

# Chronic Hypocalcemia of Vitamin D Deficiency Leads to Lower Intracellular Calcium Concentrations in Rat Hepatocytes

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## Abstract

Several lines of evidence indicate that calcium deficiency is associated with cellular defects in many tissues and organs. Owing to the large *in vivo* gradient between ionized extra- and intracellular  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_i$ ), it is generally recognized that the prevailing circulating  $\text{Ca}^{2+}$  does not significantly affect resting cytosolic  $\text{Ca}^{2+}$ . To probe the consequences of hypocalcemia on  $[\text{Ca}^{2+}]_i$ , a model of chronic hypocalcemia secondary to vitamin D (D) deficiency was used. Hepatocytes were isolated from livers of hypocalcemic D-deficient, of normocalcemic  $\text{D}_3$ -repleted, or of normal control rats presenting serum  $\text{Ca}^{2+}$  of  $0.78 \pm 0.02$ ,  $1.24 \pm 0.03$ , or  $1.25 \pm 0.01$  mM, respectively ( $P < 0.0001$ ).  $[\text{Ca}^{2+}]_i$  was measured in cell couplets using the fluorescent probe Fura-2. Hepatocytes of normocalcemic  $\text{D}_3$ -repleted and of normal controls exhibited similar  $[\text{Ca}^{2+}]_i$  of  $227 \pm 10$  and  $242 \pm 9$  nM, respectively (NS), whereas those of hypocalcemic rats had significantly lower resting  $[\text{Ca}^{2+}]_i$  ( $172 \pm 10$  nM;  $P < 0.0003$ ). Stimulation of hepatocytes with the  $\alpha_1$ -adrenoreceptor agonist phenylephrine elicited increases in cytosolic  $\text{Ca}^{2+}$  leading to similar  $[\text{Ca}^{2+}]_i$  and phosphorylase *a* (a  $\text{Ca}^{2+}$ -dependent enzyme) activity in all groups but in contrast to normocalcemia, low extracellular  $\text{Ca}^{2+}$  was often accompanied by a rapid decay in the sustained phase of the  $[\text{Ca}^{2+}]_i$  response. When stimulated with the powerful hepatic mitogen epidermal growth factor (EGF), hepatocytes isolated from hypocalcemic rat livers responded with a blunted maximal  $[\text{Ca}^{2+}]_i$  of  $237.6 \pm 18.7$  compared with  $605.2 \pm 89.9$  nM ( $P < 0.0001$ ) for their normal counterparts, while the EGF-mediated DNA synthesis response was reduced by 50% by the hypocalcemic condition ( $P < 0.03$ ). Further studies on the possible mechanisms involved in the perturbed  $[\text{Ca}^{2+}]_i$  homeostasis associated with chronic hypocalcemia revealed the presence of an unchanged plasma membrane  $\text{Ca}^{2+}$  ATPase but of a significant decrease in agonist-stimulated  $\text{Ca}^{2+}$  entry as indicated using  $\text{Mn}^{2+}$  as surrogate ion ( $P < 0.03$ ). Our data, thus indicate that, in rat hepatocytes, the *in vivo* calcium status significantly affects resting  $[\text{Ca}^{2+}]_i$ , and from this we raise the hypothesis that this lower

than normal  $[\text{Ca}^{2+}]_i$  may be linked, in calcium disorders, to inappropriate cell responses mediated through the calcium signaling pathway as illustrated by the response to phenylephrine and EGF. (*J. Clin. Invest.* 1994. 93:2159–2167.) Key words: calcium regulating hormones · hepatocytes · hypocalcemia · intracellular calcium · receptor-operated calcium channel · vitamin  $\text{D}_3$

## Introduction

*In vivo*, extracellular calcium homeostasis is under the control of three major hormones: PTH, calcitonin, and  $1,25(\text{OH})_2\text{D}$ , the hormone of the vitamin D ( $\text{D}$ )<sup>1</sup> endocrine system. Collectively these hormones interplay to maintain normal extracellular ionized calcium ( $\text{Ca}^{2+}$ ) concentrations within the very narrow range of 1.15–1.35 mM. Circulating  $\text{Ca}^{2+}$  below 1.15 mM (hypocalcemia) or above 1.35 mM (hypercalcemia) is sensed as abnormal and rapidly triggers reactive responses by the calcium regulating hormones leading to physiological adaptations in organs (intestine, kidney, bone) involved in the maintenance of calcium homeostasis. Moreover, chronic hypo- or hypercalcemia has been shown to lead to cellular defects in many tissues or organs. However, the mechanisms involved and the role played by the abnormal circulating calcium concentrations in the cellular pathophysiology of these defects have not yet been clearly identified.

At the cellular level, the response to many hormones and xenobiotics is transduced by changes in cytosolic calcium concentrations ( $[\text{Ca}^{2+}]_i$ ) which first involves mobilization of intracellular pools but which is also often associated with entry of extracellular  $\text{Ca}^{2+}$  through plasma membrane channels (1, 2). Owing to the large *in vivo* concentration gradient between extra- and intracellular  $\text{Ca}^{2+}$  ( $\sim 10,000:1$ ), it is generally recognized, however, that the prevailing extracellular  $\text{Ca}^{2+}$  does not significantly affect  $[\text{Ca}^{2+}]_i$ . Nevertheless, the observation that D deficiency and/or hypocalcemia is linked with several disease states involving inadequate cellular responses prompted us to postulate that the prevailing circulating calcium might have a significant bearing on cellular events associated with signal transduction most particularly that associated with the calcium messenger system.

The aim of the present studies was, therefore, to investigate if the *in vivo* calcium status can significantly affect intracellular calcium homeostasis. This goal was achieved by studying the consequence of hypocalcemia on  $[\text{Ca}^{2+}]_i$  in rat hepatocytes, a cell type known to be responsive to several agents acting through the calcium signaling pathway but having also the ad-

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1. Abbreviation used in this paper: D; vitamin D.

vantage of not being a direct target cell for the calcium regulating hormones. Using a model of chronic hypocalcemia secondary to D deficiency, we now report that both resting and stimulated  $[Ca^{2+}]_i$  are sensitive to the endocrine calcium status as revealed by probing short-term primary culture of hepatocytes isolated from livers of rats either (a) depleted in D thus presenting associated hypocalcemia, (b) repleted in  $D_3$  in order to normalize the D endocrine status and hence calcium homeostasis, or (c) from normal control rats.

