The calcium ionophore A23187 is a potent stimulator of the vitamin D₃-25 hydroxylase in hepatocytes isolated from normocalcaemic vitamin D-depleted rats

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INTRODUCTION

Vitamin D₃ (D₃), a natural secosteroid, has, in its native form, virtually no biological activity. The vitamin must undergo two hydroxylation reactions (at C-25 in the liver, and at C-1α in the kidney) before acquiring its full biological potential. Although the regulation of the renal C-1α hydroxylase has been the object of intensive studies, the factors involved in the regulation of the hepatic D₃-25 hydroxylase have not yet been clearly defined. Among the most important unresolved questions concerning the hepatic C-25 hydroxylation of D₃ are (1) the effect of previous dosing with D₃ [1-3]; (2) the effect of the vitamin D-hormone 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] [4-6]; as well as (3) the effect of the extra- and/or intra-cellular calcium concentrations on the formation of the hepatic metabolite, 25-hydroxyvitamin D₃ [25(OH)D₃] [6,7].

Recent studies on the effect of 1,25(OH)₂D₃ on the D₃-25 hydroxylase or on the fate of the hepatic or circulating 25(OH)D₃ have either suggested that the hormone did not significantly affect the enzyme [8,9], that it inhibited the enzyme [4-6,10], or that it accelerated the metabolic clearance rate of 25(OH)D₃ [11], or increased the biliary excretion of 25(OH)[³H]D₃-derived products [12]. Furthermore, other studies have also suggested that 1,25(OH)₂D₃ may also influence some hepatic metabolic patterns either directly or indirectly through its action on extracellular or intracellular calcium concentrations [13-17].

The aim of the present studies was, therefore, to examine (1) the role played by the hormone 1,25(OH)₂D₃, and/or by its associated physiological effect on extracellular calcium concentrations on the C-25 hydroxylation of D₃ in isolated rat hepatocytes; and (2) further the role played by 1,25(OH)₂D₃ and by calcium on the D₃-25 hydroxylase by incubating hepatocytes under conditions designed to change the extra- and/or intra-cellular calcium concentrations.

Portions of these studies were presented at the 1987 Annual Meeting of the American Society for Bone and Mineral Research, Indianapolis, June 1987, and published in abstract form [18].

MATERIALS AND METHODS

Animals and treatment procedures

The treatment procedures were as previously described [8]. Briefly, nursing female Sprague-Dawley rats with 14-
day-old litters (Charles River, St. Constant, Quebec, Canada) were fed *ad libitum* a D-deficient diet [19]. At weaning, male rats were housed in hanging stainless-steel wire cages and fed a D-deficient diet as well as demineralized water for 6–8 wk before being randomly assigned to oral calcium- or 1,25(OH)₂D₃-supplementation regimens. Oral calcium supplementation was achieved by supplying a 3% (w/v) solution of calcium gluconate as drinking water. Hormonal supplementation was achieved by intraperitoneal implantation of osmotic minipumps (Alza Corp., Palo Alto, CA, U.S.A.), which delivered constant infusions of 1,25(OH)₂D₃ at doses of 7 or 65 pmol/24 h (representing average doses of 33 or 309 pmol/kg/24 h) for 7 days. Control and calcium-supplemented animals were implanted with minipumps containing only the vehicle [ethanol/propylene glycol/saline, 3:13:4 (by vol)].

**Serum parameters of vitamin D depletion and of supplementation regimens**

Serum D metabolites were measured after extraction and chromatography as described earlier [20]. 25(OH)D was analysed by competitive-protein-binding assay using sheep serum in a dilution of 1:40000. 1,25(OH)₂D₃ was measured by competitive-protein-binding assay using a cytosolic receptor from bovine thymus [21]; the lower limit of sensitivity of the method was 1 pg/ml. Plasma calcium was measured by the colorimetric method of Grindler & King [22].

