The *de novo* synthesis of numerous proteins is decreased during vitamin D₃ deficiency and is gradually restored by 1,25-dihydroxyvitamin D₃ repletion in the islets of Langerhans of rats

P-M Boulron, A Faure-Dussert and B Billaudel

Laboratoire d'Endocrinologie, Université Bordeaux I, Avenue des Facultés, 33405 Talence Cedex, France

(Requests for offprints should be addressed to B Billaudel)

Abstract

Since both the release and *de novo* biosynthesis of insulin are severely decreased by vitamin D₃ deficiency and improved by 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) repletion following a 6-h delay in the rat, the present experiments investigated the effects of vitamin D₃ deficiency on the biosynthesis of heavier molecular weight proteins using electrophoretic separation. Gel protein staining by Coomassie blue showed very different profiles for islets protein production from 4-week vitamin D₃-deficient rats compared with normal islets. The pattern was characterised by a decrease in high molecular weight proteins, concomitantly accompanied by an increase in low molecular weight proteins. This tendency was partially reversed *in vivo* by 1,25(OH)₂D₃ repletion treatment for 7 days and was evident after only 16 h of treatment.

In parallel with these *in vivo* observations, which represent a static index of islets protein production, a kinetic study was performed *in vitro* by a double-labelling method allowing us to measure the *de novo* synthesis of proteins in islets during a strong 16.7 mM glucose stimulation. Comparison of ³H and ¹⁴C labelled samples was achieved via coelectrophoresis to avoid experimental

artefacts. The study of the ratio of d.p.m. ³H/d.p.m. ¹⁴C for each molecular weight protein in islets stimulated by 16.7 mM glucose (versus basal 4.2 mM glucose) showed an increase in the height of certain peaks: 150, 130 and 8.5 kDa.

Under the same conditions, islets from 4-week vitamin D₃-deficient rats (versus normal islets) presented a large deficit of numerous newly synthesised proteins and particularly those implicated in the response to glucose stimulation. *In vitro* repletion of 1,25(OH)₂D₃ tended to reverse, at least in part, the deleterious effect of vitamin D₃ deficiency on the *de novo* protein synthesis of islets but these effects were gradual. Indeed, there was no detectable effect at 2 h incubation, but 1,25(OH)₂D₃ increased the 60 to 65 kDa, 55 kDa, and 9 to 8 kDa molecular mass proteins at 4 h, and increased the level of most newly synthesised proteins at 6 h. These data support the hypothesis of a beneficial genomic influence of 1,25(OH)₂D₃ that occurs progressively within the islets of Langerhans and which may prepare the β cells for an enhanced response to glucose stimulation.

Journal of Endocrinology (1999) **162**, 101–109

Introduction

In the rat, as in several other species, vitamin D₃ and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), its biologically active principal metabolite, play crucial roles in the maintenance of calcium homeostasis. It is generally accepted that 1,25(OH)₂D₃ acts via specific nuclear receptors, found in target organs such as intestine, bone and kidney (Norman *et al.* 1982), but also in many non-classical target tissues (Walters 1992). The rat endocrine pancreas is one of these non-classical target tissues presenting 1,25(OH)₂D₃ receptors (Johnson *et al.* 1994), even in vitamin D₃-deficient rats (Clark *et al.* 1980,

Stumpf *et al.* 1981, Ishida & Norman 1988). Several studies have demonstrated a regulatory role for 1,25(OH)₂D₃ in improving the insulin release that is dramatically reduced by vitamin D₃ deficiency (Norman *et al.* 1980, Clark *et al.* 1981, Chertow *et al.* 1983, Labriji-Mestaghanmi *et al.* 1988). 1,25(OH)₂D₃ acts, at least in part, as a steroid in numerous tissues. Indeed, specific intracellular receptors facilitate the nuclear action of 1,25(OH)₂D₃ (Pike 1985): binding to promoter sequences in the genome causing up- or down-regulation of the transcription of various genes (Minghetti & Norman 1988), activating mRNA production coding for several *de novo* synthesised proteins (Norman *et al.* 1982), and

influencing the biosynthesis of many proteins in various tissues (Verhaeghe *et al.* 1989, Brunner & De Boland 1990, Chang & Price 1991, Moulard & Hendy 1991). The genomic actions of this steroid on β cells of the endocrine pancreas have been emphasised (Norman *et al.* 1982, Faure-Dussert *et al.* 1997). 1,25(OH)₂D₃ activates the β cell insulin response to glucose *in vivo* after a delay of 3 to 20 h (Ishida *et al.* 1983, Kadowaki & Norman 1985, Cade & Norman 1987, Ozono *et al.* 1990), or after 6 h *in vitro* (Billaudel *et al.* 1990), via an improvement of calcium handling occurring after a 4-h delay (Billaudel *et al.* 1990), increasing both Ca²⁺ entry by voltage-dependent channels and Ca²⁺ mobilisation from Ca²⁺ stores (Billaudel *et al.* 1993). However, these beneficial influences of 1,25(OH)₂D₃ can be observed more rapidly (within 45 min) when the protein kinase C (PKC) pathway of the β cell is stimulated by acetylcholine (Billaudel *et al.* 1995) or by PKC activators such as phorbol esters 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Billaudel *et al.* 1997). The existence of variable delays for the actions of 1,25(OH)₂D₃ supports the hypothesis of a genomic action of the steroid on the biosynthesis of several β cell proteins implicated in the different steps of the insulin excitation-secretion coupling.