## Methods

### Animals

To evaluate the influence of the circulating  $Ca^{2+}$  on  $[Ca^{2+}]_i$ , animals were subjected to a functional calcium deprivation through D depletion as previously described (3). Briefly, nursing female Sprague-Dawley rats were fed a D-deficient diet starting at day 7 after parturition. At weaning, male rats were fed a D-deficient diet containing 0.9% elemental calcium (4) for 6–7 wk before being assigned to placebo or to  $D_3$  repletion through intraperitoneal osmotic minipumps (Alza Corp., Palo Alto, CA) delivering  $D_3$  at  $6.5 \text{ nmol}/24 \text{ h} \times 7 \text{ d}$ . At the time of minipump implantation, a loading dose of 13 nmol  $D_3$  was administered to accelerate the establishment of steady state conditions.  $D_3$ -supplemented rats were given a 0.5% calcium gluconate solution as drinking water, whereas D-depleted animals received demineralized water. In addition to the two groups mentioned above, experiments were also carried out in normal rats fed a regular rat laboratory diet and regular tap water. All protocols were carried out in accordance with the standards of ethics for animal experimentation of the Canadian Council on Animal Care and were approved by the local animal ethics committee.

Concentrations of  $Ca^{2+}$  in blood, and in culture and experimental solutions were measured with a ICA2 ionized calcium analyzer (Radiometer, Copenhagen, Denmark).

### Isolation and primary culture of hepatocytes

Hepatocytes were obtained from livers of nonfasting rats as previously described (5). The freshly isolated cells were then suspended in Krebs' buffer containing 1% albumin, 0.5 mM glucose, sequentially filtered on 250- and 74- $\mu\text{m}$  filters, and centrifuged at 250 rpm for 3 min. The hepatocyte pellet was suspended as described above, and finally washed in culture medium taking care that extracellular  $Ca^{2+}$  ( $[Ca^{2+}]_e$ ) concentrations be as close as possible to that observed in vivo in each animal. Cell viability was evaluated by the Trypan blue exclusion test. Cell yield and viability were found to be identical in preparations from normal and D-deficient animals.

### Intracellular calcium homeostasis

**Resting and stimulated calcium measurement.** Hepatocytes showing viability 90% were plated at a density of  $3.5\text{--}5 \times 10^5$  cells/ml onto collagen-coated coverslips (no. 1 circle, 22 mm diam) in Williams E medium containing 25 mM bicarbonate, 1% BSA, and the appropriate  $[Ca^{2+}]_e$  for each experimental condition at pH 7.4,  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  atmosphere. After incubation for 60 min, cells were loaded for 30 min at  $20^\circ\text{C}$  with the fluorescent probe Fura-2 AM ( $2.5\text{--}3 \mu\text{M}$ ) (Molecular Probes, Inc., Eugene, OR) in bicarbonate-free Williams E medium supplemented with 2.5% FBS and 1% BSA. Dye-loaded cells were then transferred onto the stage of an inverted microscope (Nikon Diaphot, Nikon Corp., Tokyo, Japan) equipped for epifluorescence measurement. Coverslips formed the bottom of a specially designed plastic chamber (100  $\mu\text{l}$ ) which was perfused with a  $37^\circ\text{C}$  Krebs-Henseleit solution equilibrated with  $\text{O}_2/\text{CO}_2$  (95:5, vol:vol). Hepatocytes isolated from the different rat populations were found to adhere equally well to the collagen-coated coverslips and displayed identical gross morphological features in bright-field or phase-contrast microscopy.  $[Ca^{2+}]_i$  measurements were performed in hepatocyte couplets rather than in single cells because they provide a model where epithelial cell polarity is maintained and ion movements are tightly coupled (6).

Fluorescence signals from hepatocyte couplets were obtained with a SPEX model CM1T111 dual-excitation spectrofluorometer (Rayonics Scientific Inc., Saint-Laurent, Quebec). Excitation wavelengths were 350 and 380 nm, and fluorescence emission was measured at 505 nm every 2 s. To take into account the cellular microenvironment of the hepatocyte preparations, intracellular dye calibration was always performed in situ at the end of an experiment by perfusion of ionomycin ( $10 \mu\text{M}$ ) in a solution containing 4 mM EGTA ( $R_{\text{min}}$ ), or 4 mM  $\text{CaCl}_2$  ( $R_{\text{max}}$ ). After correction for sample autofluorescence, signal ratios ( $F_{350}/F_{380}$ ) were transformed into  $[Ca^{2+}]_i$  (7), using the software supplied by SPEX. Autofluorescence, intracellular dye spectra and loading capacities were found to be equivalent in all groups, as illustrated in Fig. 1. The average signal-to-noise ratios measured at the isobestic point of 360 nm (8) were 9.92, 9.46, and 9.54 in hepatocytes of D-deficient,  $D_3$ -repleted, and normal rat livers, respectively. The presence of nonhydrolyzed dye was periodically verified by quenching with 2 mM  $\text{MnCl}_2$  and found to be negligible when compared to autofluorescence.

$[Ca^{2+}]_i$  was measured in hepatocytes during resting conditions. In addition, studies were performed in order to investigate the possible consequences of hypocalcemia on agonist-mediated  $[Ca^{2+}]_i$  responses. Thus, hepatocytes were stimulated either with the calcium mobilizing agent phenylephrine, an  $\alpha_1$ -adrenoreceptor agonist, or with EGF, a growth factor known to elicit increases in  $[Ca^{2+}]_i$ . Hepatocytes were perfused with phenylephrine or EGF (Sigma Chemical Co, St. Louis, MO) at  $37^\circ\text{C}$  in  $\text{O}_2/\text{CO}_2$  equilibrated Krebs-Henseleit buffer at saturating concentrations of  $8 \mu\text{M}$  or 8.2 nM, respectively.

**Resting  $Ca^{2+}$  ATPase.** To evaluate the activity of the cytosolic calcium pump, liver plasma membrane vesicles were prepared from frozen livers obtained from hypo- or normocalcemic rats according to Prpic *et al.* (9). The ATP-dependent  $Ca^{2+}$  transport activity was measured as previously described by Jouniaux *et al.* (10).

**Agonist-stimulated calcium ( $Mn^{2+}$ ) entry.** In an attempt to elucidate the importance of extracellular  $Ca^{2+}$  ions in the  $[Ca^{2+}]_i$  response to agonists in extracellular conditions compatible with states of hypo- or normocalcemia in vivo,  $500 \mu\text{M}$  Mn was added to the perfusion buffer of Fura-2 AM-loaded hepatocytes obtained from hypo- and normocalcemic rat livers. The  $Mn^{2+}$ -mediated fluorescence quenching of the Fura-2 signal was then monitored at the excitation wavelengths of 334 and 360 nm. The simultaneous and parallel loss of fluorescence observed at 334 and 360 nm was taken as an indicator of  $Mn^{2+}$  entry through receptor-operated, voltage-independent  $Ca^{2+}$  channel as already reported (2).

