Influence of vitamin D_3 deficiency and 1,25 dihydroxyvitamin D_3 on *de novo* insulin biosynthesis in the islets of the rat endocrine pancreas

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Abstract

Because 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is known to activate the biosynthesis of numerous proteins in various tissues, experiments were undertaken to compare the influence of $1,25(OH)_2D_3$ *in vitro* on both the secretion and biosynthesis of insulin in islets of Langerhans from both 4-week vitamin D_3-deficient rats and normal rats. Islets were either incubated or perifused after a 6-h induction period in the presence of various concentrations of $1,25(OH)_2D_3$ from 10^{-12} M, which was inactive in controls, to 10^{-6} M. Experiments were performed in the presence of a non-labelled amino acid mixture, to favour protein synthesis. Tritiated tyrosine was added as tracer during glucose stimulation. The newly synthesised proteins, labelled with [^{3}H]tyrosine, were extracted by an acid–alcohol method and separated by gel chromatography adapted for low-molecular-weight proteins. Even in the presence of the amino acid mixture, the insulin response of the islets to 16·7 mM glucose was decreased by vitamin D_3 deficiency and improved by $1,25(OH)_2D_3$. This beneficial effect did not occur in basal conditions, but only during glucose stimulation, and was observed in both phases of insulin release. Moreover, these effects disappeared in the presence of 5×10^{-4} M cycloheximide, a protein biosynthesis inhibitor. Islets from vitamin D_3-deficient rats exhibited a general decrease in the amount of *de novo* biosynthesised proteins and of [^{3}H]tyrosine-labelled insulin and proinsulin fractions. A 6-h period of $1,25(OH)_2D_3$ induction significantly improved the amount of *de novo* biosynthesised proteins, and particularly of newly synthesised insulin in response to a 2-h glucose stimulation. Calculation of the rate of conversion of newly synthesised proinsulin-like material to insulin as the [^{3}H]insulin/[^{3}H]proinsulin-like material ratio provided evidence for a dose-dependent increase, induced by $1,25(OH)_2D_3$, that could exceed that of normal islets. These data support the hypothesis that $1,25(OH)_2D_3$ *in vitro* not only facilitated the biosynthetic capacity of the β cell – which was highly induced during a 16·7-mM glucose stimulation, via a global activation of islets protein biosynthesis – but also produced an acceleration of the conversion of proinsulin to insulin.

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Introduction

Cases of vitamin D_3 deficiency result not only from a lack of exposure to sun in wintertime (Dawson-Hughes *et al.* 1997) or from particular modes of dress (Garabedian & Ben Mekhbi 1991), but also as a result of nutritional disorders induced by unbalanced diet (Gedik & Akalin 1986) or in diabetic patients (Raghuramulu *et al.* 1992, Boucher *et al.* 1995, Rudnicki & Molsted-Pedersen 1997). One of the vitamin D_3 metabolites, 1,25 dihydroxy vitamin D_3 ($1,25(OH)_2D_3$), is considered to be the main steroid that has a crucial role in calcium homeostasis (Anderson 1991), and is able to improve the deficits in glucose tolerance observed in association with some cases of vitamin D_3 deficiency (Gedik & Akalin 1986, Raghuramulu *et al.* 1992, Boucher *et al.* 1995, Rudnicki & Molsted-Pedersen 1997).

$1,25(OH)_2D_3$ is known to exert its influence on various tissues via both genomic and more rapid non-genomic mechanisms (reviewed in Walters 1992), As calcium has a crucial role in the release of insulin in response to glucose (Grodsky & Bennett 1966), most authors agree that $1,25(OH)_2D_3$ could exert beneficial effects on the reduced insulin release caused by vitamin D_3 deficiency, mainly by an improvement of calcium handling by pancreatic islets (Billaudel *et al.* 1991); however, the respective roles of $1,25(OH)_2D_3$ and calcium in this effect are still under discussion (Beaulieu *et al.* 1993, Chertow *et al.* 1983, Tanaka *et al.* 1986). In recent studies in vitamin D_3-deficient rats, we showed that a prior exposure to low extracellular calcium *in vitro* enhances the sensitivity of the β cell to the stimulatory influence of $1,25(OH)_2D_3$ on insulin release, suggesting a predominant genomic influence (Faure-Dussert *et al.* 1997). Most of the cellular

