Calciotropic hormones during experimental hypocalcaemia and hypercalcaemia in spontaneously diabetic rats

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Abstract

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) concentrations have been found to be decreased in diabetic humans and rats. To investigate further the regulation of plasma Ca in diabetes, first we measured Ca²⁺, P, Mg, parathyroid hormone₁₋₃₄ (PTH), and total and free 1,25(OH)₂D₃ in male spontaneously diabetic rats 7 and 28 days after the onset of glycosuria. Secondly, we studied changes in the levels of PTH and 1,25(OH)₂D₃ in response to hypocalcaemia induced by an i.v. infusion of EGTA (2.5%, wt/vol.) for 24 h, and changes in the levels of 1,25(OH)₂D₃ in response to an i.v. infusion of rat PTH (10 µg over 24 h) without or with concomitant EGTA infusion (producing hypercalcaemia or normo/hypocalcaemia respectively), in diabetic and control rats.

Ca²⁺, P, Mg and PTH concentrations remained within the control ranges after 7 and 28 days of glycosuria; 1,25(OH)₂D₃ concentrations were decreased after 7, but not after 28, days of glycosuria. PTH concentrations showed a similar rise during EGTA-induced hypocalcae-

mia in control and diabetic rats compared with saline-infused rats, whereas 1,25(OH)₂D₃ concentrations were unchanged in both groups. Total and free 1,25(OH)₂D₃ levels were comparably (about 3-fold) increased during PTH, but not during combined PTH and EGTA infusion in control and diabetic rats. Total 1,25(OH)₂D₃ concentrations were lower in the diabetic groups infused with saline or PTH than in their respective controls, and there was a similar trend in the PTH+EGTA-infused group; free 1,25(OH)₂D₃ levels, however, were normal or increased in the diabetic groups, confirming our previous data.

The novel finding of this study is that, despite severe insulin deficiency and altered 1,25(OH)₂D₃ levels, the *in vivo* response of PTH levels to hypocalcaemia and the *in vivo* response of 1,25(OH)₂D₃ levels to PTH in diabetic rats are comparable with those found in nondiabetic rats.

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Introduction

There is a great deal of controversy about the effects of type 1 diabetes on circulating levels of calcium (Ca), phosphate (P), and the main calciotropic hormones parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). In those studies in which ionised calcium (Ca²⁺) was measured, a small but significant decrease has been documented in both diabetic children and adults (Fogh-Andersen *et al.* 1983, Saggese *et al.* 1988, Schwarz *et al.* 1992); this decrease persisted during a calcium infusion (Amado *et al.* 1987). Serum P concentrations were reported to be normal (Saggese *et al.* 1988, Schwarz *et al.* 1992), increased (Rødland *et al.* 1985) or decreased (Auwerx *et al.* 1988); intensifying the insulin therapy raised serum P levels (Raskin & Pak 1981). PTH concentrations, measured by immunoassays that detect the

intact hormone, were found to be within the normal range in diabetic adults (Schwarz *et al.* 1992, Gallacher *et al.* 1993), but a rise in PTH levels was noted after improvement of glycaemic control (Thalassinis *et al.* 1993); decreased levels of intact PTH were reported in diabetic children, with a sluggish response to a low-Ca diet (Saggese *et al.* 1988). Such 'functional hypoparathyroidism' has been attributed to hypomagnesaemia induced by increased Mg excretion (Saggese *et al.* 1988). Yet, PTH concentrations increased normally during a citrate-induced hypocalcaemic clamp in diabetic adults (Schwarz *et al.* 1992). 1,25(OH)₂D₃ concentrations were normal in insulin-treated diabetic adults (Storm *et al.* 1983, Nyomba *et al.* 1986, Auwerx *et al.* 1988, Gallacher *et al.* 1993); however, decreased levels of both total and free 1,25(OH)₂D₃ have been reported in diabetic children and adolescents (Frazer *et al.* 1981, Rødland *et al.* 1985,

Saggese *et al.* 1988) during diabetic ketoacidosis – reversible after treatment (Storm *et al.* 1983), and in severely insulin-deficient adult diabetics in central Africa (Nyomba *et al.* 1986).