In a previous study, 1,25(OH)₂D₃ was shown to increase insulin mRNA levels (Ozono *et al.* 1990), and we also found that it can stimulate the biosynthesis of insulin and low molecular weight proteins (Bourlon *et al.* 1999). The latter protein biosynthesis studies were performed in non-denaturing and non-reducing conditions with column chromatography separation, which is better adapted for small proteins. In the present study, we used a polyacrylamide gel electrophoresis separation in order to study the biosynthesis of other proteins within the islets of Langerhans which could be influenced by vitamin D₃ deficiency and by 1,25(OH)₂D₃ *in vivo* repletion (1 day and 7 days treatments). To investigate whether 1,25(OH)₂D₃ selectively stimulated newly synthesised islet proteins, we performed double labelling experiments which were completed by a time-course study of the effects of 1,25(OH)₂D₃ *in vitro*.

Materials and Methods

Animals and isolation of islets of Langerhans

After weaning, on the 21st post-natal day, Wistar rats (CERJ, Le Genest-Saint-Isle, France) received either a normal balanced diet (AO4, UAR, Epinay sur Orge, France) or a rachitogenic diet (US Biochemical Corporation, Cleveland, OH, USA) lacking vitamin D₃ but containing low calcium (0.50% w/w) and phosphate (0.30% w/w) for 4 weeks. Rats were housed in a dark room and had free access to food and water. As previously described (Labrijn-Mestaghami *et al.* 1988, Bourlon *et al.* 1996) such a 4-week vitamin D₃ deficiency induced

rachitism, with a smaller body weight ($\times 0.3$), hypoglycaemia ($\times 0.7$), and relative hypocalcaemia ($\times 0.8$). Pancreatic islets were isolated by collagenase digestion (Lacy & Kostianovsky 1967). *In vitro* experiments using islets could then be performed after a 30-min equilibration period.

All animal experiments were carried out in accordance with the guidelines laid down by the French Ministère de l'Agriculture et du Développement Rural.

In vivo 1,25(OH)₂D₃ administration

One group of 4-week vitamin D₃-deficient rats received 1,25(OH)₂D₃ *in vivo* (Hoffman-La Roche, Basel, Switzerland) for 7 days during the last week of vitamin D₃ deficiency. This treatment consisted of 50 μ l i.p. injections of 1 μ g/kg/day 1,25(OH)₂D₃ dissolved in ethanol and 0.9% NaCl (50% v/v), for 7 days. This group of rats was called the 1,25(OH)₂D₃-replete group (+D7). Another group of deficient rats received just a single i.p. injection of 1,25(OH)₂D₃ 16 h before the experiments, and was called 1-day treated group (+D1).

In vitro 1,25(OH)₂D₃ administration

1,25(OH)₂D₃ was added directly to isolated islets of Langerhans in the incubation medium for various times from 2 to 6 h at two concentrations: either 10⁻⁸ M which was the most commonly used, or 10⁻¹² M as a control as this dose is not considered to be biologically active (Billaudel *et al.* 1990). Previous work has shown an identical level of islets insulin release for such 10⁻¹² M controls and other controls using the vehicle alone (Bourlon *et al.* 1999). The medium with freshly prepared 1,25(OH)₂D₃ was changed every 2 h in an attempt to limit 1,25(OH)₂D₃ degradation and the well known retroinhibition exerted by insulin in a closed medium (Iversen & Miles 1971). The final concentration of ethanol (the 1,25(OH)₂D₃ vehicle) in the medium was 1% v/v.

Islet incubations

Groups of 100 islets were distributed into microvials (gassed with 95% O₂-5% CO₂ to maintain pH 7.4 of the incubation medium) at 37 °C with mild shaking. The incubation medium consisted of a Krebs bicarbonate buffer (116 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 24 mM NaHCO₃) containing 0.5 mM calcium and 0.5% bovine serum albumin (fraction V, RIA grade, Sigma, Aldrich Chimie, St Quentin Fallavier, France). It was enriched by an amino acid mixture (in mM: alanine 0.1; arginine 0.1; cysteine 0.05; histidine 0.05; isoleucine 0.2; leucine 0.2; lysine 0.2; methionine 0.05; threonine 0.2; tryptophan 0.02; tyrosine 0.1; valine 0.2) to favour protein biosynthesis. Leucine was absent in the medium of islets prepared for incorporation of labelled leucine. The medium was supplemented with 8.3 mM glucose. During the last two

hours of induction islets were either stimulated with 16.7 mM glucose or were not stimulated (4.2 mM) in the presence of labelled leucine. Aliquots for the total protein content of islets were assayed by protein dye binding (Bradford 1976) before polyacrylamide gel electrophoresis.