**Preparation of hepatocytes**

Isolated hepatocytes were prepared from the livers of fed rats by a modification of the method of Berry & Friend [23]. The liver was first washed with a Krebs–Henseleit bicarbonate buffer, pH 7.4, containing 50000 U of heparin/l, 0.25 mm-albumin (Fraction V, Sigma Chemical Co., St. Louis, MO, U.S.A.) and 5.5 mm-glucose. The liver was then perfused for 10 min with a modified Hanks’ medium containing 0.5 mm-EGTA, pH 7.4, at 37 °C (30 ml/min) under continual oxygenation. The liver was then digested with a modified Krebs buffer, containing 150000 U of collagenase/l (Worthington Biochem. Corp., Freehold, NJ, U.S.A.), 0.5 mm-glucose, 0.2 mm-albumin, pH 7.4, for 12 min at 12 ml/min. The cells were then suspended in a Krebs buffer containing 150000 U of collagenase/l, 300 mg of hyaluronidase/l, 0.2 mm-albumin, 0.5 mm-glucose, pH 7.4, 37 °C for 5 min. The cells were filtered first on a 250 μm filter and then on a 74 μm filter, and centrifuged at 250 rev./min for 3 min. The pellet was resuspended in Krebs buffer containing 6.3 mm-trypsin (Boehringer Mannheim Canada, Dorval, Quebec, Canada), 100 mg of DNAase/l (Boehringer Mannheim Canada, Dorval, Quebec, Canada), 10 mm Heps buffer, 0.2 mm-ovalbumin and 5.5 mm-glucose; after two min, 15 mg of trypsin inhibitor (Boehringer Mannheim Canada, Dorval, Quebec, Canada) was added to the cell suspension which was then centrifuged as described above. The pellet was washed twice in Swim’s 77 medium (Gibco Canada, Toronto, Ontario, Canada) before being used. Cell viability was evaluated by the Trypan Blue-exclusion test and found to be 93 ± 4%.

**Vitamin D₃-25 hydroxylase assay**

The experimental conditions for the study of the transformation of D₃ to 25(OH)D₃ in our preparation were tested for optimum temperature, substrate concentration and time of reaction to secure linear product formation. Hepatocytes [(1–1.2) × 10⁷] from rats in each of the treatment groups were pre-incubated for 5 min; 0.1 nmol [1α,25(2H)D₃] (200000 d.p.m.) (Amersham Corporation, Mississauga, Ontario, Canada) dissolved in 100 μl of 95% (v/v) ethanol, 1,2-dihydroxypropane, homologous D-deficient rat plasma, saline (1:3:8:8, by vol.) was then added, and the media incubated at 37 °C for 150 min in the presence of (1) Swim’s 77 medium with no added calcium; (2) 2.5 mm-CaCl₂ (1.9 mm-Ca²⁺); (3) 1 mm-EGTA; (4) 2 μM-Quin-2 AM (Calbiochem, San Diego, CA, U.S.A.), (5) 100 pm-1,25(OH)₂D₃ in the presence of 1.9 mm-Ca²⁺; and (6) 1 μM-calcium ionophore A23187 (Sigma Chemical Co., St. Louis, MO, U.S.A.) in the presence of 1.9 mm-Ca²⁺. The final incubation volume was 5.2 ml for all conditions, which gave a substrate concentration of 20 nM. Blank incubations were carried out under the same conditions, but in the presence of boiled hepatocytes. The reaction was stopped with ethyl acetate/toluene (9:1, v/v).

**Separation of [³H]D₃ and 25(OH)[³H]D₃**

The incubation mixture was extracted with 15 ml ethyl acetate/toluene (9:1, v/v). Radioinert D₃ (Sigma Chemical Co., St. Louis, MO, U.S.A.) (1 μg) and 25(OH)D₃ (1 μg) were added as internal standards, and the final extraction mixture was vortexed with 0.4 g of ammonium carbonate. Following centrifugation, the organic phase was collected while the aqueous phase was extracted twice more using 10 ml of ethyl acetate/methanol (9:1, v/v). The combined organic phases were evaporated under a stream of N₂.

The extracts were applied to Sephadex LH-20 columns and eluted with chloroform/n-hexane (19:31, v/v), and submitted to h.p.l.c. Separation of [³H]D₃ and 25(OH)[³H]D₃ was achieved by reverse-phase chromatography on a C-18 Vydac column (25 cm × 4.6 mm) (Mandel Scientific, Ville St-Pierre, Quebec, Canada) eluted at a flow rate of 1.5 ml/min with methanol/water (4:1, v/v) for 24 min, and methanol/water (19:1, v/v) for 20 min. In this system, 25(OH)[³H]D₃ eluted at 12 min and [³H]D₃ at 35 min with no overlap between the two compounds. Fractions were collected at 1 min intervals and counted in 10 ml Biofluor in a liquid-scintillation spectrometer (Beta LS8000, Beckman Inst., Palo Alto, CA, U.S.A.).

