muscle cells with those elicited by the natural hormone.

2 Both analogues, similarly to CT, specifically induced rapid (30–60 s) and sustained rises in $[Ca^{2+}]_i$ levels. CB1093 and GS1500 were more potent than the natural hormone at concentrations as low as 10^{-13} M (4.5 fold stimulation) and 10^{-12} M (2.5 fold), respectively, whereas higher concentrations (10^{-9}–10^{-8} M) of CT were more effective than the analogues in elevating $[Ca^{2+}]_i$. Cyclic AMP was markedly increased by both analogues pointing for a role of this messenger in the fast actions of the synthetic compounds.

3 In Ca^{2+} free medium CT and analogues elicited a transient elevation in $[Ca^{2+}]_i$. The PLC inhibitors U73122 (2 μM) and neomycin (0.5 mM), as well as depletion of intracellular stores with thapsigargin (1 μM), completely prevented CB1093/GS1500-dependent changes in $[Ca^{2+}]_i$ suggesting that, similarly to CT, these analogues mobilized Ca^{2+} from an IP_3/thapsigargin-sensitive store.

4 The voltage-dependent calcium channel (VDCC) blocker nifedipine (2 μM) reduced by 50–60% the influx phase of the $[Ca^{2+}]_i$ response to CB1093 and GS1500, indicating that VDCC contributed partially to Ca^{2+} entry. The Ca^{2+} readdition protocol suggested that analogue-dependent activation of a SOC entry pathway accounted, to the same extent as for CT, for the remaining non-VDCC mediated Ca^{2+} influx.

Keywords: Non-genomic steroid actions; 1,25$(OH)_2$-vitamin D_3; calcitriol analogues; skeletal muscle cells; intracellular calcium; vitamin D-related myopathies

Abbreviations: 1,25$(OH)_2D_3$, 1α,25-dihydroxy-vitamin-D_3; CT, calcitriol; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PLC, phosphoinositide specific phospholipase C; SOC, store operated Ca^{2+} entry; VDCC, voltage-dependent Ca^{2+} channels; VDR, vitamin D receptor

Introduction

The secosteroid hormone 1α,25-dihydroxy-vitamin-D_3 (calcitriol) modulates muscle intracellular calcium levels through both a steroid-like genomic action (long-term responses) involving a specific interaction with an intracellular receptor (VDR: vitamin D receptor) and regulation of gene expression, and a non-genomic mechanism (fast-responses) which implies direct membrane effects of the hormone (Boland *et al*., 1995). Fast actions of calcitriol involve the participation of diverse transmembrane signalling systems resulting in G-protein mediated modulation of both adenylyl cyclase, with the resultant accumulation of cyclic AMP, and phosphoinositide-specific phospholipase C (PLC) activity, causing release of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), thus promoting activation of protein kinases A and C as well as rapid release of Ca^{2+} from intracellular stores. These events drive, in an as yet unsolved cross-talking signalling network, stimulation of voltage dependent Ca^{2+} channels (VDCC) with the subsequent increase in Ca^{2+} influx from the outside (Boland *et al*., 1997; De Boland & Boland, 1994; Vazquez *et al*., 1997). These observations led to the proposal, as for other cell types, that rapid actions of calcitriol in muscle involve the existence of a putative membrane receptor for the hormone (Boland *et al*., 1995). However, the existence of such a membrane receptor still remains an open question.

Muscle weakness and altered contractility are common symptoms in vitamin D_3/calcitriol deficiency states and this myopathy has been related to abnormal muscle intracellular Ca^{2+} homeostasis (Boland, 1986). During the last few years several analogues of calcitriol have emerged which mimic its classic nuclear actions on calcium transport as well as regulation of cell proliferation and differentiation while sharing low calcemic side-effects (Bouillon *et al*., 1995a,b). Although these compounds are potentially interesting for therapeutic use in muscle pathologies related to vitamin D, their ability to activate non-genomic pathways relative to that of calcitriol has been scarcely studied. Full understanding of their pharmacology and modes of action requires knowledge on their short-term effects on target cells. In the present work we compared the fast actions of calcitriol on intracellular calcium concentration ($[Ca^{2+}]_i$) in cultured chick skeletal muscle cells with those elicited by the synthetic side-chain analogues of calcitriol CB1093 and GS1500.

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Methods

Chemicals

Calcitriol (CT; 1α,25-dihydroxy-vitamin D_3), CB1093 (22-ethoxy-23-yne-1α,25-dihydroxy-vitamin D_2) and GS1500 (9,10-seco-{1α, 3β-dihydroxy-10-methylene-5, 7-dien-17-[2′-methylenthio-[m-2″-hydroxy-isopropylphenyl]} androstane) were kindly provided by Dr L. Binderup (Department of Biochemistry, Leo Pharmaceutical Products, DK-2750 Ballerup, Denmark). Fura-2/acetoxymethyl ester (Fura-2/AM), pluronic F127, nifedipine, verapamil, neomycin, thapsigargin, Dulbecco's modified Eagle's medium and foetal bovine serum were from Sigma Chemical Co. (St.Louis, MO, U.S.A.). U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) and U73343 (1-(6-((17β-3-methoxyestra-1,3, 5 (10) -trien-17-yl) amino) hexyl) -2,5-pyrrolidine) were from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, U.S.A.). $^{45}CaCl_2$ (10 Ci g^{-1}) was purchased from New England Nuclear (Boston, MA, U.S.A.). Cyclic AMP radioimmunoassay kit was from Diagnostics Products Corporation (Los Angeles, CA, U.S.A.). All other reagents used were of analytical grade.

Cell culture

Undifferentiated, myogenic chick skeletal muscle cells (myoblasts) were isolated from the breast muscle of 13-day-old white leghorn chick embryos (Gallus gallus) essentially as described before (Vazquez & De Boland, 1993) and seeded at appropriate density (120,000 cells cm^{-2}) in Petri dishes (80 mm diameter) or multiwells (20 mm diameter) for cyclic AMP and $^{45}Ca^{2+}$ influx measurements, respectively, or onto glass coverslips (24 × 6 mm) for intracellular calcium measurements, and cultured at 37°C under humidified air/5% CO_2. Cells were allowed to grow until confluence (4–6 days after plating) before use. Under these conditions, myoblasts proliferate within the first 48 h and at day four become differentiated into myotubes expressing both biochemical (high myosin and creatine kinase levels) and morphological (>80% multinucleated) characteristics of adult skeletal muscle fibers (see Vazquez & De Boland, 1993; Capiati, Inon & Boland, submitted).