In severely insulin-deficient rats, i.e. untreated spontaneously diabetic BB rats or streptozotocin (SZ)-induced diabetic rats, blood Ca^{2+} was found to be normal in BB rats (Verhaeghe 1988). Serum PTH concentrations were reported to be increased (Schedl *et al.* 1978) or normal (Romero *et al.* 1995) after 9 days of SZ-induced diabetes, but suppressed after 25 (Romero *et al.* 1995) or 48 days (Hough *et al.* 1982) of SZ-induced diabetes. Decreased $1,25(\text{OH})_2\text{D}_3$ levels have been reported repeatedly in male SZ-induced diabetic rats (Schneider *et al.* 1977, Hough *et al.* 1982, Wilson *et al.* 1982, Nyomba *et al.* 1985, Romero *et al.* 1995) and in male spontaneously diabetic rats (Nyomba *et al.* 1989, Verhaeghe *et al.* 1990, 1993); interestingly, this is not the case in female nongravid spontaneously diabetic rats (Verhaeghe *et al.* 1988, 1989, 1994). Renal cortical slices from male diabetic rats produced less $1,25(\text{OH})_2\text{D}_3$ but more $24,25(\text{OH})_2\text{D}_3$ than control rats (Wongsurawat *et al.* 1983); however, kidneys are larger relative to body weight in diabetic rats (Pillion *et al.* 1988). We produced evidence that male diabetic rats have a higher metabolic clearance rate (MCR) of $1,25(\text{OH})_2\text{D}_3$ than control rats; in contrast, their calculated *in vivo* $1,25(\text{OH})_2\text{D}_3$ production rate per body weight remained within the control range (Verhaeghe *et al.* 1993).

In this study, we investigated (1) the longitudinal changes in Ca^{2+} , P, PTH and $1,25(\text{OH})_2\text{D}_3$ in male BB rats after 7 and 28 days of diabetes, (2) changes in the levels of PTH and $1,25(\text{OH})_2\text{D}_3$ in response to hypocalcaemia induced by an EGTA infusion for 24 h, and (3) changes in the levels of $1,25(\text{OH})_2\text{D}_3$ in response to an infusion with PTH resulting in hypercalcaemia. The renal 1α -hydroxylase activity is generally considered to be regulated by four main factors: PTH (positive), $1,25(\text{OH})_2\text{D}_3$ itself, P and Ca^{2+} (negative) (Bell 1998). In addition, insulin was found to be a necessary co-factor for PTH to stimulate $1,25(\text{OH})_2\text{D}_3$ production in cultured chick kidney cells (Henry 1981). The *ex vivo* $1,25(\text{OH})_2\text{D}_3$ production by renal slices from diabetic rats was stimulated by PTH, but less so than in insulin-treated diabetic rats (Wongsurawat & Ambrecht 1985). To differentiate between the effects of PTH and Ca^{2+} on $1,25(\text{OH})_2\text{D}_3$ production (Bushinsky *et al.* 1985), both hypercalcaemic rats infused with PTH alone and normo- or hypocalcaemic rats infused with both PTH and EGTA were studied.

Materials and Methods

Animals

The experiments described below were approved by the Ethical Committee for Animal Research at Leuven

University. Male diabetic rats were obtained from the Leuven/pfd Wistar rat colony, inbred since 1983. Between 30 and 40% of these rats (38% in 1997) develop spontaneous type 1-like diabetes at a mean age of 13 weeks. Diabetes was diagnosed by checking 5 times weekly for glycosuria, which invariably corresponds to a tail blood glucose level of >300 mg/dl. After onset of diabetes, the rats were untreated unless ketonuria was detected (checked 5 times weekly with KetoDiabur sticks from Boehringer, Mannheim, Germany), in which case 1 U Ultralente MC (Novo Nordisk, Bagsvaerd, Denmark) was administered s.c.; this regime leaves the diabetic rats untreated from a biological point of view, whilst preventing deaths due to ketosis. The rats were given tap water and a rat chow containing 0.94% Ca, 0.62% P, 0.18% Mg, and 1650 IU vitamin D_3 /kg (Arie Blok, Woerden, The Netherlands) available *ad libitum*.