**Statistical analysis**

Results are presented as mean ± s.e.m. Data variance was analysed. Factorial analyses (2 × 4) of variance were also performed to compare the response of each condition in *vitro* with data obtained under basal conditions, or in the presence of CaCl₂ alone. Correlation coefficients were performed to evaluate the influence of the circulating concentrations of calcium or 1,25(OH)₂D₃ on the response of the D₃-25 hydroxylase [24].

**RESULTS**

**Parameters of vitamin D depletion and of calcium or 1,25(OH)₂D₃ supplementation**

Table I presents parameters describing the D-nutritional and hormonal status obtained before and after calcium or 1,25(OH)₂D₃ supplementation. Body weight was not found to be significantly different between
Table 1. Parameters of the vitamin D nutritional and hormonal status

Prior to treatment, all animals were depleted in vitamin D; n was 12–14 animals per treatment group. Statistically significant differences between treatment groups were analysed by analysis of variance. N.S., not significant.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Not supplemented</th>
<th>Calcium supplemented</th>
<th>1,25(OH)2D3 infused</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 mol/day x 7</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>197±9</td>
<td>183±14</td>
<td>187±18</td>
</tr>
<tr>
<td>After treatment</td>
<td>210±11</td>
<td>236±11</td>
<td>233±19</td>
</tr>
<tr>
<td>Total serum calcium (mg/dl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>6.7±0.3</td>
<td>6.1±0.2</td>
<td>6.2±0.3</td>
</tr>
<tr>
<td>After treatment</td>
<td>6.1±0.2</td>
<td>9.7±0.2</td>
<td>9.6±0.2</td>
</tr>
<tr>
<td>Serum 25(OH)D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After treatment (ng/ml) (normal values are 12–45 ng/ml)</td>
<td>0.8±0.2</td>
<td>0.3±0.1</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>Serum 1,25(OH)2D3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After treatment (pg/ml) (normal values are 80–200 pg/ml)</td>
<td>43.8±3.3</td>
<td>41.2±4.4</td>
<td>193.9±8.5</td>
</tr>
</tbody>
</table>

Fig. 1. Transformation of D3 into 25(OH)D3 obtained under basal conditions

Hepatocytes ([1–1.2]×10³) were incubated in Swim's 77 medium for 150 min in the presence of 0.1 nmol of [3H]D3 (20 nm). All animals were depleted in vitamin D before being assigned to one of the four treatment groups which were: no supplementation (0), hypocalcaemic (n = 12), calcium gluconate as drinking water × 14 days (CaGlu) normocalcaemic (n = 14), 1,25(OH)2D3 at 7 (n = 13) or 65 (n = 13) pmol/day × 7 days. Statistically significant differences between group means were analysed by a one-way analysis of variance.

Influence of CaCl2, EGTA and of Quin-2. Table 2 presents the results obtained in the presence of CaCl2, EGTA or Quin-2 in relation to results obtained under basal conditions. Addition of CaCl2 did not significantly affect the enzyme activity, with a mean increase of 10% in hypocalcaemic rats, and mean decreases of 7%, 17% and 9% in calcium-supplemented and the 7 and 65 pmol/day 1,25(OH)2D3-supplemented groups respectively [not significant (N.S.)]. Addition of EGTA to the incubation media, however, led to significant decreases in the overall enzyme activity compared with that obtained under basal conditions (P < 0.0001) with mean decreases of 16% in hypocalcaemic rats (N.S.), 40.5% in calcium-supplemented rats (P < 0.005), and 40.0% (P < 0.006) and 30.5% (P < 0.05) in the 7 and 65 pmol/day 1,25(OH)2D3-supplemented groups respectively. The effect of EGTA could be reversed by increasing amounts of CaCl2. Addition of Quin-2 in the incubation media had no significant effect on the overall enzyme activity in
Table 2. Influence of CaCl$_2$, Quin-2 and EGTA on the transformation of D$_3$ into 25(OH)D$_3$ in relation to the transformation observed in the absence of calcium in the incubation media

Prior to treatment, all animals were depleted in vitamin D; $n$ was 12–14 animals per treatment group. Hepatocytes [(1–1.2) × 10$^6$] were incubated in Swim's 77 medium for 150 min in the presence of 0.1 nmol of [H]$^3$D$_2$. The specificity of the effect of EGTA in relation to that of calcium was checked in the calcium-supplemented group; incubation in the presence of 1 mM-EGTA and of 0, 1.0 and 2.5 mM-CaCl$_2$ resulted in activity of 18.1, 25.7 and 32 fmol 25(OH)[H]$^3$D$_2$/10$^6$ hepatocytes per min respectively. Statistically significant differences between treatment groups were analysed by 2 × 4 analyses of variance. N.S., not significant.