Intracellular calcium measurements

Intracellular Ca^{2+} changes were monitored by using the Ca^{2+}-sensitive fluorescent dye Fura-2 (Grynkiewicz *et al*., 1985). Cell dye loading was achieved by incubating the cells in buffer A containing (mM) NaCl 138, KCl 5, $MgCl_2$ 1, glucose 5, HEPES (pH 7.4) 10, $CaCl_2$ 1.5 plus 0.1% bovine serum albumin (BSA), 4 μM of the penta-acetoxymethylester derivative (membrane permeable) Fura-2/AM and 0.012% pluronic F127 in the dark during 40 min at room temperature in order to minimize dye compartmentalization. Unloaded dye was washed out and cells were maintained in buffer B (buffer A without BSA, Fura-2/AM and pluronic F-127) in the dark (room temperature) for at least 40 min prior to use to allow for complete intracellular dye deesterification. Coverslips containing confluent cells were placed into quartz cuvettes of a thermostatized (37°C) SLM Aminco 8100 spectrofluorimeter (Spectronics Inc., MO, U.S.A.) sample compartment under constant, controlled stirring. Fura-2 intracellular fluorescence intensity was monitored at an emission wavelength of 510 nm (8 nm bandpass) by alternating (300 Hz) the excitating wavelength between 340 and 380 nm (4 nm bandpass) with a dual excitation monochromator. Signals from short and long wavelength were ratioed (R = 340/380) thus making the measurement independent of variations in cellular dye content, dye leakage or photobleaching. Calibration of Fura-2 fluorescence signal to calculate $[Ca^{2+}]_i$ values was performed for each coverslip essentially as described (Vazquez *et al*., 1997). Maximal (R_{max}) and minimal (R_{min}) intracellular dye fluorescence signals were determined by adding 5 μM ionomycin plus 3 mM Ca^{2+} and EGTA (10 mM) (pH 9.0), respectively. Under these conditions of measurement, the dissociation constant (K_D) for the Ca^{2+}-Fura-2 complex was assumed to be 224 nM, and $[Ca^{2+}]_i$ according to the algorithm of Grynkiewicz and coworkers (Grynkiewicz *et al*., 1985) derives from:

$$[Ca^{2+}]_i = K_D(R - R_{min}/(R_{max} - R) \times \beta$$

β is the ratio between the specific fluorescence of the Ca^{2+}-free and Ca^{2+}-bound forms of the dye at the longer wavelength.

In some experiments, a Ca^{2+}-free extracellular medium was used. In such situations, absence of Ca^{2+} in the medium means free Ca^{2+} concentration near 1 nM, which is accomplished by preparing a nominally Ca^{2+}-free buffer B (see composition above) plus EGTA (1 mM). Free Ca^{2+} levels were calculated by using the WinMaxc program, version 1.7 (Bers *et al*., 1996). All buffers and saline solutions used were prepared with deionized water.

Measurement of $^{45}Ca^{2+}$ influx

$^{45}Ca^{2+}$ influx measurements were performed as described before (Vazquez & De Boland, 1993). At confluence, the culture medium was replaced by Krebs-Henseleit-0.2% glucose solution (KHG) and the cells were equilibrated in this solution for 20 min at 37°C. The assay was initiated by adding $^{45}CaCl_2$ (1.5 mM, 10 Ci g^{-1}) in the presence of vehicle (ethanol < 0.1%) or the indicated concentrations of calcitriol, CB1093 or GS1500. The incubation was kept to proceed for 5 min, interval in which stimulation of Ca^{2+} influx reaches a plateau level (Vazquez & De Boland, 1993; Vazquez *et al*., 1995), and then the assay was stopped by removing the medium and washing the monolayers with buffer containing (in mM) Tris (pH 7.4) 25, NaCl 140 and $LaCl_3$ 1. Cells were dissolved in 1 N NaOH/0.1% SDS and aliquots were taken for radioactivity counting by liquid scintillation spectrometry and protein determination by the Lowry procedure (Lowry *et al*., 1951).

Measurement of cyclic AMP accumulation

Confluent cells were equilibrated in KHG for 20 min at 37°C. After treatment (5 min, 37°C) with calcitriol, CB1093, GS1500 or vehicle (ethanol < 0.1%), cells were immediately frozen in liquid air and homogenized in KHG containing 1 mM isobutyl-methylxanthine. The homogenate was acidified (6% trichloroacetic acid) and centrifuged (1200 × *g*, 15 min at 4°C). The supernatant was extracted (six times) with water-satured diethyl-ether and cyclic AMP determined by radioimmunoassay with a commercially available kit.

Statistical analysis

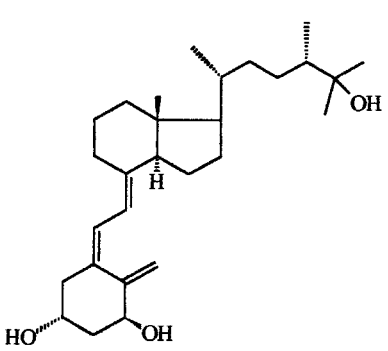
Statistical significance of data was evaluated using Student's *t*-test (Snedecor & Cochran, 1967) and probability values below 0.05 ($P < 0.05$) were considered significant. Quantitative data are expressed as means ± standard deviation (s.d.) from the indicated set of experiments.

Results

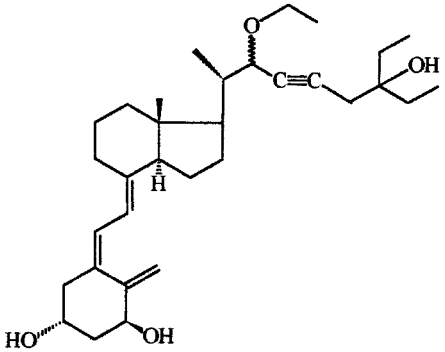
After addition of different concentrations of calcitriol (CT, $10^{-15}-10^{-6}$ M) or its side-chain analogues CB1093 and GS1500 (see Figure 1 for chemical structures) to fura-2 loaded skeletal muscle cells, changes in $[Ca^{2+}]_i$ were continuously monitored for up to 5 min. As shown in Figure 2, both analogues, similarly to CT, induced a rapid (30–60 s) rise in $[Ca^{2+}]_i$ levels which persisted elevated as long as the cells were exposed to the steroids. This action was specific for CT and its related analogues, as $1\alpha(OH)D_3$, $25(OH)D_3$ and $24,25(OH)_2D_3$, and various other unrelated steroids were not able to modify $[Ca^{2+}]_i$ in skeletal muscle cells (Table 1).