In Experiment 1, diabetic rats were studied 7 and 28 days after glycosuria was first detected; control rats were non-diabetic littermates. The rats were anaesthetized with pentobarbitone, 60 mg/kg intraperitoneally (i.p.), and a blood sample was drawn from the abdominal aorta. Ionised calcium was determined within 10 min after sampling; the sample was then centrifuged and plasma was stored at -20°C .

In Experiment 2, diabetic rats with a diabetes duration between 2 and 4 weeks were used for infusion experiments; control rats were non-diabetic littermates. The rats were anaesthetized with atropine 0.25 mg/kg, xylazine hydrochloride 10 mg/kg, and ketamine hydrochloride 50 mg/kg i.p. A catheter was inserted in the left jugular vein, and the rats were placed in a specially designed cage with the catheter connected to a swivel device that permitted sufficient movement of the rats within the cage to eat and drink *ad libitum*. For the first 24 h, all rats received 0.9% NaCl. For the next 24 h, the rats received either (1) 0.9% NaCl (saline-infused group), (2) ethylene glycol-bis (β -aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA, Sigma Chemical Co., St Louis, MO, USA) 2.5% (wt/vol.) in 0.9% NaCl, pH 7.4 (EGTA-infused group), (3) rat PTH (Calbiochem, La Jolla, CA, USA) 10 $\mu\text{g}/24$ h dissolved in 0.9% NaCl, pH 7.4 (ratPTH-infused group), or (4) the combination of EGTA and rat PTH in the same concentrations as above (ratPTH+EGTA-infused group). EGTA was used and not EDTA, because of its lower affinity for Mg (Weisinger *et al.* 1989). All infusions were given with a model AS-6C Syringe Pump (Auto-Syringe Inc., Londonderry TPKE, Hooksett, NH, USA), and the infusion volume was 3 ml/24 h in all rats. After 24 h, the rats were anaesthetized with pentobarbitone, and a blood sample was taken from the abdominal aorta, with the infusion still going.

For the rats to be included in Experiment 2 we proceeded as follows. Because we found in Experiment 1 that control as well as diabetic rats had Ca^{2+} levels between 1.16 and 1.33 mmol/l, we defined the normocalcaemic

Table 1 Body weight, blood ionised calcium and plasman values in male diabetic BB rats and their paired control littermates, 7 ($n=10$) and 28 ($n=12$) days after onset of glycosuria. Data are expressed as means (S.E.M.)

	7 Days		28 Days		P value (ANOVA)
	Control	Diabetic	Control	Diabetic	
Body weight (g)	380 (8)	318 (8)**	413 (22)	278 (16)**	<0.001
Blood Ca^{2+} (mmol/l)	1.26 (0.01)	1.25 (0.01)	1.25 (0.01)	1.24 (0.02)	0.64
Plasma P (mmol/l)	1.83 (0.03)	1.87 (0.03)	1.82 (0.01)	1.89 (0.02)	0.09
Plasma Mg (mmol/l)	0.71 (0.02)	0.74 (0.02)	0.68 (0.02)	0.74 (0.03)	0.09
Plasma glucose (mmol/l)	10.0 (0.3)	27.9 (0.9)**	10.6 (0.3)	32.0 (1.2)**†	<0.001
Plasma insulin (pmol/l)	731 (79)	65 (11)**	1083 (201)	84 (21)**	<0.001
Plasma PTH (pmol/l)	16.0 (2.1)	12.0 (1.2)	15.4 (2.5)	13.0 (2.8)	0.60
Plasma $1,25(\text{OH})_2\text{D}_3$ (pmol/l)	394 (39)	241 (12)*	344 (18)	307 (25)	0.001
Plasma DBP ($\mu\text{mol/l}$)	8.2 (0.2)	5.1 (0.6)**	8.5 (0.3)	5.0 (0.2)**	<0.001
Plasma free $1,25(\text{OH})_2\text{D}_3$ (index)	4.8 (0.4)	5.3 (0.7)	4.1 (0.3)	6.2 (0.5)*	0.02