<table>
<thead>
<tr>
<th>Conditions in vitro</th>
<th>Treatment groups . . .</th>
<th>Not supplemented</th>
<th>Calcium supplemented</th>
<th>1,25(OH)$_2$D$_3$ infused</th>
<th>Main effect of conditions in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7 pmol/day × 7</td>
<td>65 pmol/day × 7</td>
</tr>
<tr>
<td>CaCl$_2$ (1.9 mm-Ca$^{2+}$)</td>
<td></td>
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<tr>
<td>Quin-2 (2 μM)</td>
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<td></td>
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<td>EGTA* (1 mM)</td>
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<tr>
<td>Reference values (in the absence of CaCl$_2$)</td>
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</tbody>
</table>

Table 3. Influence of 1,25(OH)$_2$D$_3$ and of the calcium ionophore A23187 on the transformation of D$_3$ into 25(OH)D$_3$ in relation to the transformation observed in the presence of calcium alone in the incubation media

Prior to treatment, all animals were depleted in vitamin D; $n$ was 12–14 animals per treatment group. Hepatocytes [(1–1.2 × 10$^6$)] were incubated in Swim's 77 medium for 150 min in the presence of 0.1 nmol of [H]$^3$D$_2$. Statistically significant differences between treatment groups were analysed by 2 × 4 analyses of variance. N.S., not significant.

<table>
<thead>
<tr>
<th>Conditions in vitro</th>
<th>Treatment groups . . .</th>
<th>Not supplemented</th>
<th>Calcium supplemented</th>
<th>1,25(OH)$_2$D$_3$ infused</th>
<th>Main effect of conditions in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7 pmol/day × 7</td>
<td>65 pmol/day × 7</td>
</tr>
<tr>
<td>1,25(OH)$_2$D$_3$ (100 pm)</td>
<td></td>
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<tr>
<td>A23187 (1 μM)</td>
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<tr>
<td>Reference values (in the presence of 1.9 mm-Ca$^{2+}$)</td>
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</tbody>
</table>
Table 4. Within-group rank order of potency of the conditions used in vitro

Prior to treatment, all rats were D-depleted. Statistically significant differences between conditions in vitro were analysed by a one-way analysis of variance in each of the treatment groups. Values in parentheses represent the % mean in relation to results obtained in the presence of A23187 which were set at 100.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Rank order of potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-depleted, unsupplemented</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>EGTA ≤ 1,25(OH)(_2)D(_3) ≤ Quin-2 = Basal ≤ CaCl(_2) ≤ A23187</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>(60%) (64%) (72%) (72%) (78%) (100%)</td>
<td></td>
</tr>
<tr>
<td>D-depleted, calcium-supplemented</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>EGTA ≤ 1,25(OH)(_2)D(_3) ≤ Quin-2 ≤ CaCl(_2) = Basal ≤ A23187</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>(34%) (48%) (49%) (58%) (58%) (100%)</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)(_2)D(_3)-supplemented (7 pmol/day x 7 days)</td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>EGTA ≤ Quin-2 ≤ CaCl(_2) ≤ Basal = 1,25(OH)(_2)D(_3) ≤ A23187</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>(59%) (79%) (81%) (98%) (98%) (100%)</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)(_2)D(_3)-supplemented (65 pmol/day x 7 days)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>EGTA ≤ CaCl(_2) ≤ Basal ≤ 1,25(OH)(_2)D(_3) ≤ Quin-2 ≤ A23187</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>(56%) (73%) (80%) (91%) (96%) (100%)</td>
<td></td>
</tr>
</tbody>
</table>