The cellular entry of  $Mn^{2+}$  mediated by  $8 \mu\text{M}$  phenylephrine, or 50 nM EGF application was evaluated by calculating the slopes induced

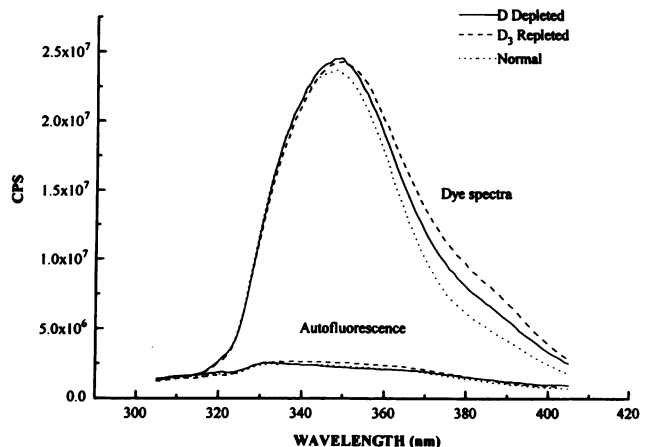


Figure 1. Representative autofluorescence, and dye spectra of Fura-2 AM-loaded hepatocytes obtained from D-deficient,  $D_3$ -repleted, or normal rat livers maintained in vitro in extracellular calcium concentrations similar to those observed in vivo. All dye calibrations were performed in situ.

by the loss of the fluorescence signal secondary to  $Mn^{2+}$  binding to the Fura-2 probe.

### Cellular responses

**Phosphorylase a activity.** Phosphorylase a activity, a calcium-dependent enzyme, was measured in order to gain insight into the functional response to phenylephrine in hypo- and normocalcemic conditions. Freshly isolated hepatocytes were diluted in Krebs-Henseleit buffer at a final density of  $9 \times 10^6$  cells/ml, and kept in extracellular  $Ca^{2+}$  concentration similar to that observed in vivo. Suspended hepatocytes were incubated for 2 min either without agonist or in the presence of 8  $\mu M$  phenylephrine. They were subsequently frozen in liquid nitrogen until ready for enzyme determination. Phosphorylase a activity was measured at 30 $^\circ$  C for 40 min as already described (11), and the reaction stopped with 10% TCA. Precipitated proteins were sedimented by centrifugation, and inorganic phosphate measured in the supernatant by the method of Fiske and Subbarow (12). One unit of phosphorylase a represents 1  $\mu mol$  of inorganic phosphate produced per minute/ $10^6$  hepatocytes.

**DNA synthesis.** The biological response to EGF was investigated by measuring its mitogenic potential in primary culture of hepatocytes obtained from hypocalcemic D-deficient or from normal rat livers. The freshly isolated hepatocytes were resuspended in Williams E medium at an ionized calcium concentration of either 0.8 (D deficient) or 1.25 mM (normals), and were supplemented with 150  $\mu g$ /liter insulin (Eli Lilly & Company, Indianapolis, IN), 4  $\mu M$  dexamethasone (Sigma Chemical Co.), 0.25  $\mu M$  pyruvate (Sigma Chemical Co.), 100 U/ml penicillin G, and 100  $\mu g$ /ml streptomycin (Gibco BRL, Burlington, Ont., Canada). They were plated in 35-mm diam dishes coated with 3  $\mu g/cm^2$  fibronectin (Sigma Chemical Co.), at a cell density of  $2.5 \times 10^5$  per dish. Cells were incubated at 37 $^\circ$ C under  $CO_2$ /air (5:95, vol/vol). After 3 h, the medium was changed to fresh Williams E medium containing 5 ng/liter insulin, 0.25  $\mu M$  pyruvate, 100 U/liter penicillin, and 100  $\mu g$ /ml streptomycin. Cells were stimulated with EGF at a concentration of 50 ng/ml.

$[^3H]$ thymidine incorporation in relation to cell protein was used to evaluate DNA synthesis. 24 h after plating, 1  $\mu Ci/ml$  [*methyl- $^3H$* ]thymidine (ICN Canada, spec act, 70–90 Ci/mmol) was added to fresh medium. DNA was precipitated 24 h later for the determination of [ $^3H$ ]thymidine incorporation according to Marceau et al. (13). Protein concentration was measured as described by Bradford (14) using crystalline BSA as standard.

Table I. Extracellular Calcium Concentrations

Parameters	Hypocalcemic D deficient	Normocalcemic		P (ANOVA)
		D <sub>3</sub> repleted	Normals	
In vivo serum ionized calcium (mM)	0.78±0.02	1.24±0.03	1.25±0.01	<0.0001
	$\overbrace{P < 0.0001}$ (between D deficient and D <sub>3</sub> repleted) $\overbrace{P < 0.0001}$ (between D deficient and Normals) $\overbrace{NS}$ (between D <sub>3</sub> repleted and Normals)			
In vitro extracellular ionized calcium (mM)	0.82±0.01	1.25±0.01	1.27±0.01	<0.0001
	$\overbrace{P < 0.0001}$ (between D deficient and D <sub>3</sub> repleted) $\overbrace{P < 0.0001}$ (between D deficient and Normals) $\overbrace{NS}$ (between D <sub>3</sub> repleted and Normals)			
Animals (n)	12	4	24	
Determinations per animal (n)	5.3±0.7	8.3±1.4	4.8±0.5	

In vivo and in vitro ionized calcium concentrations observed in the various groups of animals (in vivo), and in the solutions used during the experimental periods (in vitro). Statistically significant differences between group means were analyzed by a one-way analysis of variance; individual contrasts (bracketed P values) between group means were performed by the Tukey's test.

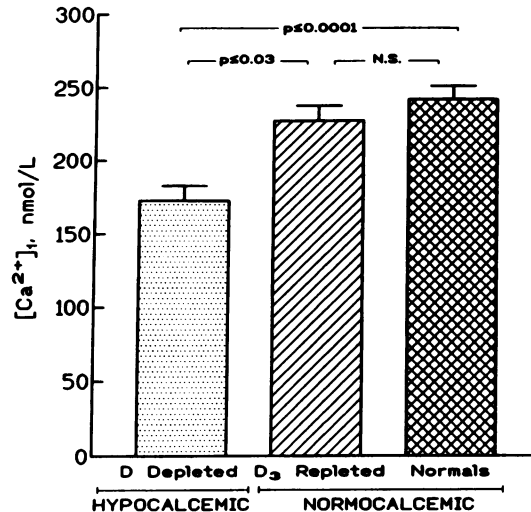


Figure 2. Resting  $[Ca^{2+}]_i$  in hepatocytes isolated from hypo- or normocalcemic rat livers. Data are presented as means±SEM. The number of animals used and number of determinations per animal are presented in Table I. Statistically significant differences between group means were analyzed by a one-way analysis of variance ( $P < 0.0003$ ) with individual contrasts (bracketed values) between group means performed by the Tukey's test.