* $P<0.01$, ** $P<0.001$ vs respective control; † $P<0.05$ vs diabetic 7 days.

range for the purpose of this Experiment as being between 1.16 and 1.35 mmol/l (0.20 mmol/l range). The target hypercalcaemic range for the rats infused with ratPTH was defined as a Ca^{2+} level between 1.36 and 1.60 mmol/l (0.25 mmol/l range); the hypocalcaemic range for the rats infused with EGTA was defined as a Ca^{2+} level between 0.91 and 1.15 mmol/l (0.25 mmol/l range). The response in the group infused with both EGTA and PTH was predictably variable, and all rats with a Ca^{2+} of 1.35 mmol/l or less (i.e. either normocalcaemic or hypocalcaemic) were included. Only those rats having the target Ca^{2+} values as defined entered the study, and their plasma was stored at -20°C for further analysis. Three control and six diabetic PTH-infused, as well as six control and five diabetic PTH+EGTA-infused rats were thus excluded.

Assays

Blood Ca^{2+} was measured by the ion selective electrode method on the Ciba Corning Z88 Blood Gas System (Chiron Diagnostics, Zaventem, Belgium). Plasma P was measured by colorimetry with ammonium molybdate (Daly & Ertingshausen 1972), and Mg was measured by colorimetry with xylidylblue on the BM/Hitachi Auto-Analyzer (Boehringer). Plasma glucose was measured by the glucose-oxidase method with a YSI 2300 Stat Plus glucometer (Yellow Springs, OH, USA). Insulin was measured by radioimmunoassay using a rat insulin standard (Verhaeghe *et al.* 1986). PTH was measured by an immunoradiometric assay kit that is directed against the amino-terminal region (1–34) of the rat PTH molecule (Immunotopics, San Clemente, CA, USA). The sensitivity of this assay is 1 pg/ml (0.25 pmol/l), and the intra- and interassay coefficients of variation are $\leq 4.7\%$; this assay has been validated *in vivo* in rats injected with calcium

chloride and EDTA (Rucinski *et al.* 1995). $1,25(\text{OH})_2\text{D}_3$ was measured by radioimmunoassay after HPLC purification (Bouillon *et al.* 1980); one of us (I.J.) has performed this assay since its description. The purification procedure we have used in this study was identical as described *ibidem*, but we have used an equilibrium assay rather than a non-equilibrium assay, with improved sensitivity: the specific activity of the radiolabelled $1,25(\text{OH})_2(^3\text{H})\text{D}_3$ was 180 Ci/mmol, and the antiserum was diluted at 1:100 000. As a result, less plasma is needed (0.5–0.75 ml in general). The detection limit is around 18 pg per 1 ml sample. The vitamin D-binding protein (DBP) was measured by single radial immunodiffusion (Bouillon *et al.* 1978); the 'free $1,25(\text{OH})_2\text{D}_3$ index' was calculated as the molar $1,25(\text{OH})_2\text{D}_3/\text{DBP}$ ratio $\times 10^5$.

Statistical analysis

A software program (NCSS, Kaysville, UT, USA) was used. For Experiments 1 and 2, the overall differences were examined by one-way ANOVA; if $P<0.05$, Scheffé's *post hoc* test was used to detect significant ($P<0.05$) differences between groups. Unpaired *t*-tests were then used to quantify the level of significance between these groups.

Results

Seven days after onset of glycosuria, spontaneously diabetic BB rats weighed 84% of their nondiabetic littermates, and were severely hyperglycaemic and hypo-insulinaemic; 28 days after onset of glycosuria, body weight was 67% of controls, and the diabetic rats were slightly more hyperglycaemic than diabetic rats at 7 days (Table 1). There were no significant differences in blood Ca^{2+} or plasma P and Mg levels, nor in plasma PTH concentrations. DBP

Table 2 Body weight and plasma values in infused BB rats. Data are expressed as means (S.E.M.)