At low concentrations ($10^{-13}-10^{-11}$ M), both CB1093 and GS1500 were significantly more active than the natural hormone following the efficacy order: CB1093 > GS1500 > CT (Figure 3). CT-induced increments in $[Ca^{2+}]_i$ become detectable from 10^{-11} M (1.2 fold over basal levels, $P<0.05$), but marked differences with respect to basal $[Ca^{2+}]_i$ were reached at 10^{-10} M (1.5 fold stimulation, $P<0.01$). CB1093 and GS1500 were more potent than CT at concentrations as low as 10^{-13} M (4.5 fold) and 10^{-11} M (3.5 fold), respectively. Similarly to the natural hormone, both CB1093 and GS1500 exhibited bell-shaped dose-response profiles, with an evident downturn phase when the optimal dose was exceeded. Notoriously, the above observed order of efficacy changed when the concentration increased (CT > CB1093 > GS1500, at 10^{-8} M; $P<0.05$ and $P<0.01$ for CT vs CB1093 and GS1500 actions, respectively). However, when the influence of these analogues on $^{45}Ca^{2+}$ influx in muscle cells was studied, a different correlation was observed, as increasing the concentration of either CT, CB1093 or GS1500 resulted in a more pronounced action of these compounds on $^{45}Ca^{2+}$ influx (Figure 4). Similar results were obtained when cyclic AMP generation was measured (data not shown). At 10^{-9} M, an optimum concentration for CT-mediated stimulation of both $^{45}Ca^{2+}$ influx and $[Ca^{2+}]_i$ changes, cyclic AMP accumulation was stimulated 5.5, 4.7 and 2 fold by CB1093, GS1500 and CT, respectively (Figure 5).

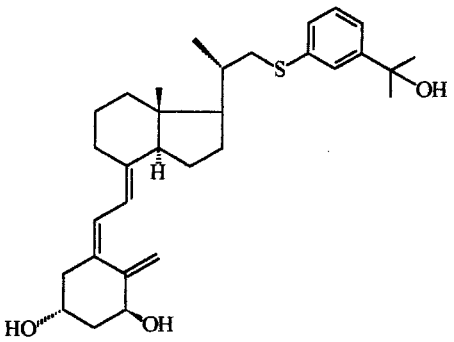
In skeletal muscle cells, the rapid, non-genomic $[Ca^{2+}]_i$ response to CT is composed of an initial fast sterol-induced Ca^{2+} release from endogenous thapsigargin-sensitive stores which is followed by cation influx from the extracellular milieu. This cation entry pathway accounts for the sustained, long-lasting $[Ca^{2+}]_i$ phase, which has been shown to be contributed by both the well established L-type VDCC-mediated Ca^{2+} entry, and a novel store-operated Ca^{2+} entry (SOC) pathway (Vazquez *et al*., 1997; 1998). Experiments were then performed to determine if similar Ca^{2+} routes were involved in the effect of CT analogues on $[Ca^{2+}]_i$ reported here. As previously shown (Vazquez *et al*., 1997) the response to CT in Ca^{2+}-free medium (Figure 6) was rapid (30–60 s) and transient (2.5 fold at peak), temporally paralleling the well characterized CT-induced IP_3-response in these cells (Morelli *et al*., 1993; Vazquez *et al*., 1998). Under the same conditions, the actions of CB1093 and GS1500 followed a profile similar to that of the



Calcitriol



CB1093



GS1500

Figure 1 Chemical structures of calcitriol and its side-chain analogues CB1093 and GS1500. Note the altered stereochemistry at carbon 20 (20-epi) for both analogues. See text for details.

natural hormone. However, while the CT-induced $[Ca^{2+}]_i$ transient seemed to be entirely due to mobilization from endogenous stores without contribution of cation influx (there were no significant differences in average peak $[Ca^{2+}]_i$ when Ca^{2+} or VDCC blockers (see Vazquez *et al*., 1997; 1998) were present in the bath prior to sterol stimulation), the effect of the analogues was significantly reduced by the absence of extracellular Ca^{2+} (74 and 75% reduction in $[Ca^{2+}]_i$ 1 min after exposure to CB1093 and GS1500, respectively). As for the natural hormone, this analogue-induced Ca^{2+} transient was totally blocked by pretreatment with the PLC inhibitors U73122 (2 μM) or neomycin (0.5 mM) (Table 2) but not by U73343 (not shown), an analogue of U73122 without effect on PLC (Bleasdale *et al*., 1990).

When intracellular muscle Ca^{2+} stores were depleted by inhibition of the sarcoplasmic reticulum Ca^{2+}-Mg^{2+}-ATPase with 1 μM thapsigargin, the response to either CT, CB1093 or GS1500, was completely blocked (Figure 7). The involvement of Ca^{2+} channels in the Ca^{2+} response to the CT analogues was evaluated. Pretreating muscle cells with the VDCC blockers nifedipine (2 μM, Table 2) or verapamil (not shown), as occurs for CT, only partially (50–60%) reduced the $[Ca^{2+}]_i$ increase induced by CB1093 and GS1500. As for CT, the effect of VDCC blockade was evident at the influx phase of the response, while the early $[Ca^{2+}]_i$ transient remained unaltered (not shown).