Labelling of newly synthesised proteins

A radiolabelled amino acid, leucine, was incorporated into newly synthesised islet proteins during the last 2-h incubation in the presence of 1,25(OH)₂D₃ induction and 16.7 mM glucose stimulation. To compare the protein biosynthetic capacities of islets from different groups with rigorously similar conditions, we used a double labelling method (Drittanti *et al.* 1989), one group being labelled with 111 kBq L-[4,5-³H]leucine, the other with 37 kBq L-[U-¹⁴C]leucine (Amersham International, Amersham, Bucks, UK). After washing with a cold medium containing 5 mM non-labelled leucine to eliminate free radioactivity (not incorporated into proteins), [³H]leucine- and [¹⁴C]leucine-labelled samples were mixed in equal amounts of non-labelled proteins, and determined by total protein assay before protein separation by coelectrophoresis.

Protein separation by polyacrylamide gel electrophoresis

Samples of islets were dissolved in electrophoresis buffer (0.05 M Tris, 4% sodium-dodecyl-sulphate (SDS-Page), 12% glycerol, 15 mM dithiothreitol, 0.01% Bromophenol blue from Bio-Rad, Richmond, CA, USA), homogenised with an ultrasonic probe (Sonics & Materials Inc., Danbury, CT, USA), and heated at 100 °C for 1 min. Aliquots containing 10 µg protein were applied onto 0.3% SDS polyacrylamide gel in 3 M Tris (pH 8.45). Electrophoretic runs (Laemmli 1970) were performed using 5 to 6 h of isoelectric focusing (40 mA constant amperage).

Gel protein staining by Coomassie blue

Gels were stained in a fixative solution of 10% acetic acid, 4% formaldehyde containing 50% methanol and 0.1% Coomassie blue R-250 (Sigma, Aldrich Chimie, St Quentin Fallavier, France) for 1 h and revealed by a destaining medium containing 30% methanol and 7.5% acetic acid applied for 24 h with shaking. Gels were calibrated with molecular weight markers; standards ranged from 6.5 to 200 kDa (Bio-Rad). The different fractions were analysed by optical densitometry.

Detection of labelled proteins

Gel lanes were sliced into 2-mm fractions and dissolved in 50% H₂O₂ (Prolabo, France). The ³H and ¹⁴C radioactivities were measured in a liquid scintillator (Emulsifier Safe, Packard, Rungis, France) using a β

spectrometer (Packard-Tricarb, Rungis, France). Both ³H and ¹⁴C radioactivities could be measured on the same slice, thus proteins of the same molecular weight from the two groups of islets could be compared.

Presentation of the results from double labelling experiments

The d.p.m. measured from the [³H]leucine-labelled or [¹⁴C]leucine-labelled material within islets can vary from one experiment to another one. Thus, the electrophoretic patterns are qualitative, and cannot be quantitatively compared. However the d.p.m. ³H/d.p.m. ¹⁴C ratio of values measured together from islets of different groups, run in coelectrophoresis, allow a rigorous analysis of the variations in the amounts of newly synthesised proteins.

Results

Electrophoresis of total islet proteins

Groups of 100 islets from each group of rats were isolated, dried, and replaced in a minimal distilled water aliquot for sonication with an ultrasonic probe. As previously described (Bourlon *et al.* 1999) the total protein content of islets was not statistically different between the various groups of rats. In any case, in order to avoid the interference of any such variations, the protein content of islets groups was measured using the Bio-Rad method in order to obtain equal amounts of total proteins within each well of the electrophoresis gel. After electrophoretic migration, proteins were revealed by Coomassie blue and their molecular weights determined using standard markers as shown in Fig. 1 (St). The relative distribution of proteins in each lane was compared between islets from vitamin D₃-deficient rats (−D) and islets from normal rats (N) (Fig. 1). This showed that −D islets proteins presented a significant deficit in most of the high molecular mass proteins over 15 kDa (versus N), except around 66 kDa. The intense staining observed at 15 kDa in N islets was also observed in −D islets, whereas the lower molecular weight proteins were more intensely stained in −D islets particularly between 12 and 7 kDa. *In vivo* repletion of 1,25(OH)₂D₃, as compared with the −D lane, tended to reverse these effects of vitamin D₃ deficiency; the effect was seen as soon as the 1st day of treatment (+D1) and was more pronounced after 7 days (+D7). It enhanced the staining of most of the high molecular weight proteins over 15 kDa, did not change the staining of proteins around 15 kDa and decreased the staining of the bands around 66 and 12 to 7 kDa.