	Saline		EGTA		RatPTH		RatPTH+EGTA	
	Control	Diabetic	Control	Diabetic	Control	Diabetic	Control	Diabetic
Body weight (g)	368 (21)	264 (16)*	382 (14)	271 (13)**	358 (18)	266 (17)*	386 (15)	264 (9)**
Plasma glucose (mmol/l)	7.4 (0.6)	28.8 (1.1)**	9.4 (0.4)	33.7 (1.2)**§	8.0 (0.6)	29.0 (1.6)**	9.0 (0.3)	28.0 (1.2)**
Plasma insulin (pmol/l)	851 (116)	46 (12)**	861 (203)	42 (13)**	655 (91)	45 (10)**	630 (86)	25 (9)**
Plasma DBP (µmol/l)	9.2 (0.3)	5.1 (0.4)**	9.2 (0.3)	6.1 (0.5)**	8.7 (0.4)	5.1 (0.3)**	9.0 (0.2)	5.4 (0.6)**
Plasma Mg (mmol/l)	0.59 (0.02)	0.64 (0.04)	0.62 (0.02)	0.57 (0.02)	0.69 (0.02)†	0.69 (0.03)	0.75 (0.04)†	0.71 (0.04)

Because there were no significant differences in the results of the normo- and hypocalcaemic ratPTH+EGTA-infused rats (see Fig. 1), the results of the 2 subgroups were combined. **P*<0.01, ***P*<0.001 vs respective control; †*P*<0.01 vs saline control; §*P*<0.01 vs saline diabetic and ratPTH+EGTA diabetic.

concentrations were 63 and 59% of the respective control levels in diabetic rats after 7 and 28 days of glycosuria respectively. Total 1,25(OH)₂D₃ concentrations were lower after 7, but not after 28, days of glycosuria. Consequently, the 1,25(OH)₂D₃/DBP molar ratio was normal after 7 days of glycosuria, but was slightly increased after 28 days of glycosuria.

In the infusion experiments, the diabetic groups had body weights that were significantly lower than in the respective control groups, and all diabetic groups were severely hyperglycaemic and hypo-insulinaemic; the plasma glucose level was slightly higher in the diabetic group infused with EGTA than in the diabetic groups infused with either saline or ratPTH+EGTA (Table 2). Plasma DBP levels were uniformly decreased in all diabetic groups (between 55 and 66% of the respective control values).

Blood Ca²⁺ levels are depicted in Fig. 1: all rats infused with EGTA had Ca²⁺ levels between 0.98 and 1.11 mmol/l; the rats infused with PTH had Ca²⁺ levels between 1.39 and 1.60 mmol/l; in the rats infused with PTH and EGTA, Ca²⁺ levels were between 1.17 and 1.34 mmol/l (normocalcaemic group) or between 0.83 and 1.12 mmol/l (hypocalcaemic group).

In the rats infused with EGTA and PTH, there was no significant difference in any parameter (weight, glucose, insulin, DBP, P, PTH, total and free 1,25(OH)₂D₃) other than the Ca²⁺ level between the normocalcaemic and hypocalcaemic subgroups, both in control and diabetic rats (unpaired *t*-tests, data not shown). Consequently, the results of both subgroups were combined in the subsequent analyses. The only significant difference was that Mg levels were lower (*P*=0.02) in hypocalcaemic (1.61 (0.11)) compared with normocalcaemic

Figure 1 Individual blood Ca²⁺ levels and means ± S.E.M. in the infused BB rat groups. Numbers of animals in each group are shown in parentheses. In the ratPTH+EGTA groups, hypocalcaemic (○) and normocalcaemic (■) subgroups were defined. †*P*<0.001 compared with respective saline-infused control/diabetic group.

Figure 2 Box plots of the plasma levels of phosphate (left upper panel), log₁₀ PTH (right upper panel), log₁₀ 1,25(OH)₂D₃ (left lower panel) and free 1,25(OH)₂D₃ (right lower panel) in the infused BB rat groups. Because there were no significant differences in the results of normo- and hypocalcaemic ratPTH+EGTA-infused subgroups (see Fig. 1), the results of the 2 subgroups were combined. Numbers of animals in each group are shown in parentheses. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with respective control group; †*P*<0.05, ††*P*<0.01, †††*P*<0.001 compared with respective saline-infused control/diabetic group; §§§*P*<0.001 compared with respective EGTA-infused control/diabetic group.