Finally, in order to evaluate if, as is the case for CT, SOC channels were involved in the remaining analogue-induced non-VDCC mediated Ca^{2+} entry, we used the Ca^{2+} readdition protocol to preliminary address this point. Fura-2 loaded muscle cells were stimulated with either CT, CB1093 or GS1500 in Ca^{2+}-free medium and in the presence of 2 μM nifedipine and 5 μM verapamil (added 3 min prior to CT/analogue stimulation); once the rapid and transient elevation in $[Ca^{2+}]_i$ occurred, Ca^{2+} readdition (1.5 mM) was performed after $[Ca^{2+}]_i$ fell down to basal levels (around 2 min after peak response). At this point, readdition of Ca^{2+} resulted in a fast (30–40 s) and sustained $[Ca^{2+}]_i$ rise, thus evidencing Ca^{2+} influx from the outside through a preactivated pathway (Figure 6, right arrow on each trace). Ca^{2+} readmission to

Table 1 Specificity of the action of calcitriol and calcitriol-analogues on $[Ca^{2+}]_i$ stimulation in skeletal muscle cells

	$[Ca^{2+}]_i$ (%)
Control	100
CT	240±6*
CB1093	384±12**
GS1500	256±7**
1α(OH)D_3 (10^{-9}–10^{-7} M)	103±5
25(OH)D_3 (10^{-9}–10^{-7} M)	105±3
24,25$(OH)_2D_3$ (10^{-9}–10^{-7} M)	108±3
17β-estradiol (10^{-10}–10^{-7} M)	107±4
Dihydrotestosterone (10^{-10}–10^{-7} M)	97±5
Progesterone (10^{-10}–10^{-8} M)	110±8
β-sitosterol (10^{-10}–10^{-8} M)	99±5

Fura-2 loaded skeletal muscle cells were treated with vehicle (ethanol < 0.1%, Control), CT (10^{-9} M), CB1093 (10^{-12} M), GS1500 (10^{-11} M) or the indicated concentrations of vitamine D_3-derived compounds or other steroids, and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured as described under Methods. When stimulation of $[Ca^{2+}]_i$ occurred, it was quantitated when the corresponding response stabilized (plateau phase). Results are expressed as per cent of control (100%) to allow comparison among different treatment conditions, and are the average of three independent experiments ± s.d.; *P < 0.001; **P < 0.01.

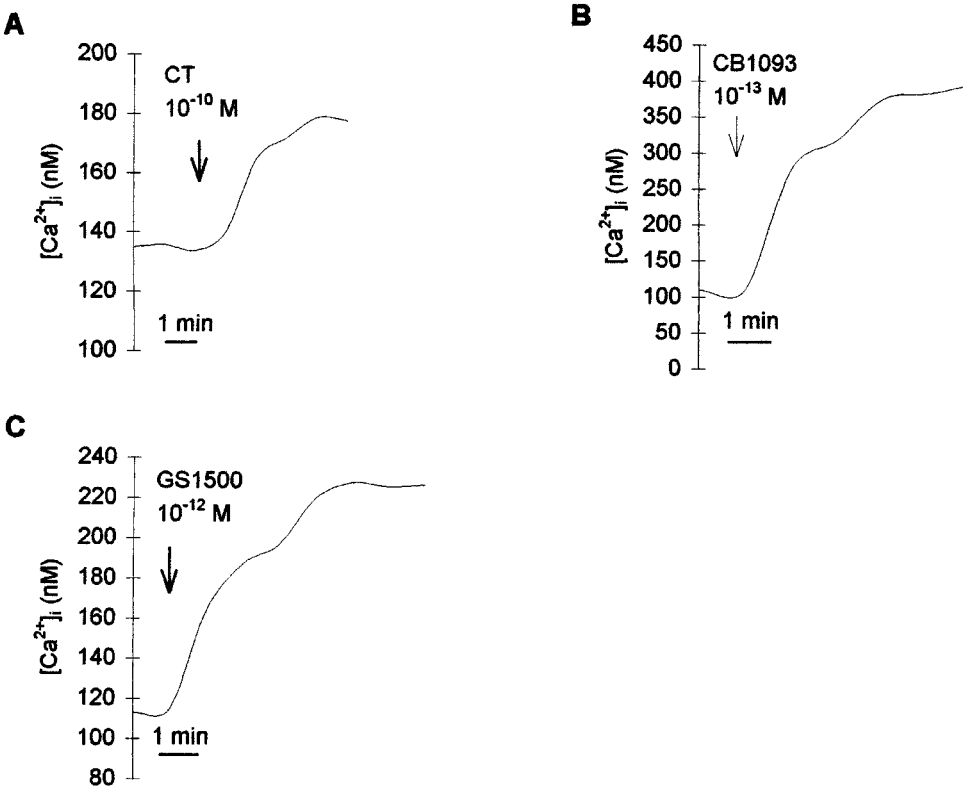


Figure 2 Effects of calcitriol side-chain analogues CB1093 and GS1500 on intracellular Ca^{2+} levels in skeletal muscle cells. $[Ca^{2+}]_i$ changes were monitored after addition (arrows) of CT (A), CB1093 (B) or GS1500 (C). Representative time-traces from three independent experiments, corresponding to the lowest stimulating-dose, are shown.

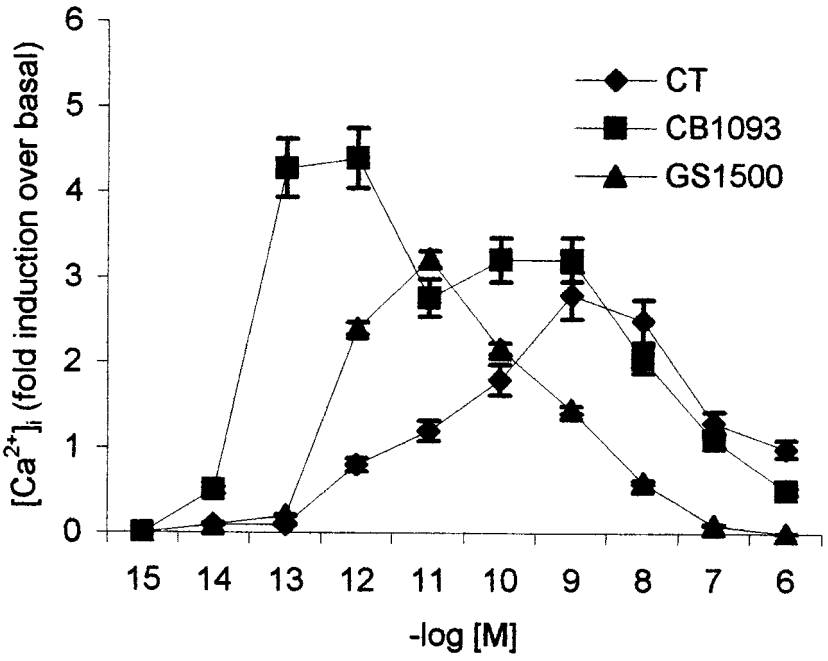


Figure 3 Dose-response profiles for the effects of calcitriol, CB1093 and GS1500 on skeletal muscle cell $[Ca^{2+}]_i$ levels. $[Ca^{2+}]_i$ changes were monitored after addition of different concentrations of CT, CB1093 or GS1500 into the measurement cuvette. Data represent fold-induction over the corresponding basal values after 3–4 min of hormone or analogue addition (plateau phase) and are representative from $[Ca^{2+}]_i$ recordings performed on at least three coverslips of five different cultures, for each assay condition.