Newly synthesised proteins within islets: validation of the double labelling method in normal islets

In the present experiments, the method of coelectrophoresis which was previously applied to skeletal muscle

Figure 1 Influence of vitamin D₃ deficiency and 1,25(OH)₂D₃ treatment on the separation of total proteins from rat islets of Langerhans by electrophoresis and Coomassie blue staining. Equal amounts of total islet proteins were used from the different groups of rats. N, normal rats; -D, 4-week vitamin D₃-deficient rats; +D7 and +D1, 1,25(OH)₂D₃-replete rats *in vivo* for 7 days or 1 day respectively. Standard molecular weight markers (St) were used from 6.5 to 200 kDa. The seven lanes were run in parallel and represented one of four separate experiments.

cells (Drittanti *et al.* 1989) was adapted to isolated islets of Langerhans. Two groups of 100 islets from normal rats were incubated for 2 h in the presence of basal 4.2 mM glucose and an amino acid mixture in which leucine was either ³H or ¹⁴C labelled, for incorporation into newly synthesised proteins. After washing and sonication, ³H and ¹⁴C labelled samples, mixed in equal amounts of non-labelled protein content, were run in coelectrophoresis, thus generating rigorously identical experimental conditions. Analysis of radioactivity as a function of electrophoretic mobility (see Fig. 2A and B) showed that both groups of islets from normal rats, either labelled with ³H or ¹⁴C, presented the same electrophoretic pattern, with maximum leucine incorporation into proteins for which the molecular mass was around 60 kDa, 48 kDa and 24 kDa. Figure 2C presents the d.p.m. ³H/d.p.m. ¹⁴C ratio which showed a rather constant value with slight variations included between the two dotted lines of the Fig. 2C. These limits obtained for islets from normal rats were used as a reference for experimental variations on all the following figures presenting ratio studies.

Influence of glucose stimulation on the incorporation of radiolabelled leucine into islets

Two groups of 100 islets from normal rats were incubated for 2 h either in the presence of basal 4.2 mM glucose and

Figure 2 Electrophoresis of newly synthesised proteins within normal islets in the presence of basal 4.2 mM glucose as control, using a double labelling method. Equal amounts of total islet proteins either [³H]leucine-labelled (A) or [¹⁴C]leucine-labelled (B) were run in coelectrophoresis. The d.p.m. ³H/d.p.m. ¹⁴C ratio (C) determined the limits (dotted lines) of experimental variations as a reference for other experiments (*n*=4).

[¹⁴C]leucine labelling or in the presence of 16.7 mM glucose (providing a strong specific stimulation of bio-synthesis) in the presence of [³H]leucine labelling. As described above, equal protein samples were run in coelectrophoresis. Figure 3A showed the same variations in the d.p.m. patterns induced by glucose stimulation especially around 9 kDa and over 66 kDa. Analysis of the d.p.m. ³H/d.p.m. ¹⁴C ratio in Fig. 3B showed more precisely that glucose stimulation induced increases in several peaks of the electrophoresis pattern over the reference limits (dotted lines). This graph shows that glucose stimulation increased the neo-synthesis of some

Figure 3 Influence of a 16.7 mM glucose stimulation versus 4.2 mM basal glucose (as control) in coelectrophoresis on newly synthesised proteins in islets from normal rats (N). Classical d.p.m. patterns are shown in (A). The d.p.m. ³H/d.p.m. ¹⁴C ratio (B) shows the specific peaks of molecular weight proteins which were increased over the reference level by 16.7 mM glucose stimulation in [³H]leucine-labelled islets compared with [¹⁴C]leucine-labelled control islets (*n*=4).

³H labelled protein as compared with ¹⁴C labelled proteins of islets in basal conditions. These increments corresponded mainly to 150 kDa, 130 kDa, and 8.5 kDa, and to a lesser degree to 75 kDa, 45 kDa, 30 kDa, 22 kDa, 10 kDa and 5.5 kDa. The 8.5 kDa molecular mass species may correspond to proinsulin-like material.

Influence of vitamin D₃ deficiency on newly synthesised islet proteins

One group of 100 islets from normal rats (N) was incubated for 2 h in the presence of a 16.7 mM glucose stimulus and [³H]leucine. Another group of 100 islets from 4-week vitamin D₃-deficient rats (–D) was also incubated for 2 h in the presence of 16.7 mM glucose but with [¹⁴C]leucine. Both protein samples, mixed equally as previously described, were run in coelectrophoresis. Examination of the d.p.m. ³H/d.p.m. ¹⁴C ratio in Fig. 4

Figure 4 Influence of 4-week vitamin D₃ deficiency (–D) on newly synthesised proteins in [¹⁴C]leucine-labelled islets during a 16.7 mM glucose stimulation, as compared with [³H]leucine-labelled normal islets (N) run in coelectrophoresis. The d.p.m. ³H/d.p.m. ¹⁴C ratio shows peaks over the reference level (dotted lines), thus corresponding to the molecular weights of the proteins of normal islets which were decreased during vitamin D₃ deficiency (–D). Points under the reference correspond to increased levels of newly synthesised proteins in islets from –D islets versus N islets (note the different scales in Figs 3 and 4 in relation to the low incorporation into –D islets) (*n*=4).