### Statistical analysis

Data are presented as means±SEM. Statistically significant differences between group means were analyzed by analysis of variance, or by Student's *t* tests (15) as indicated in the table or figure legends.

### Results

**Extracellular calcium conditions.** The in vivo serum  $Ca^{2+}$  and in vitro extracellular  $Ca^{2+}$  concentrations used for the determination of  $[Ca^{2+}]_i$  are presented in Table I. Serum  $Ca^{2+}$  was significantly lower in D deficient rats but D<sub>3</sub> supplementation contributed to normalize circulating  $Ca^{2+}$  to concentrations similar to those observed in normal rats. In vitro, hepatocytes were maintained in an extracellular  $Ca^{2+}$  milieu similar to that observed in vivo with concentrations compatible with hypocalcemia in D deficient or with normocalcemia in D<sub>3</sub> repleted and normal rats.

Serum electrolytes, vitamin D metabolites, and hepatic enzymes in similarly treated animals have been reported previously and indicate that liver functions are normal in these animals, whereas serum 25-hydroxyvitamin D (25(OH)D) are at or near undetectable levels and 1,25(OH)<sub>2</sub>D concentrations significantly decreased (3, 16, 17). This animal model is regularly used as an experimental paradigm for human vitamin D deficiency and calcium deprivation (18–20).

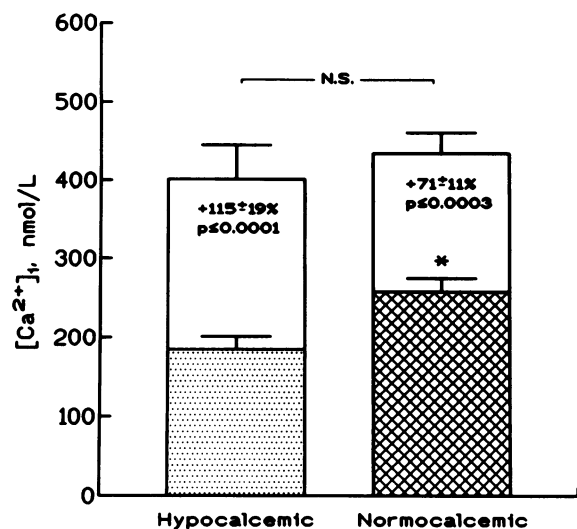
**Resting intracellular calcium concentrations.**  $[Ca^{2+}]_i$  in hepatocytes obtained from hypo- or normocalcemic rats are illustrated in Fig. 2. Hepatocytes from hypocalcemic D-deficient rats exhibited significantly lower resting  $[Ca^{2+}]_i$  (172±10 nM) than those obtained from D<sub>3</sub> repleted ( $P < 0.03$ ) or from normal rats ( $P < 0.0001$ ); no significant difference between hepatocytes of D<sub>3</sub>-repleted and normal rat livers was observed with  $[Ca^{2+}]_i$  of 227±10 and 242±9 nM, respectively. The coefficient of variation in the measurement of  $[Ca^{2+}]_i$  did not vary significantly between groups and was 25.1%, 24.2%, and 20.3% in the

D-deficient, D<sub>3</sub>-repleted, and normal rats respectively, a range compatible with that of others (21). Owing to the equivalence in basal [Ca<sup>2+</sup>]<sub>i</sub> between hepatocytes isolated from D<sub>3</sub>-repleted and normal rat livers, all subsequent studies were performed using hepatocytes obtained from normal rats as representative of the normocalcemic group.

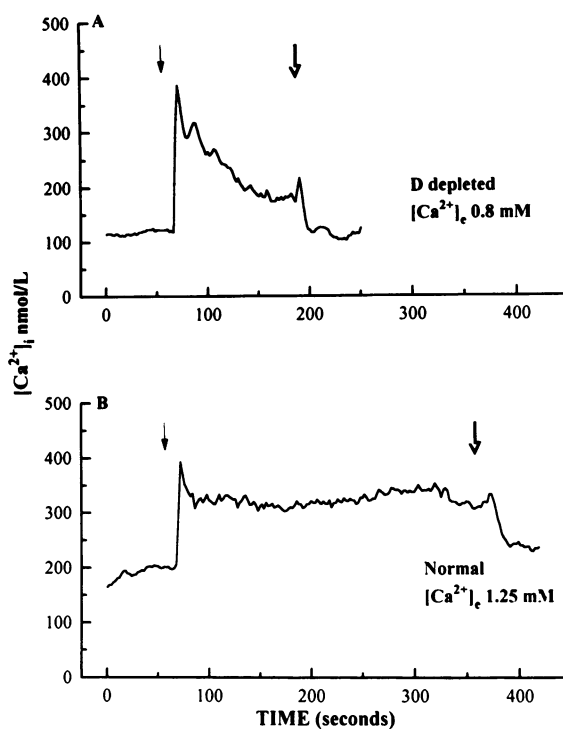
When paired hepatocytes were equilibrated in vitro in [Ca<sup>2+</sup>]<sub>e</sub> compatible with states of hypo- or normocalcemia, it was found that the in vitro extracellular Ca<sup>2+</sup> milieu did not significantly influence [Ca<sup>2+</sup>]<sub>i</sub>. In hepatocytes from normal rat livers, resting [Ca<sup>2+</sup>]<sub>i</sub> was 230±13 and 241±17 nM (*n* = 7) (NS) in the presence of extracellular Ca<sup>2+</sup> of 0.84±0.01 and 1.27±0.01 mM, respectively. Similarly, when hepatocytes of hypocalcemic rats were equilibrated in vitro in extracellular Ca<sup>2+</sup> concentrations of 0.82±0.02 or 1.24±0.05 mM, [Ca<sup>2+</sup>]<sub>i</sub> remained stable at 110±2 and 105±19 nM (*n* = 2) (NS).

**Agonist-stimulated intracellular calcium responses.** To evaluate the effect of the prevailing in vivo ionized calcium concentrations on cell responses, [Ca<sup>2+</sup>]<sub>i</sub> was evaluated in cell preparations kept in vitro at extracellular Ca<sup>2+</sup> concentrations similar to those observed in each animal.