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actions of 1,25(OH)$_2$D$_3$ are mediated via the interaction of this secosteroid with the vitamin D_3 receptor, which is expressed and localised, not only in the classical target tissues such as intestine, kidney and bone, but also in many other tissues and cell lines not primarily connected with mineral metabolism, such as the endocrine pancreas (reviewed in Walters 1992). Certain characteristics of its mechanism of action are similar to those of steroid hormones: specific intracellular receptors facilitating nuclear uptake of 1,25(OH)$_2$D$_3$ (Pike 1985), binding to promoter sequences in the genome, causing up- or down-regulation of the transcription of various genes (Minghetti & Norman 1988), and mRNA production coding for several *de novo* synthesised proteins (Norman *et al.* 1982). Indeed, it has been shown that 1,25(OH)$_2$D$_3$ influences the biosynthesis of numerous proteins in various tissues (Verhaeghe *et al.* 1989, Brunner & De Boland 1990, Chang & Price 1991, Mouland & Hendy 1991).

Because, in the rat, the insulin response to glucose is impaired by vitamin D_3 deficiency and can be improved by 1,25(OH)$_2$D$_3$ both *in vivo* and *in vitro* (Norman *et al.* 1980, Clark *et al.* 1981, Chertow *et al.* 1983, Labriji-Mestaghanmi *et al.* 1988), we have examined the hypothesis of an effect of 1,25(OH)$_2$D$_3$ on biosynthesis of insulin by the β cells, in which 1,25(OH)$_2$D$_3$ receptors have been observed (Clark *et al.* 1980, Stumpf *et al.* 1981, Johnson *et al.* 1994). Indeed, this hypothesis was supported by some of our previous studies showing that a 6-h delay was necessary for the beneficial influence of 1,25(OH)$_2$D$_3$ on the release of insulin (Billaudel *et al.* 1990). Moreover, 1,25(OH)$_2$D$_3$ did not affect the basal insulin response, but affected exclusively the insulin response of β cells to a glucose stimulation (Billaudel *et al.* 1990), which engages both insulin exocytosis and insulin biosynthesis (Portha 1991). For these reasons, we have studied both the content and secretion of insulin by the islets after a 6-h induction period in the presence, or not, of various concentrations of 1,25(OH)$_2$D$_3$, paying particular attention to the amount of insulin newly synthesised during a glucose stimulation. Use of a labelled amino acid, [^{3}H]tyrosine, enabled us to follow the production of *de novo* synthesised proteins, and in particular proinsulin and insulin fractions, which were separated by chromatography. We compared islets from normal rats with those from 4-week vitamin D_3-deficient rats.

Material and Methods

Animals

Experiments were run in parallel using two groups of Wistar rats (CREJ, Le Genest-Saint-Isle, France): normal rats and 4-week vitamin D_3-deficient (D_3-deficient) rats. All animals were housed in a dark room for the 8 weeks after birth and had free access to food and water. After weaning (3 weeks old), the group of normal rats received a balanced diet (AO4, UAR, Epinay sur Orge, France) with carbohydrates (59·7% w/w), proteins (16% w/w) and lipids (3% w/w) and containing calcium (0·64% w/w), phosphate (0·64% w/w) and vitamin D_3 (2000 IU/kg), whereas D_3-deficient rats received a rachitogenic diet (US Biochemical Corporation, Cleveland, OH, USA) devoid of vitamin D, but containing low calcium (0·5% w/w) and low phosphate (0·3% w/w), for 4 weeks. The D_3-deficient rats exhibited rachitis and hypocalcaemia as verified in our previous studies (Labriji-Mestaghanmi *et al.* 1988, Bourlon *et al.* 1996).

Isolation of islets and 1,25(OH)$_2$D$_3$ induction

Pancreatic islets of Langerhans were isolated by the collagenase (Boehringer Co, Mannheim, Germany) method (Lacy & Kostianovsky 1967). Batches of 50 islets from either normal rats or D_3-deficient rats were used; those from the latter were divided into five groups for a 6-h period of induction in the presence of various concentrations of 1,25(OH)$_2$D$_3$. Cycloheximide (5×10^{-4} M, Sigma), a potent inhibitor of protein synthesis, was added to the incubation medium in a control experiment. Inductions were run for 6 h at 37 °C in Krebs–Ringer bicarbonate (KRB) medium with 0·5% bovine albumin (fraction V, RIA grade, Sigma) and 0·5 mM Ca^{2+}, and gassed with 95% oxygen/5% carbon dioxide. This medium also contained various non-labelled amino acids (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). The medium was changed every 2 h, dividing the 6-h induction period into three incubation periods of 2 h each, in an attempt to limit the well known feedback inhibition exerted by insulin in a closed medium (Iversen & Miles 1971). Glucose was present in a concentration of 8·3 mM for the two first periods and 16·7 mM for the last incubation period, providing a strong specific stimulation of the biosynthesis of insulin compounds (proinsulin, split proinsulins, insulin) and proteins necessary for the secretion of insulin by the β cells of the islets.