showed a larger neo-synthetic activity in N islets than within –D islets since many peaks appeared over the dotted reference lines. Thus vitamin D₃ deficiency was observed to impair the neo-synthesis of many islet proteins: those of 200 to 113 kDa, 92 kDa, 65 to 60 kDa, 36 kDa, 28 kDa, 17 kDa, 12 kDa, 9 to 8 kDa and 5.5 kDa molecular mass. Some of these impairments corresponded to the proteins which are highly solicited during a glucose stimulus, particularly 150 to 130 kDa and 9 to 8 kDa, as shown by the comparison of Figs 3 and 4 (note the very different scales). The higher peaks, representing the larger differences between N and –D groups and thus the larger deficit in neo-synthesis, were around 200 kDa, 65 kDa and 8.5 kDa.

It was only for the low molecular mass range around 7 kDa that the experimental ratio curve was below the reference line, corresponding to an increment of ¹⁴C labelled material (–D) as compared with the ³H labelled normal material (N). These data suggested an increase in *de novo* biosynthesis of the corresponding small proteins in islets from vitamin D₃-deficient rats as compared with normal rats.

Time-course of the effect of 1,25(OH)₂D₃ in vitro on islets from vitamin D₃-deficient rats

Three different periods of 1,25(OH)₂D₃ induction were studied (2 h, 4 h, 6 h) in three separate experiments. Groups of 100 islets from vitamin D₃-deficient rats

received either 10^{-8} M 1,25(OH)₂D₃ and [³H]leucine, or 10^{-12} M 1,25(OH)₂D₃ as control with [¹⁴C]leucine added directly to the incubation medium. Then, equivalent amounts of islet proteins were treated as previously described before coelectrophoresis. The 2-h induction study was performed in the presence of 16.7 mM glucose, 0.5 mM calcium and labelled leucine; the 4-h and 6-h induction studies were performed in the presence of 8.3 mM glucose for the first hours, followed by a 16.7 mM glucose stimulation for the last 2 h for the incorporation of labelled leucine as above.

The main observation as shown in Fig. 5 was that the influence of 10^{-8} M 1,25(OH)₂D₃ induction was gradual. Indeed, no influence of 1,25(OH)₂D₃ could be seen at 2 h since the radioactivity ratios stayed within the reference limits (Fig. 5A). Some newly synthesised proteins appeared after a 4-h induction with 10^{-8} M 1,25(OH)₂D₃ as shown by the d.p.m.³H/d.p.m.¹⁴C peaks over the reference (Fig. 5B) for 60 to 65 kDa, 55 kDa and 9 to 8 kDa molecular masses. On the other hand 10^{-8} M 1,25(OH)₂D₃ decreased the neo-synthesis of 7 kDa proteins, the ratio of which dropped below the reference curve. After 6 h of 10^{-8} M 1,25(OH)₂D₃ induction the activation of protein neo-synthesis was more pronounced and involved more numerous proteins. Indeed the d.p.m.³H/d.p.m.¹⁴C ratio (Fig. 5C) showed many peaks appearing over the reference curve: 120 to 110 kDa, 92 kDa, 70 kDa, 60 to 56 kDa, 40 kDa, 35 kDa, 27 kDa to 25 kDa, 22 kDa, 15 kDa, 12 to 8 kDa and 5.5 kDa molecular mass. The radioactivity ratio was below the reference level for 7 to 6.5 kDa molecular mass species suggesting a negative regulatory effect of 10^{-8} M 1,25(OH)₂D₃ on the biosynthesis of these small molecular mass proteins (which are increased during vitamin D₃ deficiency). This effect was more pronounced at 6 h than at 4 h. A control experiment was performed with 10^{-8} M 1,25(OH)₂D₃ for 4 h in the presence or not of $5 \cdot 10^{-4}$ M cycloheximide (Fig. 6). This protein synthesis inhibitor almost completely suppressed the incorporation of labelled leucine into the islets, thus confirming that 1,25(OH)₂D₃ influenced the neo-synthesis of proteins induced during a glucose stimulus.

Discussion

There is evidence that in the islets of Langerhans which contain 1,25(OH)₂D₃ receptors, 1,25(OH)₂D₃ may act, at least in part, as a steroid via a nuclear mechanism rendering the β cell more competent as concerns its insulin response to glucose. Indeed, in previous studies we found that the beneficial influence of 1,25(OH)₂D₃ on insulin release is only seen when the β cells are stimulated and not in basal conditions. It is observable only after 6 h of induction (Billaudel *et al.* 1990) and it cannot occur in the presence of cycloheximide, a transcriptional inhibitor (Bourlon *et al.*

Figure 5 Time-course study of 1,25(OH)₂D₃ effects (1,25) added *in vitro* on newly synthesised proteins relative to proteins of islets from vitamin D₃-deficient rats (–D). The [³H]leucine-labelled islets during a 16.7 mM glucose stimulation correspond to various 10^{-8} M 1,25(OH)₂D₃ induction times: (A) 2 h, (B) 4 h, (C) 6 h. Aliquots were each run in coelectrophoresis with [¹⁴C]leucine-labelled control islets from vitamin D₃-deficient rats. The d.p.m.³H/d.p.m.¹⁴C ratio shows peaks corresponding to the molecular weight of proteins the neo-synthesis of which was increased by 1,25(OH)₂D₃ ($n=4$ for each time point studied).