(2.04 (0.11)) control rats infused with PTH and EGTA; in diabetic rats, there was no significant difference (*P*=0.44).

The P levels were decreased by the EGTA infusion and by the PTH infusion compared with saline-infused rats, in both control and diabetic rats (although this was not significant in the control EGTA-infused group); the effects of EGTA and PTH infusion were not additive, however (Fig. 2). Plasma Mg levels were higher in control rats infused with PTH or PTH+EGTA (i.e. only the normocalcaemic subgroup) than in saline-infused control rats; there were no significant differences in plasma Mg in the diabetic groups versus the control groups, nor in the PTH-infused diabetic groups versus the saline-infused diabetic group (Table 2).

Because of the large variations in the individual PTH and 1,25(OH)₂D₃ concentrations (particularly in the PTH-infused groups), log corrections were used for statistical analysis and data presentation (Fig. 2). Log₁₀ PTH concentrations were increased in EGTA-infused control and diabetic groups compared with their respective saline-infused groups. As expected, the log₁₀ PTH concentrations were even higher in the control and diabetic PTH-infused groups; the log₁₀ PTH concentrations were lower in the diabetic PTH-infused group than in the control PTH-infused group, but there

was no difference between the control and diabetic PTH+EGTA-infused groups. Log₁₀ 1,25(OH)₂D₃ concentrations were increased in the PTH-infused control and diabetic groups compared with their respective saline-infused groups (mean values were increased 3.4-fold in the control group and 2.6-fold in the diabetic group), but not in the PTH+EGTA-infused groups. Log₁₀ 1,25(OH)₂D₃ concentrations were lower in the saline- and PTH-infused diabetic groups than in the respective control groups, and there was a similar trend in the PTH+EGTA-infused diabetic group compared with the respective control group (*P*=0.06). The molar 1,25(OH)₂D₃/DBP index was increased in the PTH-infused control and diabetic groups compared with their respective saline-infused groups, but again this was not observed in the PTH+EGTA-infused groups. There were no significant differences in the free 1,25(OH)₂D₃ index between the control and diabetic groups. In a multiple regression analysis of the three independent variables (log₁₀ PTH, P and Ca²⁺) that predicted log₁₀ 1,25(OH)₂D₃ concentrations (dependent variable), Ca²⁺ was a significant (*t*=5.01, *P*<0.001) predictive variable and P tended to be predictive (*t*=−1.71, *P*=0.097) in control rats; in diabetic rats, P was the only significant predictive variable (*t*=−2.41, *P*=0.02).

Discussion

This study demonstrates that there are no abnormalities in the plasma concentrations of Ca^{2+} , P and Mg, as well as PTH, in male diabetic BB rats up to 4 weeks after the onset of diabetes, whereas total $1,25(\text{OH})_2\text{D}_3$ concentrations are generally lower in the diabetic groups. Furthermore, this study shows that hypocalcaemia, induced by an infusion with EGTA, results in a similar rise in PTH concentrations in diabetic and nondiabetic rats, and that an infusion with homologous PTH results in a comparable rise in total and free $1,25(\text{OH})_2\text{D}_3$ levels in diabetic and nondiabetic rats. These results indicate that severe insulin deficiency does not hamper the *in vivo* parathyroid response to a decrement in the Ca^{2+} level, nor the renal 1α -hydroxylase activity response to PTH. Previous data reported by Wilson *et al.* (1982) and by some of us (Verhaeghe *et al.* 1988) have documented higher $1,25(\text{OH})_2\text{D}_3$ concentrations in diabetic rats with chronic dietary restriction of Ca and/or P. We have also demonstrated a normal tissue-organ response to exogenous $1,25(\text{OH})_2\text{D}_3$ in diabetic rats, i.e. the mucosal-serosal Ca transport and concentrations of calbindin- $\text{D}_{9\text{K}}$ in the duodenum, and urinary Ca excretion and concentrations of calbindin- $\text{D}_{28\text{K}}$ in the kidneys (Verhaeghe *et al.* 1993). Hence, plasma Ca regulatory mechanisms – the negative feedback by Ca^{2+} on PTH concentrations, the stimulation of $1,25(\text{OH})_2\text{D}_3$ concentrations by PTH, the stimulation of duodenal Ca absorption by $1,25(\text{OH})_2\text{D}_3$ – are functioning normally *in vivo* despite severe insulin deficiency. These data are in apparent disagreement with previous *in vitro* and *ex vivo* findings (Henry 1981, Wongsurawat & Armbrecht 1985) that the renal 1α -hydroxylase activity is insulin dependent. Further work should verify whether the down-regulation of PTH secretion by $1,25(\text{OH})_2\text{D}_3$ (Silver *et al.* 1986) is operative in insulin-deficient rats *in vivo*.