Fig. 3 presents the mean maximum [Ca<sup>2+</sup>]<sub>i</sub> achieved after stimulation with phenylephrine while typical [Ca<sup>2+</sup>]<sub>i</sub> responses for the two groups studied are illustrated in Fig. 4. Application of 8 μM phenylephrine stimulated the mobilization of [Ca<sup>2+</sup>]<sub>i</sub> in both groups. Peak [Ca<sup>2+</sup>]<sub>i</sub> was similar in both groups reaching 401.6±43.3 (+115±19% over paired basal values, *P* < 0.0001) in hepatocytes obtained from hypocalcemic D-defi-



**Figure 3.** Peak [Ca<sup>2+</sup>]<sub>i</sub> attained after stimulation of rat hepatocyte couplets with 8 μM phenylephrine. Freshly isolated hepatocytes were kept in short-term primary culture (1 h) and maintained in vitro in extracellular Ca<sup>2+</sup> concentrations similar to those observed in vivo. Data are illustrated as basal (□, ▨), and phenylephrine-stimulated (□) values. Hepatocytes were obtained from hypocalcemic D-deficient (□, ▨; *n* = 14), or normal (▨, □; *n* = 8) rat livers. The number indicated in each column represents the mean percent increase over paired basal values for each group of animals studied. Statistically significant differences between group means were evaluated by the unpaired Student's *t* test while the increase over respective basal values was evaluated by the paired Student's *t* test. The bracketed value represents the significance in peak [Ca<sup>2+</sup>]<sub>i</sub> achieved under phenylephrine stimulation; \*Difference between the two groups in basal [Ca<sup>2+</sup>]<sub>i</sub>, *P* < 0.02.



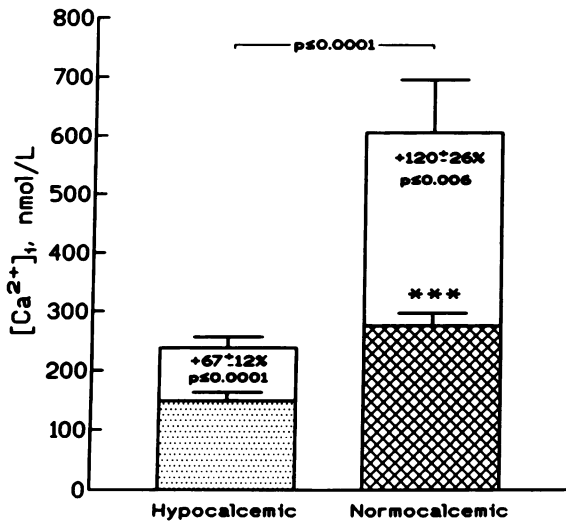
**Figure 4.** Illustration of a typical [Ca<sup>2+</sup>]<sub>i</sub> responses after stimulation with phenylephrine. Hepatocyte couplets were obtained from livers of (A) hypocalcemic D-deficient, maintained in extracellular Ca<sup>2+</sup> of 0.8 mM or (B) normal rats maintained in extracellular Ca<sup>2+</sup> of 1.25 mM. (↔) and (→) indicate the beginning and the end of phenylephrine perfusion, respectively.

cient rats, and 433.6±27.6 nM (+71±11%, *P* < 0.0003) in those obtained from normal rats (NS). In several cases (~ 50%), chronic hypocalcemia modified the pattern of response to phenylephrine whereby [Ca<sup>2+</sup>]<sub>i</sub> rapidly decayed after an initial peak as opposed to a consistently sustained elevation observed in cells from normocalcemic animals (Fig. 4).

Figs. 5 and 6 present the [Ca<sup>2+</sup>]<sub>i</sub> response after EGF stimulation in hypocalcemic D-deficient and in normal rat hepatocytes. Although EGF stimulated increases in [Ca<sup>2+</sup>]<sub>i</sub> in both groups, calcium deficiency highly influenced the response to EGF with mean [Ca<sup>2+</sup>]<sub>i</sub> peaks reaching only 237.6±18.7 (+67±12% over paired basal values, *P* < 0.0001) as compared to 605.2±89.9 nM (+120±26%, *P* < 0.006) in hepatocytes obtained from normal rat livers (*P* < 0.0001) where a sustained response was also observed during the course of EGF stimulation as illustrated in Fig. 6.

The data obtained on the evaluation of Ca<sup>2+</sup> entry using Mn<sup>2+</sup> as indicator are presented in Fig. 7 and in Table II. As illustrated in the representative traces (Fig. 7), extracellular Ca<sup>2+</sup> influenced the entry of Mn<sup>2+</sup> into hepatocytes after stimulation by both phenylephrine and EGF as indicated by the Mn<sup>2+</sup>-mediated fluorescence quenching of the Fura-2 signal. Calculation of the slopes of the decay in the Fura-2 fluorescence signal after phenylephrine or EGF exposure (Table II) shows that for both agonists Mn<sup>2+</sup> entry was significantly decreased in low [Ca<sup>2+</sup>]<sub>e</sub> compared to that observed in normal [Ca<sup>2+</sup>]<sub>e</sub> condition (*P* < 0.03).

**Biological responses.** The ATP-dependent Ca<sup>2+</sup> transport observed in liver plasma membrane vesicles of hypo- and nor-



**Figure 5.** Peak  $[Ca^{2+}]_i$  attained after stimulation of rat hepatocyte couplets with 8.2 nM EGF. Freshly isolated hepatocytes were kept in short-term primary culture (1 h) and maintained in vitro in extracellular  $Ca^{2+}$  concentrations similar to those observed in vivo. Data are illustrated as basal ( $\square$ ), and EGF-stimulated ( $\square$ ) values. Hepatocytes were obtained from hypocalcemic D-deficient ( $\square$ ), or normal ( $\square$ ) rat livers. The number indicated in each column represents the mean percent increase over paired basal values for each animal studied. Statistically significant differences between group means were evaluated by the Student's *t* test while the increase over respective basal values was evaluated by the paired Student's *t* test. The bracketed value represents the significance in peak  $[Ca^{2+}]_i$  achieved under EGF stimulation; \*\*\*Difference between the two groups in basal  $[Ca^{2+}]_i$ ,  $P < 0.0003$ .

mocalcemic rats is presented in Fig. 8. As illustrated, no significant difference in the cytoplasmic  $Ca^{2+}$  ATPase was observed between the two groups with  $K_m$  values of  $7.20 \pm 6.53$  and  $15.33 \pm 4.51$  nM while  $B_{max}$  was estimated at  $1.55 \pm 0.39$  and  $1.88 \pm 0.18$  nmol  $^{45}Ca$  per 30 s/mg protein after normal and hypocalcemic conditioning, respectively.