1999). Moreover, in this recent study, we show that the total islets proteins is not statistically modified by vitamin D₃ deficiency or by 1,25(OH)₂D₃, whereas the amount of newly synthesised labelled proteins during a glucose stimulation exhibits some variation. The amount of tritiated tyrosine incorporated into the total islet proteins during a glucose stimulation is decreased during vitamin

Figure 6 Influence of a protein synthesis inhibitor, cycloheximide (cyclo, $5 \cdot 10^{-4}$ M) on the neo-synthesis of proteins induced by 10^{-8} M 1,25(OH)₂D₃ (+1,25) in vitamin D₃-deficient islets. Both d.p.m. patterns were obtained during a 2 h glucose stimulation performed after 2 h induction, corresponding to the 4 h induction experiment *in vitro* as in Fig. 5B. Islets incubated in the presence of cycloheximide were labelled by [¹⁴C]leucine versus islets without cycloheximide which were [³H]leucine labelled ($n=4$).

D₃ deficiency and can be re-activated by 1,25(OH)₂D₃ induction (Bourlon *et al.* 1999). The present study (Fig. 3) showed the numerous proteins required for insulin synthesis, maturation, storage and/or exocytosis implicated in β cell stimulation-secretion coupling by glucose. The strong specific stimulation exerted by 16.7 mM glucose increased the neo-synthesis of several proteins in islets in agreement with similar findings using insulin secretory granules (Guest *et al.* 1991): this involved two groups of proteins over 98 kDa and a group of proteins with a molecular mass around 9 kDa.

The present experiments, examining vitamin D₃ deficiency and 1,25(OH)₂D₃ repletion, also revealed the implication of different proteins after migration by two methods. The first, using Coomassie blue staining for total islets proteins gives an idea of the global static state of protein synthesis without glucose stimulation. The second technique used double-labelling coelectrophoresis to compare newly synthesised proteins from two groups of islets during a glucose stimulation. Since the two experimental groups of labelled islets were run together, any external artefactual interference on protein migration can be discarded. So these comparative experiments showed specific variations in the amount of newly synthesised proteins *in vitro* during a glucose stimulation. Since these effects disappeared in the presence of cycloheximide, an inhibitor of protein synthesis, the increase in protein labelling was the result of an increment in biosynthesis.

These coelectrophoresis experiments showed that vitamin D₃ deficiency considerably altered the patterns of

protein migration on gel electrophoresis, decreasing the amount of most islet proteins, especially the heaviest proteins and enhancing certain low molecular weight proteins particularly around 7 kDa. The latter method with N and -D labelled islets in a coelectrophoresis excluded any artefact of an experimental degradation of heavy proteins into smaller fragments.

The *in vivo* administration of 1,25(OH)₂D₃ tended to reverse the influence of 4 weeks of vitamin D₃ deficiency on the relative distribution between heavy and low proteins, increasing the amount of heavier molecular weight proteins and lowering that of small molecular weight proteins, as revealed by Coomassie blue. Similar to this static observation, the kinetic study of the *in vitro* influence of 1,25(OH)₂D₃ on labelled islet proteins, which were thus newly synthesised during a glucose stimulation, demonstrated that this effect was progressive. It was detectable *in vitro* after either a 4 h or a 6 h induction period, but not as early as 2 h. Indeed, during the first 2 h of 1,25(OH)₂D₃ induction, the neo-synthesis of islet proteins was either not affected by the steroid, or was not detectable. However, this observation does not exclude rapid or intermediate effects of 1,25(OH)₂D₃ that may be likely to involve both membrane-initiated rapid actions and transcriptional effects on early genes that do not require the nuclear receptor, such as in osteoblasts (Farach Carson & Ridall 1998) or in islets (Billaudel *et al.* 1995, 1997).

During the later periods of 1,25(OH)₂D₃ induction, the steroid progressively increased the *de novo* synthesis of numerous islet proteins, some of these being already activated at 4 h (60 to 65 kDa, 55 kDa, 9 to 8 kDa). Among the numerous proteins whose synthesis is activated by 1,25(OH)₂D₃ in the islets of Langerhans, some of them (presenting an equivalent molecular weight) were also found to be increased by 1,25(OH)₂D₃ in skeletal muscle cells: a glycoprotein of 55 kDa molecular mass and several calcium binding components of 100, 40, 17 and 9 kDa (Drittanti *et al.* 1989); others such as those of 17, 20, 30, 38, 89 kDa were also found to be PKC substrates in islets (Howell 1994). Among the proteins whose synthesis is increased by the glucose stimulus some of them such as those with molecular masses of 150 to 130 kDa and 8.5 kDa were observed to be decreased during vitamin D₃ deficiency and re-activated by 1,25(OH)₂D₃. Thus, these proteins may play a crucial role in the events implicated in transcription or transduction or during the process of insulin exocytosis. The 1,25(OH)₂D₃-induced neo-synthesis of 8.5 kDa proteins may be proinsulin-like materials, but the experimental conditions used during the electrophoresis process did not allow the detection of insulin (6 kDa). In fact, the denaturing and reducing conditions are not adapted for proteins containing disulphide bridges, such as insulin, since the two A and B chains of insulin may be separated. However, in a previous column chromatography study we showed that