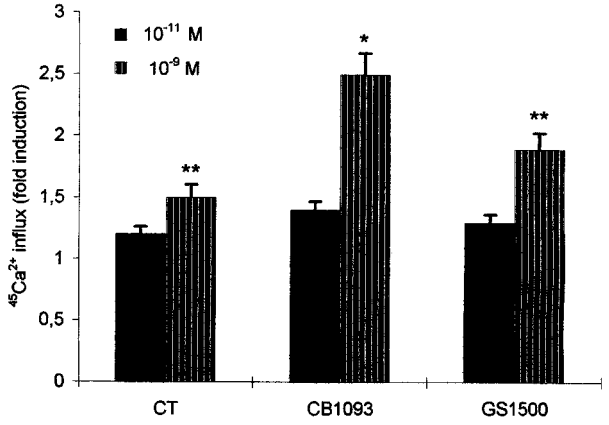


Figure 4 Effects of calcitriol and its side-chain analogues CB1093 and GS1500 on muscle cell $^{45}Ca^{2+}$ influx. Cells were incubated for 5 min at 37°C in KHG solution containing $^{45}CaCl_2$ (2 μCi ml^{-1}) in the presence of vehicle (ethanol<0.1%: Control) or the indicated concentrations of CT, CB1093 or GS1500. $^{45}Ca^{2+}$ influx was then determined as described in Methods. Data are expressed as fold-induction respect to control and represent the mean±s.d. of values from three independent experiments performed in quadruplicate. *P<0.001 and **P<0.01 for 10^{-9} M and 10^{-11} M respectively.

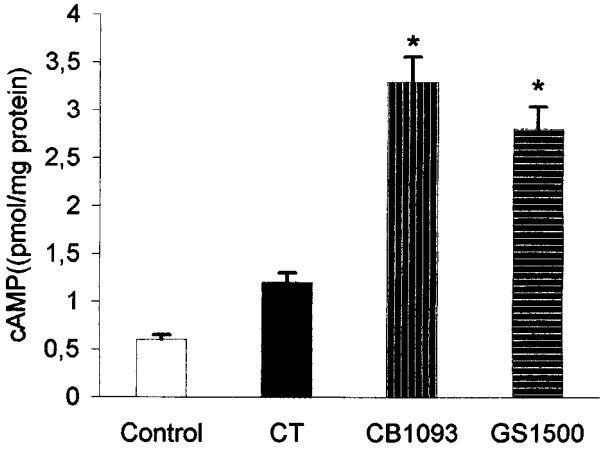


Figure 5 Effects of calcitriol and analogues CB1093 and GS1500 on cyclic AMP levels in skeletal muscle cells. Cells were incubated (5 min at 37°C) in the presence or absence (ethanol<0.1%: Control) of 10^{-9} M of either CT, CB1093 or GS1500. Cyclic AMP accumulation was then determined as described in Methods. Data represent the mean±s.d. of values from three independent experiments performed in triplicate. *P<0.001 for both analogues with respect to CT.

cells not previously exposed to the steroids resulted in no detectable Ca^{2+} influx (not shown). Inclusion of both nifedipine and verapamil at concentrations known to effectively block VDCC-mediated Ca^{2+} influx in our cell system (see Table 2, and Vazquez & De Boland, 1993) was done because functional isolation of a SOC entry pathway in excitable cells requires suppression of the large Ca^{2+} influx that normally occurs through VDCC. Thus, the Ca^{2+} entry observed by the Ca^{2+} free/Ca^{2+} back protocol mainly reflects Ca^{2+} entering the cell *via* SOC channels. Under these conditions, CB1093 and GS1500 stimulated SOC influx within the same extent as CT (2.5, 1.7 and 2.0 fold-induction of Ca^{2+} influx following Ca^{2+} readmission respect to basal for CB1093, GS1500 and CT, respectively).

Discussion

The present work provides for the first time information on the rapid effects of synthetic side-chain analogues of calcitriol (CT) on intracellular calcium levels in skeletal muscle cells. We used here the CT analogues CB1093 and GS1500 which belong to