The basal and phenylephrine-stimulated phosphorylase *a* activities are presented in Table III. As indicated, resting phosphorylase *a* activity was similar in hepatocytes obtained from normo- and hypocalcemic rat livers. Phenylephrine increased enzyme activity in both groups by  $0.45 \pm 0.09$  ( $P < 0.042$ ) and  $0.55 \pm 0.05$  ( $P < 0.009$ )  $\mu\text{mol } P_i/\text{min per } 10^6$  hepatocytes in the hypo- and normocalcemic group, respectively, thus indicating similar stimulation of phosphorylase *a* activity above baseline in response to phenylephrine.

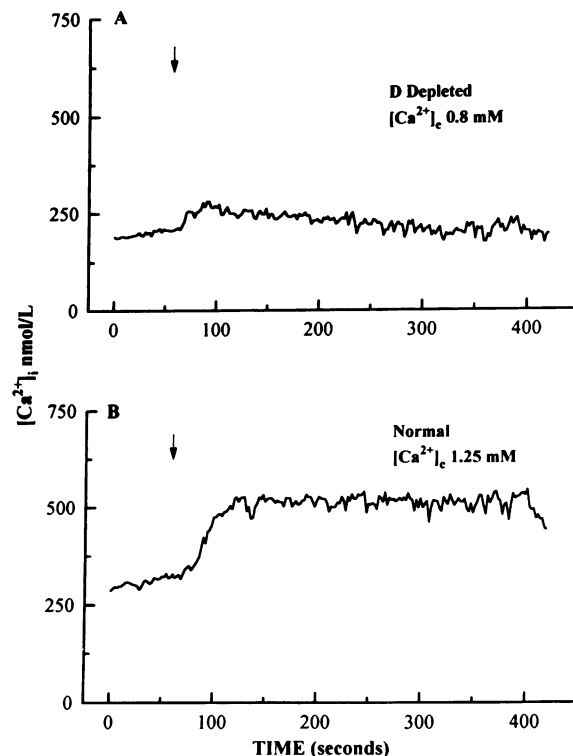
The biological response to EGF was measured by its capacity to stimulate DNA synthesis and is presented in Table IV. Hypocalcemia significantly impaired EGF-induced DNA synthesis as evidenced by a 50% decrease in  $[^3H]$ thymidine incorporation compared to the response observed in hepatocytes obtained from normocalcemic animals ( $P < 0.03$ ).

## Discussion

The data obtained during the present studies show, for the first time, that long-lasting hypocalcemia secondary to D deficiency can lead to a significant lowering of resting  $[Ca^{2+}]_i$  in rat hepatocytes maintained in vitro in extracellular  $Ca^{2+}$  concentrations similar to those observed in vivo. Correction of hypocal-

cemia by repletion with  $D_3$  (leading to normal circulating  $D_3$  metabolites as already reported [3]) contributes in normalizing both the circulating calcium and the  $[Ca^{2+}]_i$  to a level similar to that observed in normal rats. These data clearly illustrate that despite the large concentration gradient between extra- (millimolar range) and intracellular (nanomolar range)  $Ca^{2+}$ , a physiologically relevant lowering of extracellular  $Ca^{2+}$  concentration in vivo has indeed a significant impact on resting  $[Ca^{2+}]_i$ . Although surprising, others have, in fact, reported a  $K_m$  for extracellular  $Ca^{2+}$  as high as 10 mM for the plasma membrane  $Ca^{2+}$  pump (*trans* activity) (22), which suggests that, in the hypo- to normocalcemic range, substantial changes in the kinetics of  $Ca^{2+}$  movement can be expected. Interestingly, in vitro variations in extracellular  $Ca^{2+}$  do not seem to influence resting  $[Ca^{2+}]_i$  as short-term (1–2 h) equilibration of paired hepatocytes in extracellular  $Ca^{2+}$  concentrations in vitro compatible with states of hypo- or normocalcemia in vivo did not significantly influence  $[Ca^{2+}]_i$  in hepatocytes obtained from normal or D-deficient rats. This observation indicates that the in vivo calcium status is the main determinant of the resting  $[Ca^{2+}]_i$ , and may explain why acute in vitro changes in  $[Ca^{2+}]_e$  have, up to now, been reported not to influence  $[Ca^{2+}]_i$ . It is not excluded, however, that longer equilibration periods in vitro and/or the presence of active metabolic demands on the cells may be required to fully simulate the in vivo influence of extracellular  $Ca^{2+}$  on the hepatocyte.

In an attempt to investigate the mechanisms involved in the resting as well as in the suboptimal  $[Ca^{2+}]_i$  responses induced by hypocalcemia, the activity of the plasma membrane ATP-



**Figure 6.** Illustration of a typical  $[Ca^{2+}]_i$  responses after stimulation with EGF. Hepatocyte couplets were obtained from livers of (A) hypocalcemic D-deficient, maintained in extracellular  $Ca^{2+}$  of 0.8 mM or (B) normal rats, maintained in extracellular  $Ca^{2+}$  of 1.25 mM. ( $\rightarrow$ ) indicates the beginning of EGF perfusion.