The plasma concentrations of total $1,25(\text{OH})_2\text{D}_3$ were decreased in some, but not all, diabetic groups in this study, i.e. in the saline- and ratPTH-infused diabetic groups of the infusion experiments, and in the diabetic group sampled after 7 days of glycosuria. However, normal levels were found in the EGTA-infused group, and in the diabetic group sampled after 28 days. We have no explanation for these discordant findings. In most studies that have used male diabetic rats, total $1,25(\text{OH})_2\text{D}_3$ concentrations were found to be lower than in the control group in SZ-induced diabetes (Schneider *et al.* 1977, Hough *et al.* 1982, Wilson *et al.* 1982, Nyomba *et al.* 1985, Romero *et al.* 1995) as well as in spontaneous diabetes (Nyomba *et al.* 1989, Verhaeghe *et al.* 1990, 1993). However, we have repeatedly found that $1,25(\text{OH})_2\text{D}_3$ concentrations in female nonpregnant diabetic BB rats remain within the control range, both in rats fed a standard diet and in rats fed a restricted Ca-P diet (Verhaeghe *et al.* 1988, 1989, 1994); in contrast, pregnant and lactating

diabetic rats do have lower $1,25(\text{OH})_2\text{D}_3$ concentrations (Verhaeghe *et al.* 1986, 1988, 1989). Interestingly, both male and nonpregnant female diabetic rats exhibit lower active duodenal Ca absorption (assessed by the everted gut sac technique) and concentrations of calbindin- $\text{D}_{9\text{K}}$ in duodenal mucosa (Nyomba *et al.* 1989, Verhaeghe *et al.* 1990, 1993, 1994). We have previously documented that the MCR of $1,25(\text{OH})_2\text{D}_3$ is increased in male diabetic BB rats, and shows an inverse correlation with concentrations of DBP, the main plasma binding protein of the vitamin D metabolites. This is compatible with recent findings that the clearance of $25(\text{OH})\text{D}_3$ is markedly accelerated in DBP-deficient transgenic mice (Safadi *et al.* 1999). Since male and female diabetic rats have comparably low levels of DBP, the sex difference in $1,25(\text{OH})_2\text{D}_3$ levels cannot be explained by differences in binding of $1,25(\text{OH})_2\text{D}_3$ to DBP. In addition, endogenous sex steroids appear to be unrelated to this observation, because there is no difference between plasma $1,25(\text{OH})_2\text{D}_3$ concentrations in ovariectomised diabetic and control rats, either untreated or treated with 17β -oestradiol (Verhaeghe *et al.* 1994, 1997). Further experiments should be designed to study the *in vivo* production and catabolism of $1,25(\text{OH})_2\text{D}_3$ in diabetic rats, and the regulation by sex-specific and environmental factors. In contrast to lower total $1,25(\text{OH})_2\text{D}_3$ concentrations, the free $1,25(\text{OH})_2\text{D}_3$ level either remains normal or is increased in male as well as in female diabetic rats (Nyomba *et al.* 1985, 1989, Verhaeghe *et al.* 1988, 1990, 1993, 1994). More specifically, we have found that the free $1,25(\text{OH})_2\text{D}_3$ index is normal or increased in male diabetic compared with control rats after short-term (6–10 days) (Table 1, Nyomba *et al.* 1985) and medium-term (2–4 weeks) diabetes (Table 1 and Fig. 2, Nyomba *et al.* 1989, Verhaeghe *et al.* 1993), and is normal after long-term (12 weeks) diabetes (Verhaeghe *et al.* 1990). Although the free $1,25(\text{OH})_2\text{D}_3$ concentration is generally considered to be the biologically active $1,25(\text{OH})_2\text{D}_3$ fraction, the discrepancy between normal/increased free $1,25(\text{OH})_2\text{D}_3$ and decreased duodenal Ca absorption in diabetic rats should be investigated further.