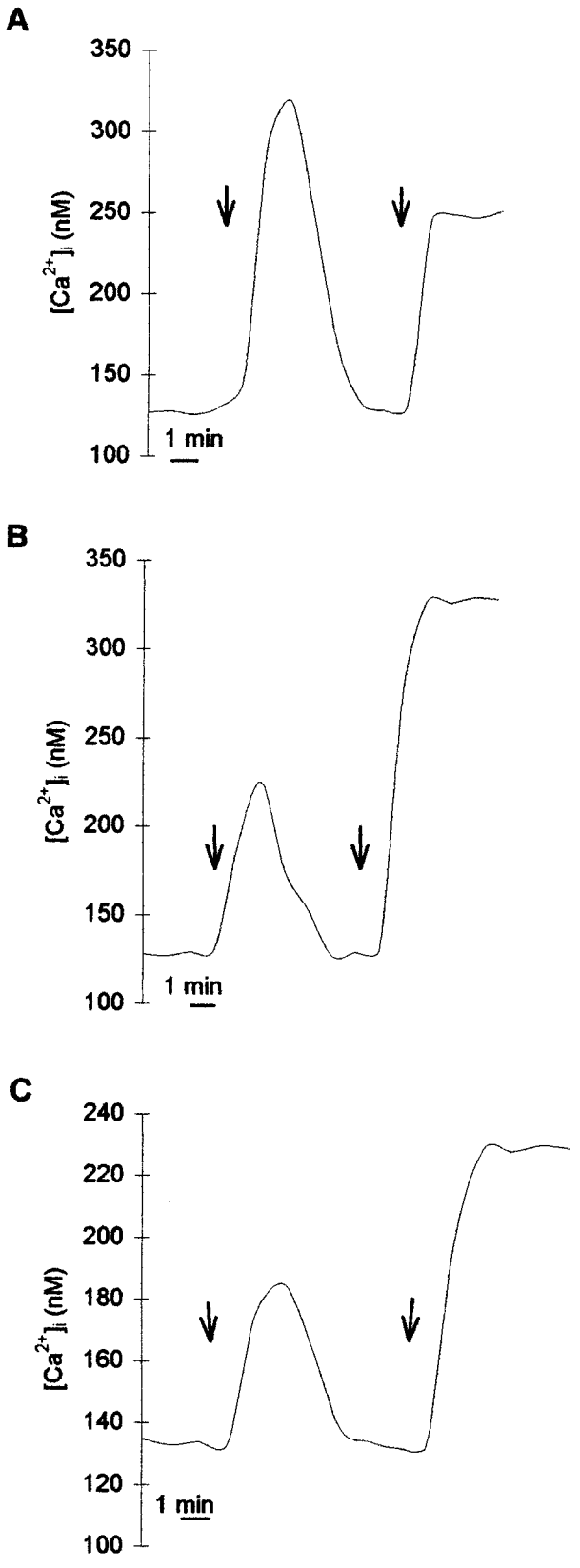


Figure 6 Mobilization of Ca^{2+} from endogenous stores by CB1093 and GS1500: activation of a store-operated Ca^{2+} (SOC) influx pathway. Cells were incubated in Ca^{2+}-free extracellular medium and then stimulated with either CT (10^{-9} M; A), CB1093 (10^{-12} M; B) or GS1500 (10^{-11} M; C), as indicated by left arrows on each trace. Nifedipine (2 μM) and verapamil (5 μM) were added 3 min prior to CT/analogue stimulation, to eliminate the large Ca^{2+} influx that normally occurs through VDCC and functionally isolate the SOC entry pathway (see text). Right arrows indicate Ca^{2+} (1.5 mM) readdition to the medium to evidence the existence of a preactivated (store-operated) Ca^{2+} influx pathway. Each panel shows representative time-traces from three independent experiments.

Table 2 Effects of both voltage-dependent Ca^{2+} channel and phospholipase C inhibition on calcitriol and calcitriol-analogue induced $[Ca^{2+}]_i$ responses in skeletal muscle cells

	$[Ca^{2+}]_i$ (%)	$\Delta[Ca^{2+}]_i$ (%)	% *Inhibition*
Control	100	–	–
CT	275±10*	175±10	–
CB1093	425±8**	325±8	–
GS1500	250±5**	150±12	–
U73122 (or neomycin)+CT:			
1 min	109±3 (100±2)	–	98 (100)
5 min	100±4 (100±1)	–	100 (100)
U73122 (or neomycin)+CB1093:			
1 min	99±5 (100±1)	–	99 (100)
5 min	100±6 (98±4)	–	100 (100)
U73122 (or neomycin)+GS1500:			
1 min	102±3 (98±3)	–	100 (100)
5 min	100±6 (98±4)	–	100 (100)
Nif.+CT	179±5*	79±5	55
Nif.+CB1093	259±11*	159±11	51
Nif.+GS1500	163±4*	63±14	58

Fura-2 loaded skeletal muscle cells were treated with vehicle (ethanol<0.1%, Control), CT (10^{-9} M), CB1093 (10^{-12} M) or GS1500 (10^{-11} M) and intracellular Ca^{2+}concentration ($[Ca^{2+}]_i$) was measured as described under Methods. Unless otherwise indicated, $[Ca^{2+}]_i$ stimulation was evaluated at the plateau phase of the hormone/analogue-induced response (see Figure 2). When used, both nifedipine (2 μM) and the PLC inhibitors U73122 (2 μM) or neomycin (0.5 mM, data in parentheses) were added into the measurement cuvette 3 min before stimulation. In the PLC-inhibition assay $[Ca^{2+}]_i$ values measured at 1 and 5 min after stimulation are given. Results are expressed as per cent of control (100%) to allow comparison among different assay conditions, and are the average of three independent experiments±s.d. *$P<0.001$; **$P<0.01$. Per cent (%) inhibition refers to the decrease in $\Delta[Ca^{2+}]_i$.

the 20-epi-CT analogue group characterized by an altered stereochemistry at carbon 20 of the side chain. These compounds are considerably more potent regulators of cellular growth, differentiation and immune responses than CT, while having a longer half-life than other analogues of this group, thus being more suitable for systemic use (Binderup *et al*., 1991). Compared to CT, GS1500 is characterized by the presence of both an aromatic ring and a sulphur atom at position 23 in the side chain, whereas CB1093 has an ethoxy group at position 22 and a triple 23-24 bond (23-yne) (see Figure 1). Interestingly, both compounds have almost completely lost their ability to bind to the intracellular vitamin D receptor (Binderup *et al*., 1994).

We have shown here that, similarly to CT, both CB1093 and GS1500 were able to induce a fast and sustained rise in $[Ca^{2+}]_i$ levels in chick skeletal muscle cells. The analogues are particularly more effective than CT at low doses, which was not observed when $^{45}Ca^{2+}$ influx or cyclic AMP generation were measured. We noted here that, as for CT, both CB1093 and GS1500 exhibited bell-shaped dose-response relationships, with a marked downturn phase when concentrations exceeding the optimal ones were used. This type of response is by no means unusual in the pharmacology of many drugs (for a review see Pliska, 1994). Although not specifically addressed in this study, two main mechanisms leading to this phenomenon could be hypothesized here: dose-dependent variations in the interaction between the steroid and the putative membrane