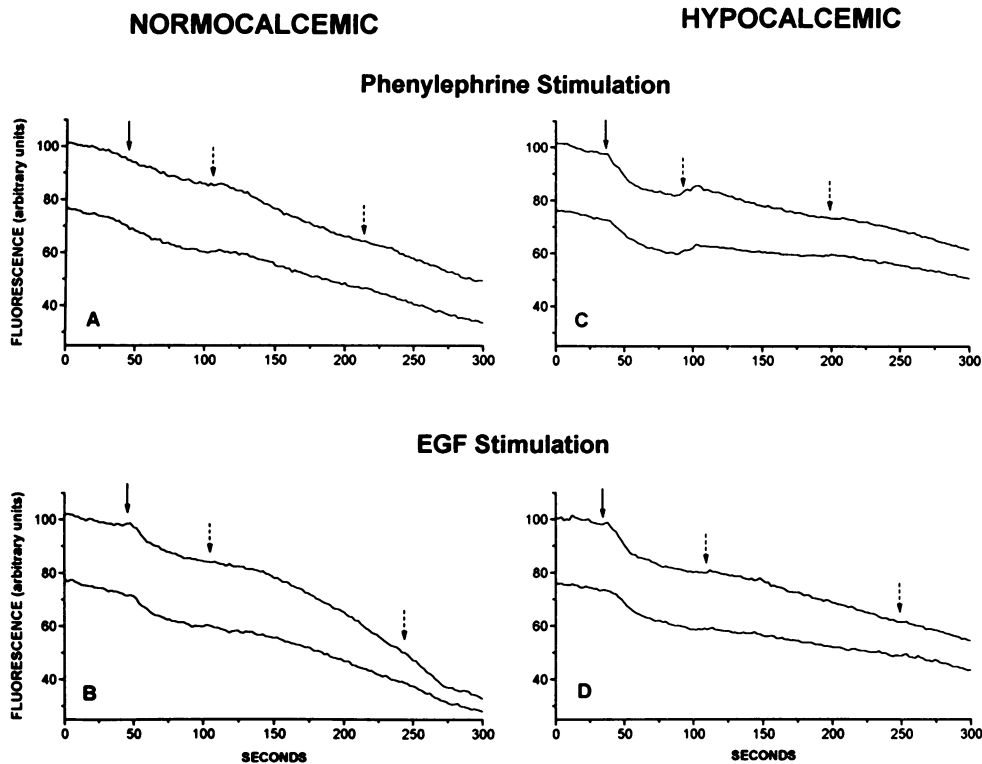


Figure 7. Representative traces of the extracellular  $Mn^{2+}$ -mediated fluorescence quenching of the Fura-2 signal evaluated at 334 (top trace) and 360 nm (bottom trace). Hepatocytes were isolated from hypo- or normocalcemic rat livers and maintained for 1 h in primary culture in an  $[Ca^{2+}]_e$  environment similar to that observed in vivo. Extracellular Mn was added at a concentration of 500  $\mu M$  as indicated by the solid arrows ( $\rightarrow$ ). Agonist application was added as indicated by the dashed arrows ( $---\rightarrow$ ).

dependent  $Ca^{2+}$  transport has been investigated. Our data indicate that the *cis*-affinity of the  $Ca^{2+}$  ATPase is in the order of 1–2 nM in hepatic membranes obtained from both normal and hypocalcemic rats, a value entirely comparable to that reported previously (23). The  $Ca^{2+}$  ATPase capacity of these membranes was also found to be unchanged by chronic hypocalcemia. These observations indicate that the cytoplasmic  $Ca^{2+}$  pump remains unchanged by hypocalcemia and cannot, therefore, be identified as responsible for the lower than normal resting  $[Ca^{2+}]_i$  in D-deficient, hypocalcemic animals. The state of the intracellular calcium pools and their involvement in the resting cytoplasmic  $Ca^{2+}$  concentration is not presently known and further studies will certainly have to address these questions

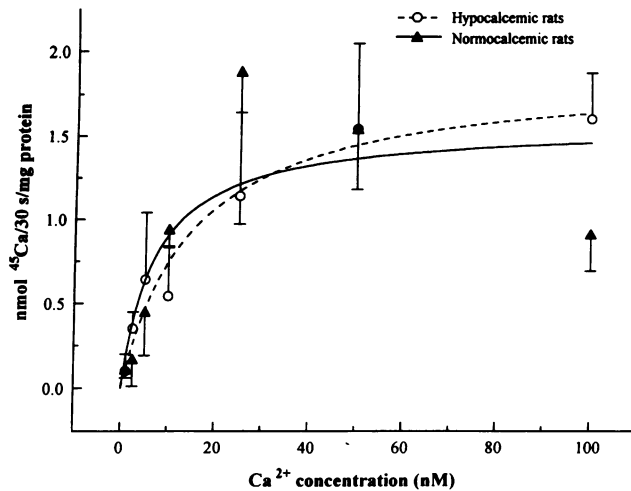
Table II. Slopes of the Extracellular  $Mn^{2+}$ -mediated Fluorescence Quenching of the Fura-2 Signal upon Agonist Stimulation

Stimulation	Group		P
	Normocalcemic (slopes)	Hypocalcemic (slopes)	
Phenylephrine (8 $\mu M$ )	$-0.2151 \pm 0.0057$	$-0.1528 \pm 0.0016$	<0.03
EGF (8.2 nM)	$-0.3729 \pm 0.0226$	$-0.1674 \pm 0.0271$	<0.03

Hepatocytes were isolated from hypo- or normocalcemic rat livers and maintained for one hour in primary culture containing  $[Ca^{2+}]_e$  similar to that observed in vivo. The Fura-2 fluorescence quenching by  $Mn^{2+}$  was obtained as mentioned in Fig. 7. Data represent mean  $\pm$  SEM, and were obtained in hepatocytes isolated from three animals. Statistically significant differences between group means were evaluated by the Student's *t* test for each agonist studied.

most particularly in the light that emptying of the intracellular calcium stores initiates an accelerated entry of extracellular  $Ca^{2+}$  into the cellular compartment (24).

The respective role of D deficiency and of its induced functional calcium deprivation in the observed lowering of resting and agonist-stimulated  $[Ca^{2+}]_i$  have not been addressed during the present studies. Indeed, the aim of our studies was to carefully evaluate in vitro the cellular consequences of the prevailing in vivo ionized calcium on the hepatocyte  $[Ca^{2+}]_i$  taking great care not to disturb the extracellular steady-state  $Ca^{2+}$  environment. It is postulated that most likely both the D and the calcium status contribute in maintaining the  $[Ca^{2+}]_i$  homeostasis in vivo. Indeed, the D endocrine system, acting through specific genomic actions, or through effects on calcium homeostasis, may be partly responsible for the observed effect on  $[Ca^{2+}]_i$  as 1,25(OH) $_2D_3$  has been reported to influence basal  $[Ca^{2+}]_i$  in vitro in the HL-60 cell line (25) and to stimulate calcium mobilization in several cell types (26–30). At the membrane level, the D endocrine system is also known to influence membrane fluidity (31), phospholipid composition (32), phosphoinositide turnover (26), several protein kinases (26, 33, 34), as well as the transcription of the inositol trisphosphate receptor gene (35), all of which may influence cellular calcium metabolism and the transduction of signals linked to  $Ca^{2+}$  mobilization. On the other hand, in other cell types, extracellular  $Ca^{2+}$  alone can influence  $[Ca^{2+}]_i$  as exemplified in keratinocytes where  $[Ca^{2+}]_i$  has been shown to be sensitive in vitro to small changes in extracellular calcium concentrations (36), and to be influenced by the state of differentiation of the cells (37). Moreover, extracellular calcium, independently of the D status, has also been reported to influence liver EGF receptor density and autophosphorylation indicating that the observed perturbed  $[Ca^{2+}]_i$  response to EGF stimulation may be partly



**Figure 8.** Hepatic ATP-dependent  $\text{Ca}^{2+}$  transport. Plasma membrane vesicles were prepared from hypo- or normocalcemic rat livers and ATP-dependent  $\text{Ca}^{2+}$  transport activity measured. Data are the mean  $\pm$  SEM of triplicate determinations. Values for  $K_m$  were  $7.20 \pm 6.5$  and  $15.33 \pm 4.51$ , nM, while  $B_{\max}$  was estimated at  $1.55 \pm 0.39$  and  $1.88 \pm 0.18$  nmol  $^{45}\text{Ca}$  per 30 s/mg protein after normal and hypocalcemic conditioning, respectively.

due to a failure in the EGF receptor transduction apparatus brought about by hypocalcemia (38). In intact animals, however, it is difficult to dissociate the effect of calcium from that of the calcium regulating hormones as they are intimately interrelated with changes in any of the parameters of calcium homeostasis triggering compensatory changes in the others. Ongoing experiments indicate, however, that the in vitro correction of the  $[\text{Ca}^{2+}]_e$  environment of hepatocytes obtained from hypocalcemic rat livers influences neither the  $[\text{Ca}^{2+}]_i$  nor the cellular response to EGF as evaluated by its capacity to induce DNA synthesis over a period of 24–48 h (unpublished data).