1,25(OH)₂D₃ increases both the amount of newly synthesised insulin and proinsulin-like materials, accelerating more particularly the neo-conversion of proinsulin into insulin when the β cell is highly solicited by glucose (Bourlon *et al.* 1999).

In conclusion, the present data lend support to the hypothesis of genomic effects of 1,25(OH)₂D₃ on islets from vitamin D₃-deficient rats, in agreement with observations on the 1,25(OH)₂D₃-induced increase in proinsulin mRNA found by other authors (Ozono *et al.* 1990), but this is the first time that these beneficial effects were shown to occur gradually, and on numerous neo-synthesised proteins which may prepare the β cells for an enhanced insulin response to glucose.

Acknowledgements

We thank Drs Kaiser and Fisher (Hoffman-La Roche, Basel, Switzerland) for their generous gift of 1,25(OH)₂D₃ and Dr T Durkin for correcting the English. This study was supported by grants from Fondation pour la Recherche Médicale and Conseil Régional d'Aquitaine.

References

- Billaudel BJL, Faure AG & Sutter BCHJ 1990 Effect of 1,25-dihydroxyvitamin D₃ on isolated islets from vitamin D₃-deprived rats. *American Journal of Physiology* **258** E643–E648.
- Billaudel BJL, Delbancut PA, Sutter BCHJ & Faure AG 1993 Stimulatory effect of 1,25-dihydroxyvitamin D₃ on calcium handling and insulin secretion by islets from vitamin D₃-deficient rats. *Steroids* **58** 335–341.
- Billaudel BJL, Bourlon P-MD, Sutter BCHJ & Faure-Dussert A 1995 Regulatory role of 1,25-dihydroxyvitamin D₃ on insulin release and calcium handling via the phospholipid pathway in islets from vitamin D-deficient rats. *Journal of Endocrinological Investigation* **18** 673–682.
- Billaudel B, Bourlon P-M, Sutter BCHJ & Faure-Dussert A 1997 1,25-Dihydroxyvitamin D₃ (1,25-(OH)₂D₃) improves islets insulin via signal transduction pathways. In *Vitamin D: Chemistry, Biology and Clinical Applications of the Steroid Hormone*, pp 391–392. Eds AW Norman, R Bouillon & M Thomasset. Riverside, California: University of California.
- Bourlon P-M, Faure-Dussert A, Billaudel B, Sutter BCHJ, Tramu G & Thomasset M 1996 Relationship between calbindin D28K levels in the A and B cells of the rat endocrine pancreas and the secretion of insulin and glucagon: influence of vitamin D₃ and 1,25-dihydroxyvitamin D₃. *Journal of Endocrinology* **148** 223–232.
- Bourlon P-MD, Billaudel BJL & Faure-Dussert A 1999 Influence of vitamin D₃ deficiency and 1,25(OH)₂D₃ on *de novo* insulin biosynthesis in the islets of the rat endocrine pancreas. *Journal of Endocrinology* **160** 87–95.
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254.
- Brunner A & De Boland AR 1990 1,25-Dihydroxyvitamin D₃ affects the synthesis, phosphorylation and *in vitro* calmodulin binding of myoblast cytoskeletal proteins. *Zeitschrift für Naturforschung* **45** 1156–1160.
- Cade C & Norman AW 1987 Rapid normalization/stimulation by 1,25-dihydroxyvitamin D₃ of insulin secretion and glucose tolerance in the vitamin D-deficient rat. *Endocrinology* **120** 1490–1497.
- Chang PL & Price CW 1991 1- α ,25-Dihydroxyvitamin D₃ stimulates synthesis and secretion of nonphosphorylated osteopontin (secreted phosphoprotein 1) in mouse JB6 epidermal cells. *Cancer Research* **51** 2144–2150.
- Chertow BS, Sivitz WI, Baranetsky NG, Clark SA, Waite A & Deluca HF 1983 Cellular mechanisms of insulin release: the effects of vitamin D deficiency and repletion on rat insulin secretion. *Endocrinology* **113** 1511–1517.
- Clark SA, Stumpf WE, Sar M, Deluca HF & Tanaka Y 1980 Target cells for 1,25-dihydroxyvitamin D₃ in the pancreas. *Cellular Tissue Research* **209** 515–520.
- Clark SA, Stumpf WE & Sar M 1981 Effect of 1,25-dihydroxyvitamin D₃ on insulin secretion. *Diabetes* **30** 382–386.
- Drittanti LN, Boland RL & De Boland AR 1989 Induction of specific proteins in cultured skeletal muscle cells by 1,25-dihydroxyvitamin D₃. *Biochimica et Biophysica Acta* **1012** 16–23.