Influence of 1,25(OH)\(_2\)D\(_3\) and of the calcium ionophore A23187. Table 3 presents the data obtained in the presence of CaCl\(_2\) in addition to the hormone 1,25(OH)\(_2\)D\(_3\), or the calcium ionophore A23187. Results show that addition of 1,25(OH)\(_2\)D\(_3\) in vitro did not significantly affect the overall enzyme activity in relation to the activity observed in the presence of CaCl\(_2\) alone, nor did it lead to significant differences between groups. By contrast, addition of A23187 led to highly significant increases in the overall activity of the enzyme (P < 0.0002), as well as to significant differences between groups in the magnitude of the response (P < 0.0005). Further statistical analysis of the data using multiple comparisons between treatment means revealed that the D-depleted calcium-supplemented group, with a mean increase of 87% over the activity observed in the presence of CaCl\(_2\) alone, is the group solely responsible for the overall statistically-significant increase in enzyme activity (P < 0.001), while 1,25(OH)\(_2\)D\(_3\) treatment in vitro (P < 0.001) or low serum calcium concentrations (P < 0.01) blunted the response to A23187 compared with the value obtained in D-depleted normocalcaemic rats.

Rank order of potency of conditions in vitro on the transformation of D\(_3\) into 25(OH)D\(_3\): differences within groups

Analysis of the rank order of potency of the various conditions in vitro indicated that the highest mean enzyme activity was obtained, in all treatment groups, in the presence of A23187, while the lowest mean enzyme activity was obtained, also in all treatment groups, in the presence of EGTA. The rank order of potency of the other compounds used varied from one group to another as illustrated in Table 4. Within-group comparison showed that in the two D-depleted groups, A23187 led to significantly higher enzyme activity than for all other conditions except CaCl\(_2\) in hypocalcaemic rats where no significant difference between CaCl\(_2\) alone and CaCl\(_2\) + A23187 was found. In 1,25(OH)\(_2\)D\(_3\)-supplemented rats, EGTA led to lower enzyme activity than basal conditions, 1,25(OH)\(_2\)D\(_3\), A23187, as well as Quin-2 in the 65 pmol/day group; in this group, CaCl\(_2\) also led to significantly lower enzyme activity than Quin-2 and A23187.

Influence of the circulating Ca\(^{2+}\) and 1,25(OH)\(_2\)D\(_3\) concentrations

In an attempt to evaluate further the effect of the calcium or D status on the response of the D\(_3\)-25 hydroxylase, correlation coefficients between the

Vol. 255
enzyme activity and the circulating serum calcium or 
\[ 1,25(OH)_2D_3 \] concentrations were measured. Serum 
calcium concentrations were found to be only weakly 
related to the value obtained in the presence of 
EGTA (r of -0.328, \( P < 0.1 \)), while serum 
\[ 1,25(OH)_2D_3 \] concentrations were inversely related to 
the values obtained in the presence of A23187 (r of -0.361, 
\( P < 0.05 \)). No other correlation was found to be 
statistically significant.

**DISCUSSION**

The results obtained from the present studies show 
that vitamin D-depletion was achieved in all animals 
before the start of the treatment regimens, as indicated 
by the low serum calcium and 25(OH)D concentrations. 
The results also show that oral calcium gluconate was 
successful in normalizing total serum calcium 
concentrations without significantly changing the circulating 
\[ 1,25(OH)_2D_3 \] concentrations. Administration of 
\[ 1,25(OH)_2D_3 \] at doses of 7 and 65 pmol/24 h for 7 days 
also normalized serum calcium in both groups of animals. 
Moreover, despite circulation levels of the hormone far 
above normal in the group infused with the 65 pmol/ 
24 h dose, serum calcium concentrations remained 
within the normal range, indicating that the hormone 
had no apparent toxic effect in these animals.

The results also indicate that the basal activity of the 
\[ \Delta^2 \] hydroxylase remained unaltered by the treatment 
regimens used. This observation confirms results 
obtained in vitro, as well as in hepatic microsomal 
fractons from similarly treated rats [8]. The originality 
of the studies, however, resides in the observation that 
magnitude of response to various challenges in vitro was 
greatly influenced by the conditioning in vivo of the 
animals. Indeed, while the stimulatory or inhibitory 
effects of A23187 and EGTA respectively are quite clear 
in all groups, the magnitude of the response, not only to 
these two chemicals but also to all other compounds, was 
found to differ between groups. This observation suggests 
that subtle modulations in vitro of the enzyme and/or of 
its environment are able to lead to stimulated or blunted 
responses in the presence of these agents. The results 
obtained in the presence of A23187 and EGTA suggest 
that, in all groups, calcium is required for maximal 
enzyme activity.