In order to determine the level of *de novo* protein biosynthesis, during the last 2 h of induction (during the 16·7-mM glucose stimulation) the islets were incubated with radiolabelled L-[1,4 ^{3}H]tyrosine (1850 Gbq/mmol; Amersham, Amersham, UK). This amino acid is a useful marker because it is incorporated into proinsulin, but not into the carboxypeptide segment resulting from the proteolytic cleavage of proinsulin (Schuit *et al.* 1991). Thus [^{3}H]tyrosine will be incorporated into proinsulin, split proinsulins, and insulin, each of them including four tyrosines. Islets were then rinsed twice with KRB medium containing 5 mM non-labelled tyrosine, to eliminate free radioactivity (not incorporated into protein). Islets were ready for the separation and determination of *de novo* biosynthesised proinsulin and insulin, or for insulin

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release studies using static incubation experiments or dynamic perifusion experiments as previously described (Billaudel *et al.* 1990).

Separation of proteins

Extraction procedure After the period of 1,25(OH)$_2$D$_3$ induction and [^{3}H]tyrosine labelling, islets were treated for 24 h at 5 °C with 100 µl ethanol–acid mixture (ethanol 74% v/v; distilled water 24·6% v/v; hydrochloric acid 1·4% v/v). Small peptides, such as insulin (6·5 kDa) or proinsulin (9 kDa), remain in the soluble supernatant fraction, whereas heavier peptides form precipitates (Best *et al.* 1969). The incorporation of the ^{3}H radioactivity into the small proteins was measured on aliquots of the supernatant, after addition of scintillation liquid (Emulsifier safe, Packard, Rungis, France), using a Packard Tricarb spectrometer. Similarly, the radioactivity of the heavy proteins was measured on the precipitate. The calculation of the total amount of *de novo* synthesised radiolabelled proteins was obtained as the sum of these two values.

Separation of insulin material Insulin compounds were separated by a chromatographic method, which was more precise for low-molecular-weight proteins, rather than by electrophoresis, which is better adapted for higher-molecular-weight proteins. Aliquots of the soluble fraction, after extraction with ethanol–acid mixture, were placed on top of a gel chromatography column (Sephadex G50, Pharmacia, Sweden). Elution was performed with KRB buffer containing 0·5% albumin. Effluents were collected in 1-ml fractions in which the total anti-insulin immunoreactive material was assayed by RIA. Insulin RIA was performed using a charcoal–dextran method (Herbert *et al.* 1965), with rat insulin as standard (Novo, Paris, France). Measurements of the [^{3}H]tyrosine radioactivity, corresponding only to newly synthesised materials, were also made on the same fractions, as described above. Calibration of the column was made using ^{125}I-radiolabelled proinsulin and insulin as standard. The elution profile showed that insulin was eluted from fraction 34 to fraction 45 of the effluents, with a maximal peak at fraction 38, and proinsulin-like material at fraction 30 (proinsulin and split proinsulins have equivalent molecular weights).

Total islet protein content was determined with islets in aqueous solution by the Bio Rad protein–dye binding technique (Bradford 1976).

Statistical analysis

Results are expressed as means ± S.E.M. The number of experiments performed on each occasion with islets from two normal rats and six D$_3$-deficient rats is given as *n*. Data were analysed by analysis of variance (ANOVA) using the Bonferroni/Dunn complementary test for a statistical significance of at least <0·05. Student's *t*-test for unpaired samples was used for comparison of two groups such as D$_3$-deficient and normal islets, using three levels of significance (<0·05,<0·01 and <0·001).