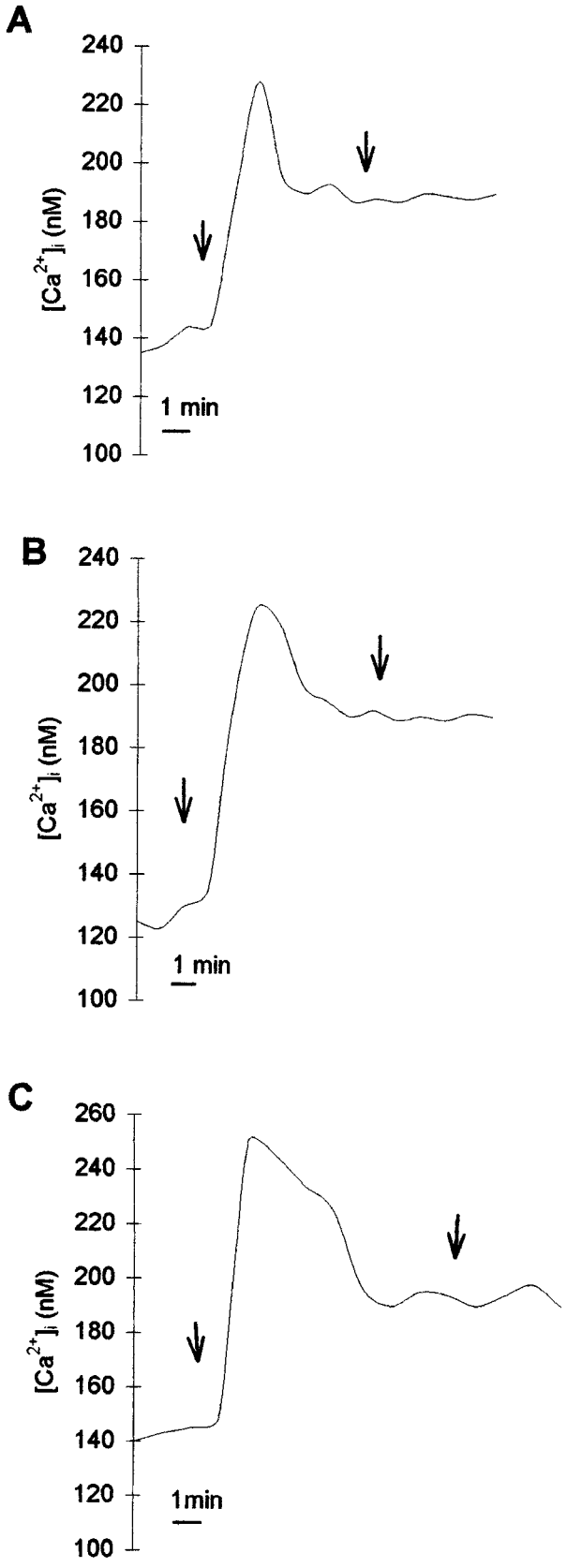


Figure 7 Thapsigargin depletion of skeletal muscle cell intracellular Ca^{2+} stores blocks CT-analogue effects on intracellular Ca^{2+}. Skeletal muscle cells were treated with thapsigargin (1 μM, left arrow in each panel) to deplete intracellular Ca^{2+} stores. A typical response due to inhibition of the sarcoplasmic Ca^{2+}-ATPase is observed, with a fast, transient rise in $[Ca^{2+}]_i$ (store depletion) and a sustained phase corresponding to the influx pathway. When $[Ca^{2+}]_i$ reached the plateau, CT (10^{-9} M), CB1093 (10^{-12} M) or GS1500 (10^{-11} M) were added (right arrow, A, B and C, respectively) and $[Ca^{2+}]_i$ was monitored over at least 5 min. Each panel shows time-traces representative from three independent experiments.

receptor, or counteracting compensatory responses coming from effector entities, all of them related to the dynamics of the cell-signalling system. It is also possible that different second messenger systems could become activated at different steroid concentrations, as it has been observed for other steroid hormones (Civitelli *et al*., 1990; Picotto *et al*., 1996).

As we previously reported for CT (Vazquez *et al*., 1995), our results point for a role of the cyclic AMP pathway in the fast actions of these two analogues. Various lines of evidence have suggested that CT regulation of Ca^{2+} channel activity in muscle involves cyclic AMP-mediated phosphorylation of membrane proteins, either constitutive of the channel itself or tightly associated, regulatory ones. However, no dose-response correlations could be established between the magnitude of analogue-induced cyclic AMP generation and that of $[Ca^{2+}]_i$ elevation. The contribution of the cyclic AMP cascade to analogue potency on $[Ca^{2+}]_i$ stimulation needs additional investigation. A similar apparent discrepancy between CT/analogue effects on $^{45}Ca^{2+}$ influx and $[Ca^{2+}]_i$ was observed. However, it must be kept in mind that under the conditions of loading and measurement used here, changes in fura-2 fluorescence mainly report changes in cytosolic Ca^{2+}, while the $^{45}Ca^{2+}$ uptake technique, although widely used to evaluate the action of CT analogues on Ca^{2+} influx (see for instance, Farach-Carson *et al*., 1998; Yukihiro *et al*., 1994), allows radiotracer accumulation into Ca^{2+} sequestering cellular organelles (e.g., mitochondria, sarcoplasmic reticulum) thus seriously affecting the suitability of the method to allow fine analysis of cytosolic variations in Ca^{2+}. We think that information provided by each of these methods should be handled independently and attempts to get combined interpretations must be avoided.

The observed profile for both the CB1093 and GS1500 $[Ca^{2+}]_i$ responses was highly similar to that of CT, involving an initial rapid analogue-induced Ca^{2+} mobilization from thapsigargin-sensitive endogenous stores, followed by cation influx from the extracellular millieu which finally accounts for the sustained $[Ca^{2+}]_i$ phase. The concept that, as for CT, the rapid analogue-induced $[Ca^{2+}]_i$ transient is due to mobilization of the cation from IP_3-sensitive stores, is strongly supported by the Ca^{2+} mobilizing effect of the analogues when acting in a Ca^{2+} free medium and the blocking effect of the PLC inhibitors U73122 and neomycin, both acting at different sites of PLC activity (Prentki *et al*., 1986; Yule & Williams, 1992). These results also show that such analogue-induced Ca^{2+} release from internal stores does not last long enough to be responsible *per se* for the non-VDCC mediated Ca^{2+} entry phase seen after steroid stimulation of muscle cells in Ca^{2+} containing medium under conditions of VDCC blockade.