- Farach Carson MC & Ridall AL 1998 Dual 1,25-dihydroxyvitamin D₃ signal response pathways in osteoblasts: cross-talk between genomic and membrane-initiated pathways. *American Journal of Kidney Disease* **31** 729–742.
- Faure-Dussert AG, Delbancut APA & Billaudel BJL 1997 Low extracellular calcium enhances β cell sensitivity to the stimulatory influence of 1,25-dihydroxyvitamin D₃ on insulin release by islets from vitamin D₃-deficient rats. *Steroids* **62** 554–562.
- Guest PC, Baylies EM, Rutherford NG & Hutton JC 1991 Insulin secretory granule biogenesis. Co-ordinate regulation of the biosynthesis of the majority of constituent proteins. *Biochemical Journal* **274** 73–78.
- Howell S 1994 Regulation of insulin secretion: the role of second messengers. *Diabetologia* **37** S30–S35.
- Ishida H & Norman AW 1988 Demonstration of a high affinity receptor for 1,25-dihydroxyvitamin D₃ in rat pancreas. *Molecular and Cellular Endocrinology* **60** 109–117.
- Ishida H, Seino Y, Seino S, Tsuda K, Takemura J, Nishi S, Ishizuka S & Imura H 1983 Effect of 1,25-dihydroxyvitamin D₃ on pancreatic B and D cell function. *Life Science* **33** 1779–1786.
- Iversen J & Miles DW 1971 Evidence of a feedback inhibition of insulin on insulin secretion in the isolated perfused canine pancreas. *Diabetes* **20** 1–9.
- Johnson JA, Grande JP, Roche PC & Kumar R 1994 Immuno-histochemical localization for the 1,25(OH)₂D₃ receptor and calbindin D_{28K} in human and rat pancreas. *American Journal of Physiology* **267** E356–E360.
- Kadowaki S & Norman AW 1985 Time course study of the insulin secretion after 1,25-dihydroxyvitamin D₃ administration. *Endocrinology* **117** 1765–1771.
- Labrijn-Mestaghanmi H, Billaudel B, Garnier B, Malaisse WJ & Sutter BCHJ 1988 Vitamin D and pancreatic islet function. I. Time course for changes in insulin secretion and content during vitamin D deprivation and repletion. *Journal of Endocrinological Investigation* **11** 577–584.
- Lacy PE & Kostianovsky M 1967 Method for the isolation of intact islets of Langerhans from pancreas. *Diabetes* **16** 35–39.
- Laemmli UK 1970 Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685.
- Minghetti PP & Norman AW 1988 1,25(OH)₂-Vitamin D₃ receptors: gene regulation and genetic circuitry. *FASEB Journal* **2** 3043–3053.
- Moulend AJ & Hendy GN 1991 Regulation of synthesis and secretion of chromogranin-A by calcium and 1,25-dihydroxycholecalciferol in cultured bovine parathyroid cells. *Endocrinology* **128** 441–449.
- Norman AW, Frankel BJ, Heldt AM & Grodsky GM 1980 Vitamin D deficiency inhibits pancreatic secretion of insulin. *Science* **208** 823–825.
- Norman A, Roth J & Orci L 1982 The vitamin D endocrine system: steroid metabolism, hormone receptors and biological response (calcium binding protein). *Endocrine Review* **3** 331–366.

- Ozono K, Seino Y, Yano H, Yamaoka K & Seino Y 1990 1,25-Dihydroxyvitamin D₃ enhances the effect of refeeding on steady state preproinsulin messenger ribonucleic acid levels in rats. *Endocrinology* **126** 2041–2045.
- Pike JW 1985 Intracellular receptors mediate the biologic action of 1,25-dihydroxyvitamin D₃. *Nutrition Review* **43** 161–168.
- Stumpf WE, Sar M & De Luca HF 1981 Sites of action of 1,25(OH)₂ vitamin D₃ identified by thaw-mount autoradiography. In *Hormone Control of Calcium Metabolism*, pp 222–229. Eds DV Cohn, RV Taladge & J Matthews. Amsterdam: Excerpta Medica.
- Verhaeghe J, Suiker AMH, Nyomba BL, Visser WJ, Einhorn TA, Dequeker J & Bouillon R 1989 Bone mineral homeostasis in spontaneously diabetic BB rats. II. Impaired bone turnover and decreased osteocalcin synthesis. *Endocrinology* **124** 573–582.
- Walters MR 1992 Newly identified actions of the vitamin D endocrine system. *Endocrine Review* **13** 719–764.

Received 1 December 1998

Accepted 8 March 1999