The short- and long-term consequences of lower than normal  $[\text{Ca}^{2+}]_i$  on cell function are not known. The question

**Table III.** Influence of the In Vivo Calcium Status on the Activity of the Hepatic Phosphorylase a

	Group		P
	Normocalcemic	Hypocalcemic	
	<i>μmol P<sub>i</sub>/min per 10<sup>6</sup> cells</i>		
Basal	0.42 $\pm$ 0.19	0.66 $\pm$ 0.25	NS
Phenylephrine-stimulated (8 $\mu\text{M}$ )	0.97 $\pm$ 0.15	1.11 $\pm$ 0.15	NS

Hepatocytes were obtained from hypocalcemic or normocalcemic rat livers and maintained in vitro at all times at  $[\text{Ca}^{2+}]_e$  similar to those observed in vivo. All determinations were done in duplicate in hepatocytes obtained from three hypo- and three normocalcemic rats. Statistically significant differences between group means (hypocalcemic vs normocalcemic) were evaluated by the Student's *t* test. Differences between basal and stimulated mean values were evaluated by the paired Student's *t* test: Hypocalcemic  $P < 0.05$ ; Normocalcemic  $P < 0.009$ .

**Table IV.** Influence of the In Vivo Extracellular Calcium Concentration on the EGF-mediated DNA Synthesis Response

	Experimental condition		P
	Normocalcemic	Hypocalcemic	
In vivo $[\text{Ca}^{2+}]_e$ (mM)	1.26 $\pm$ 0.02	0.78 $\pm$ 0.05	<0.0001
In vitro $[\text{Ca}^{2+}]_e$ (mM)	1.24 $\pm$ 0.01	0.78 $\pm$ 0.02	<0.001
$[\text{^3H}]$ thymidine incorporation (percentage of base value)	411 $\pm$ 52	206 $\pm$ 53	<0.03

Hepatocytes were obtained from hypocalcemic or normocalcemic rat livers and maintained in vitro at all times at  $[\text{Ca}^{2+}]_e$  similar to those observed in vivo. DNA synthesis was evaluated in primary hepatocyte culture. All determinations were done in duplicate in hepatocytes obtained from three hypocalcemic and six normocalcemic rats. Statistically significant differences between group means was evaluated by the Student's *t* test.

arises, however, as to the impact of calcium deficiency on the integrity of the many calcium dependent metabolic pathways, the cell secretory apparatus as well as the full integrity of the receptor-operated cell responses which are mediated through the calcium signaling cascade. The data obtained during the present studies may contribute to explain some of these observations by clearly showing that chronic hypocalcemia of D deficiency leads to abnormal responses in the intensity as well as in the pattern of the  $[\text{Ca}^{2+}]_i$  movements after stimulation with both EGF, and the  $\alpha_1$ -adrenergicreceptor agonist phenylephrine. Recent studies have, indeed, shown that hypocalcemia of D deficiency perturbs compensatory hepatic growth in vivo after two-thirds partial hepatectomy (3) while the present studies indicate that the EGF-mediated rise in  $[\text{Ca}^{2+}]_i$  as well as in DNA synthesis are greatly dampened in vitro by the in vivo hypocalcemic status. These observations reinforce the contention that several key pathways linked to intracellular calcium homeostasis may be perturbed by chronic hypocalcemia. Indeed, EGF is known to act through autophosphorylation of its membrane receptor (39) subsequently leading to changes in intracellular calcium fluxes partly through activation of phospholipase C $\gamma$  (40, 41) but also via the participation of extracellular calcium (42). Phenylephrine, on the other hand, is a classical calcium mobilizing agent acting through receptor mediated phosphatidyl inositol phosphate turnover and intracellular calcium mobilization via the  $\text{IP}_3$  responsive domains of the endoplasmic reticulum (43). Our data using  $\text{Mn}^{2+}$  as a  $\text{Ca}^{2+}$  surrogate suggest that  $[\text{Ca}^{2+}]_e$  in the hypocalcemic range is associated with a decrease in extracellular  $\text{Ca}^{2+}$  contribution to the  $[\text{Ca}^{2+}]_i$  response following both phenylephrine and EGF application. Collectively, these observations indicate that chronic hypocalcemia is associated not only with a lowering of resting  $[\text{Ca}^{2+}]_i$  but also with changes in cellular responses linked to signals needing the calcium transduction system for full activation as exemplified by the response to EGF (40, 44) and phenylephrine (45, 46).

Contrary to the hepatocyte EGF-dependent DNA synthesis, phosphorylase a activity was found to remain unchanged by hypocalcemia. This observation was not unexpected as the

activity of phosphorylase *a* is known to be very sensitive to  $[Ca^{2+}]_i$  surges. It is therefore postulated that the enzyme most likely responded to the phenylephrine-induced initial rise in cytosolic  $Ca^{2+}$  which was found to remain unchanged in hypocalcemia. Cell activity demanding more sustained calcium responses such as those involved in endogenous secretion may, on the other hand, be perturbed by hypocalcemia. Bile secretion, for example, has indeed been found to be stimulated in rats subjected to  $1,25(OH)_2D_3$  administration leading to normocalcemia when compared to animals kept in a state of D deficiency and hypocalcemia (47).

The observation that the resting  $[Ca^{2+}]_i$  is sensitive to long standing hypocalcemia in hepatocytes, a cell type not considered as a classical target for the calcium regulating hormones, also raises the possibility that other cell types may be sensitive to the endocrine calcium/vitamin D status in vivo most particularly those cells responsive to PTH,  $1,25(OH)_2D_3$ , and calcitonin. In conclusion, our data show that in unstimulated rat hepatocytes, the in vivo endocrine calcium status has a significant bearing on  $[Ca^{2+}]_i$ . This raises the possibility that a lower than normal  $[Ca^{2+}]_i$  may be linked, in calcium disorders, to inappropriate cell responses mediated through the calcium signaling pathway as illustrated, in the present studies, by the response to phenylephrine and EGF.

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