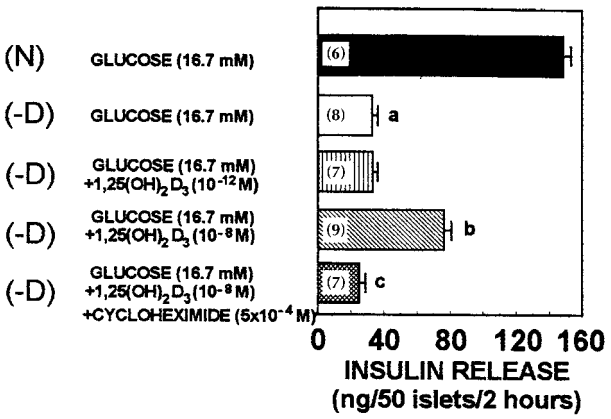

Figure 1 Influence of vitamin D$_3$ deficiency and 1,25(OH) $_2$D$_3$ *in vitro* on 50 islets. Insulin response to 16·7 mM glucose during a 2-h incubation following a 6-h pre-incubation, in the presence or not of 1,25(OH)$_2$D$_3$. (N), islets from normal rats; (−D), 4-weeks vitamin D$_3$-deficient rats, in the presence of a biologically inactive 10^{-12} M concentration as control or an active 10^{-8} M concentration without or with a biosynthesis inhibitor, 5×10^{-4} M cycloheximide. Values are means ± S.E.M.; number of experiments is shown within the bars, in parentheses. [a]*P*<0·001 compared with N; [b]*P*<0·001 compared with (−D) control or 10^{-12} M; [c]*P*<0·001 compared with 10^{-8} M group (Student's *t*-test).

Results

Total insulin content of islets and global insulin secretion

Vitamin D$_3$ deficiency did not significantly affect the total protein content of 50 islets compared with that of normal rats (0·91 ± 0·08 µg/islet, *n*=10, compared with 1·01 ± 0·09 µg/islet, *n*=9, respectively). However, vitamin D$_3$ deficiency decreased the total insulin content of 50 islets compared with that of normal rats, expressed either as ng/islet (25·72 ± 3·5, *n*=10, compared with 44·92 ± 4·28, *n*=9, respectively; *P*<0·01) or as ng/µg of proteins (28·2 ± 1·5, *n*=10, compared with 44·47 ± 3·18, *n*=9, respectively; *P*<0·001).

The release of insulin in response to a 16·7 mM glucose stimulation was studied using groups of 50 islets incubated for 2 h in the presence or not of 1,25(OH)$_2$D$_3$, following a 6-h preincubation period that was performed in the presence or not of 1,25(OH)$_2$D$_3$ as an induction period. As shown in Fig. 1, the insulin response of islets from D$_3$-deficient rats was very much lower than that of normal islets (*P*<0·001). Islets from D$_3$-deficient rats did not show any variation in their insulin release after a 6-h preexposure to a 10^{-12} M concentration of 1,25(OH)$_2$D$_3$,

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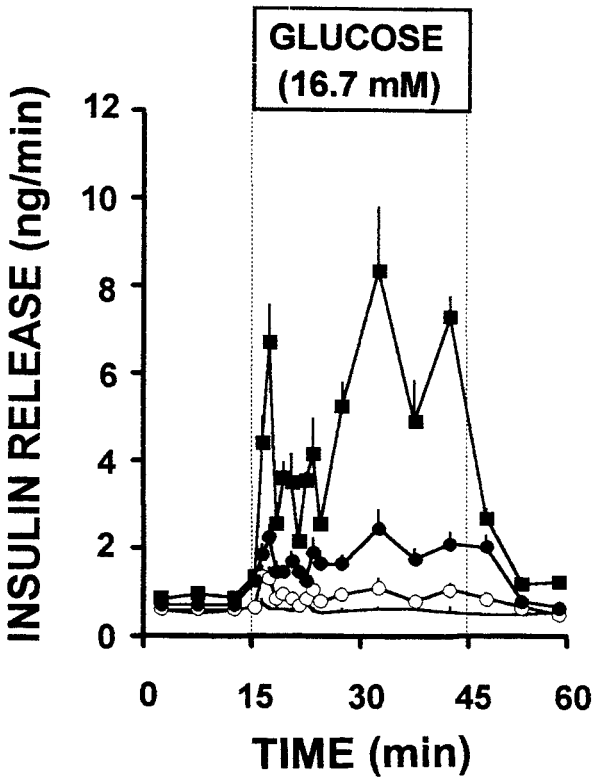

Figure 2 Influence of vitamin D_3 deficiency and 1,25(OH)$_2$D$_3$ *in vitro*, on the kinetics of the insulin response to 16·7 mM glucose of 50 islets during a perifusion performed after a 6-h pre-incubation, both being performed in the presence or not of 1,25(OH)$_2$D$_3$. The perifusion design consisted of a 15-min basal (4·2 mM glucose) prestimulatory recovery period, followed by a 30-min (16·7 mM) glucose stimulation and a 15-min post-stimulatory recovery period returning to basal secretion. Islets from normal rats (■; n=6) or from vitamin D_3-deficient rats with 10^{-12} M inactive 1,25(OH)$_2$D$_3$ as control (○; n=7) or with 10^{-8} M 1,25(OH)$_2$D$_3$ (●; n=9) or 10^{-8} M 1,25(OH)$_2$D$_3$ in the presence of 5×10^{-4} M cycloheximide (–; n=7). Values are means ± S.E.M. from n experiments.