The infusion experiments with PTH and EGTA described here were modelled on the work of Bushinsky *et al.* (1985) and Weisinger *et al.* (1989), who presented data that $1,25(\text{OH})_2\text{D}_3$ concentrations correlate inversely with blood Ca^{2+} levels in chronically (24-h) infused rats. Further evidence that would support a negative regulation of $1,25(\text{OH})_2\text{D}_3$ levels by Ca^{2+} was produced by Fox (1992) in an elegant hypocalcaemic clamp experiment, and by Trechsel *et al.* (1980) who found higher $1,25(\text{OH})_2\text{D}_3$ concentrations in parathyroidectomised (PTX) rats fed a low versus normal Ca-containing diet. The present study, however, shows that $1,25(\text{OH})_2\text{D}_3$ concentrations were up-regulated in the ratPTH-infused groups, which also demonstrated higher Ca^{2+} but lower P levels. In contrast, total and free $1,25(\text{OH})_2\text{D}_3$

concentrations were not up-regulated in the ratPTH+EGTA-infused group with similar P and PTH but lower Ca^{2+} levels, which suggests either PTH resistance in the proximal tubules of the kidneys or increased $1,25(\text{OH})_2\text{D}_3$ catabolism in the latter group. Future studies should, therefore, measure the renal 1α - and 24R -hydroxylase activities in these rats. The differences between previously published results and our observations, based on a comparable experimental protocol, are difficult to explain. Factors that need to be taken into account include: the use of a radioligand versus radioreceptor assay for $1,25(\text{OH})_2\text{D}_3$; we only used parathyroid-intact rats; we used homologous PTH for all infusions. In addition, inconsistencies in some of the published data need further clarification. For example, mean serum $1,25(\text{OH})_2\text{D}_3$ concentrations varied 22-fold within a narrow $0.23 \text{ mmol/l } \text{Ca}^{2+}$ range produced by infusions with bovine (b) PTH or EGTA+bPTH in parathyroid-intact rats (Bushinsky *et al.* 1985), but varied only 9-fold within a wider $0.53 \text{ mmol/l } \text{Ca}^{2+}$ range produced by calcium chloride (CaCl_2) or EGTA infusions in ratPTH-treated PTX rats. Matsumoto *et al.* (1987) also reported alterations in $1,25(\text{OH})_2\text{D}_3$ levels that correlated negatively with variations in the Ca^{2+} level produced by CaCl_2 or EGTA infusions in bPTH-treated PTX rats; however, severely hypocalcaemic untreated PTX rats had markedly lower, not higher, $1,25(\text{OH})_2\text{D}_3$ levels than hypercalcaemic bPTH-treated PTX rats. Clearly, more data are warranted on the interactive effects of PTH, P and Ca^{2+} on the production and catabolism of $1,25(\text{OH})_2\text{D}_3$ *in vivo*.

In conclusion, the novel finding of this study is that, despite severe insulin deficiency and altered levels of DBP and total $1,25(\text{OH})_2\text{D}_3$, PTH levels increase normally during EGTA-induced hypocalcaemia in diabetic rats, and that $1,25(\text{OH})_2\text{D}_3$ levels change comparably in diabetic and control rats in response to exogenous PTH, alone or in combination with EGTA.

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