We previously showed that in skeletal muscle cells, CT induces a fast (30–60 s) and monophasic generation of IP_3 (Morelli *et al*., 1993; Vazquez *et al*., 1998). Although the present data suggest that a similar mechanism of endogenous Ca^{2+} mobilization might operate for these two analogues, the effects of both CB1093 and GS1500 on phospholipid metabolism in skeletal muscle cells remain to be investigated. Interestingly, the CT side chain analogue MC903 exhibits a considerably greater ability than CT to rapidly stimulate Ca^{2+} influx into chick skeletal muscle cells (Sellés *et al*., 1997) and it has been also shown to be more effective than the parental hormone in increasing the IP_3 production in Caco-2 cells (Tien *et al*., 1993). As polyphosphoinositide turnover could be directly or indirectly involved in the control of Ca^{2+} influx from outside the cell (Berridge, 1989; Irvine, 1992), it is tempting to speculate that differences in the extent of inositol phosphate liberation account for the greater stimulation of

Ca^{2+} entry through Ca^{2+} channels by analogues CB1093 and GS1500 here reported, even when Ca^{2+} mobilization, as seen in Ca^{2+} free medium, is significantly lower for the analogues than for CT. This hypothesis is under current investigation.

We observed here that the Ca^{2+} influx phase of the Ca^{2+} response to CB1093 and GS1500 was partially abolished by VDCC blockers, suggesting that modulation of voltage-dependent channels is involved in the non-genomic action of both analogues in muscle cells as previously established for CT in these as well as other cell systems (Tornquist & Tashjian, 1989; Vazquez & De Boland, 1993; Vazquez *et al*., 1997; Walters, 1995). Moreover, by using the Ca^{2+} readdition protocol, which has been shown to be a sensitive procedure to measure changes in Ca^{2+} influx through the SOC pathway (Xu *et al*., 1995; Zhu *et al*., 1996), we obtained evidence indicating that CB1093 and GS1500 are able to promote Ca^{2+} influx through SOC channels, both analogues being equipotent with respect to CT. As Ca^{2+} entry through the SOC pathway is proportional to the degree of depletion of the endogenous cation store (Llopis *et al*., 1992), the magnitude of SOC entry measured by the Ca^{2+} readdition protocol, which imposes store depletion without refilling chance until the cation is added back to the medium, is exacerbated when compared with that of the total (VDCC plus SOC) Ca^{2+} entry observed under physiological stimulation with the steroids. As the early $[Ca^{2+}]_i$ transient in the analogue $[Ca^{2+}]_i$ response was significantly reduced in Ca^{2+}-free medium but not by VDCC blockade, it is tempting to speculate that activation of SOC influx at an early step of the analogue action could be accounting for such differences. Whether the mechanism underlying analogue modulation of SOC entry into muscle cells follows the kinetics and features of that for CT (see Vazquez *et al*., 1998), remains to be established.

Previous studies from our laboratory showed that CT stimulates DNA synthesis in proliferating myoblasts, e.g. at the early (first 48 h) stages of culture prior to cell fusion, whereas it inhibits such process during the subsequent phase of myoblast differentiation (Drittanti *et al*., 1989; Marinissen *et al*., 1998). Changes in other biochemical parameters such as creatine kinase activity and myosin expression were also consistent with a role of the hormone in the modulation of muscle cell proliferation and differentiation. It has been recently reported that the side chain analogues MC903 and MC1288 are able to exert a more pronounced antiproliferative and differentiative effect than CT in chick muscle cells (Sellés *et al*., 1997). Moreover, CB1093 is more potent than CT in stimulating differentiation of skeletal muscle cells in culture, whereas GS1500 seems to be equipotent with respect to the natural hormone (Sellés & Boland, unpublished observations). It is conceivable that these properties are related, at least in part, to the greater potency of the analogues in promoting activation of Ca^{2+} influx, in view of the role of Ca^{2+} in CT regulation of muscle cell proliferation (Bellido *et al*., 1993). However, considering the present results, mediation of the cyclic AMP signalling pathway in the genomic actions of CT and its analogues, independently of changes in intracellular Ca^{2+}, cannot be excluded.

Over the past few years a myriad of calcitriol analogues have been developed (reviewed by Bouillon *et al*., 1995a), the clinical interest of which expanded from the traditional fields of bone, kidney and parathyroid disorders, to the non-classical psoriasis, cancer and autoimmune diseases (Binderup *et al*., 1994). Clinical entities related to vitamin D deficiency and/or abnormal metabolism, such as renal osteodystrophy, chronic renal failure and osteomalacia among others, exhibit moderate to severe muscle weakness and abnormal contractility (Boland, 1986; Traba-Villameytide, 1993), which seem to be related, at least in part, to deregulation of muscle Ca^{2+} homeostasis. Considering that appropriate regulation of fast changes in Ca^{2+} levels are inherent to normal muscle function, it is tempting to speculate that an abnormal bioavailability of, or response to CT could contribute to the ethiology of such muscle pathological states. Development of CT analogues has been directed towards the reduction in calcemic effects in order to avoid side effects such as hypercalciuria and hypercalcemia. However, neither their possible side-actions on muscle nor their potentiality as therapeutic agents for treatment of CT-related muscle dysfunctions has been studied. It is therefore important to expand our knowledge of the mechanism(s) of action of CT analogues on muscle cells. In the present as well as in previous studies, chick skeletal muscle cells have been chosen as the experimental system. The high homology existing in the signalling mechanisms mediating CT rapid actions in both mammalian and chick muscle cells (Boland *et al*., 1995; Vazquez & De Boland, 1993; Vazquez *et al*., 1995) has evidenced that avian skeletal muscle cells in culture represent an appropriate model system to evaluate calcitriol actions on muscle.

In conclusion, the results obtained here with the synthetic compounds CB1093 and GS1500 further confirm previous observations made with analogues MC903 and MC1288 (Sellés *et al*., 1997), indicating that side-chain modifications in the CT molecule strongly increase its ability to modulate muscle cell Ca^{2+} levels by a rapid, non-genomic mechanism which seems homologous to that of the natural hormone. These findings are potentially relevant for the development of analogues with therapeutic value in the management of vitamin D_3-related muscle disorders.

This research was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas, Agencia Nacional de Promocion Cientifica y Tecnologica, the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires and Universidad Nacional del Sur (Argentina)

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(Received October 2, 1998
Accepted January 5, 1999)