The inhibitory effect of EGTA on the activity of the 
enzyme does not reveal whether its action was solely 
exerted through its chelating effect on extracellular 
calcium, or whether it was mediated via a drain on 
intracellular calcium reserves. Addition of \( CaCl_2 \) alone 
to the incubation media did not, however, significantly 
stimulate the enzyme in any of the groups; this 
observation would tend to suggest that while extracellular 
calcium is needed for maximal enzyme activity, it does 
not seem to be the main determinant of its activity. On 
the one hand, the combined addition of \( CaCl_2 \) and 
A23187 led to significant increases in enzyme activity in 
the D-depleted animals, but most particularly in the D-
depleted calcium-supplemented group. This suggests 
that upward spikes in intracellular cytosolic calcium 
and/or movements of calcium from an A23187-sensitive 
pool, such as the endoplasmic reticulum [25] which is the 
site of the physiologically responsive \( \Delta^2 \) hydroxylase 
[26,27], might be necessary to stimulate the enzyme when 
the latter is in optimum condition(s) to respond (i.e. in a 
state of D-depletion, and in the presence of sufficient 
ambient calcium). On the other hand, excessive amounts 
of calcium seem to decrease rather than increase the 
activity of the \( \Delta^2 \) hydroxylase as seen from the 
tendency of \( CaCl_2 \) to increase enzyme activity in D-
depleted hypocalcaemic rats compared with the tendency 
to lower enzyme activity in all normocalcaemic animals, 
but most particularly in animals treated with 
\[ 1,25(OH)_2D_3 \]. The lack of effect of Quin-2 in most groups 
may be an indication that chelation of intracellular 
calcium resulting in a simple lowering of the resting 
cytosolic calcium is insufficient to affect enzyme activity, 
and that transient movements of intracellular calcium 
and/or calcium mobilization from external or internal 
sources are necessary to modulate enzyme activity.

The different response to \[ 1,25(OH)_2D_3 \] addition in vitro in the D-depleted and the \[ 1,25(OH)_2D_3 \]-treated 
groups is also very interesting. Indeed, it has been 
reported that \[ 1,25(OH)_2D_3 \] could act as a calcium 
ionophore in hepatocytes isolated from D-depleted rats [6]. However, contrary to the action of A23187, 
\[ 1,25(OH)_2D_3 \] failed to increase significantly the activity 
of the enzyme in D-depleted animals, whereas in 
\[ 1,25(OH)_2D_3 \] treated animals its action was similar to 
that obtained under basal conditions or in the presence of 
A23187. It is not impossible that the hormone acted as a 
competitor of \( D_3 \) in D-depleted rats, leading then to a 
decrease in enzyme activity, and that administration of 
\[ 1,25(OH)_2D_3 \] in vitro conceals further inhibition in vitro. 
Alternatively the intracellular calcium reserves, and thus 
the intracellular calcium-sensitive pool(s) may be 
different in D-depleted and \[ 1,25(OH)_2D_3 \]-treated rats, 
allowing different responses in the presence of agents 
able to mobilize calcium selectively from these pools. The 
situation may then be analogous to that for \( GH \) 
pituitary cells, where the endoplasmic reticulum has been 
shown to be insensitive to \[ 1,25(OH)_2D_3 \] despite a 
\[ 1,25(OH)_2D_3 \] -enhanced intracellular calcium response to 
thyrotropin-releasing hormone [28]. Interestingly, in 
these cells, the action of \[ 1,25(OH)_2D_3 \] was also blocked 
by EGTA. It is important to mention, however, that 
\[ 1,25(OH)_2D_3 \] administration in vitro also leads to physio-
logical and/or biochemical effects other than that of 
intracellular calcium mobilization [29-31]; some of these 
effects may also be responsible for the difference in 
response in D-depleted and \[ 1,25(OH)_2D_3 \]-treated animals 
which was observed during our studies.

These studies show, for the first time, that only D-
depleted rats seem to have a significant hepatocytic 
reserve for the C-25 hydroxylation of \( D_3 \). This reserve 
is uncovered by A23187 in animals treated with dietary 
calcium, but the response is dampened by calcium 
depression in vitro, and totally blunted by \[ 1,25(OH)_2D_3 \] 
treatment. Based on the between-, as well as the within-
group differences in response to the other conditions 
studied in vitro, it is postulated that the cellular 
mechanism(s) involved in the failure to respond to 
A23187 is (are) most likely different in hepatocytes 
obtained from D-depleted hypocalcaemic rats, and in 
hepatocytes obtained from \[ 1,25(OH)_2D_3 \]-treated animals.

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Calcium and D$_3$-25 hydroxylase

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