which is considered not to be biologically active. In contrast, the most commonly used 10^{-8} M concentration improved their insulin response (P<0·001 compared with D_3-deficient islets). This enhancing effect of 10^{-8} M 1,25(OH)$_2$D$_3$ on the insulin response to 16·7 mM glucose disappeared (Fig. 1) in the presence of 5×10^{-4} M cycloheximide, a protein biosynthesis inhibitor.

To complement to these static measurements, a kinetic study was performed (Fig. 2), using islets in perifusion after the 6-h pre-incubation period. Islets from normal rats increased their insulin release at the beginning of the 16·7 mM glucose stimulation (P<0·001 compared with their own basal secretion) and exhibited the two well-described phases of insulin secretion: a rapid first peak and a longer lasting second phase, returning to basal levels of secretion when glucose stimulation was replaced by the 4·2 mM basal glucose concentration. To compare the various groups of islets, we evaluated the amplitude of their insulin response to glucose by comparison of the area under the insulin curves over their own basal secretion by planimetry, thus including both phases of insulin release (Billaudel *et al.* 1988, 1990). Islets from D_3-deficient rats, exposed to the 10^{-12} M inactive 1,25(OH)$_2$D$_3$ concentration as control, presented a very low insulin response to glucose compared with that in normal islets (respective insulin curves areas: 0·99 ± 0·08 ng/min, n=7 and 5·46 ± 0·51 ng/min, n=6; P<0·01). After 1,25(OH)$_2$D$_3$ induction with a 10^{-8} M concentration, we observed an increase of the area under the insulin curve: 1·89 ± 0·19 ng/min, n=8; P<0·01 compared with the 10^{-12} M control group). The addition of 5×10^{-4} M cycloheximide to 10^{-8} M 1,25(OH)$_2$D$_3$ suppressed the beneficial effect of 1,25(OH)$_2$D$_3$ during the 16·7 mM glucose stimulation. Both phases of insulin release were decreased by vitamin D_3 deficiency and improved by 10^{-8} M 1,25(OH)$_2$D$_3$. In contrast, basal insulin secretion, both before and after glucose stimulation, was not significantly affected, whatever the group of islets.

De novo *biosynthesis studies*

The glucose-induced incorporation of a labelled amino acid, [^{3}H]tyrosine, into the total proteins from 50 islets was measured after a 6-h induction with or without 1,25(OH)$_2$D$_3$ (Table 1). The amount of these *de novo* synthesised labelled proteins was decreased by vitamin D_3 deficiency (P<0·001 for islets from D_3-deficient rats exposed to 10^{-12} M inactive 1,25(OH)$_2$D$_3$ compared with normal islets), but improved by 1,25(OH)$_2$D$_3$. This beneficial influence of the various concentrations of 1,25(OH)$_2$D$_3$ was analysed in comparison with the D_3-deficient group using a 10^{-12} M inactive concentration as control. This action of 1,25(OH)$_2$D$_3$ was not significant at a 10^{-10} M concentration, but was significant for 10^{-8} M and 10^{-6} M (P<0·05).

The incorporation of labelled tyrosine into small proteins (acid–alcohol soluble), including newly synthesised insulin and proinsulin-like material (proinsulin and split proinsulins), like that into total labelled proteins, was decreased by vitamin D_3 deficiency (P<0·001) and improved by 1,25(OH)$_2$D$_3$, with a maximal influence at concentrations of 10^{-8} M and 10^{-6} M (P<0·05; Table 1).

Insulin and proinsulin-like fractions studies

A chromatographic separation, performed after the glucose-induced incorporation of [^{3}H]tyrosine into groups of 50 islets, allowed us to compare more specifically the insulin and proinsulin-like chromatography peaks. The study of the effluent medium allowed us to determine both the total anti-insulin immunoreactive material contained

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Table 1 Influence of vitamin D_3 deficiency (−D) and 1,25(OH)$_2$D$_3$ on glucose-induced, newly synthesised islet proteins ([^{3}H]tyrosine-labelled) separated by chromatography: study of insulin peak (Ins, fraction 38) and proinsulin-like peak (PIns, fraction 30). Various concentrations of 1,25(OH)$_2$D$_3$ were used, from 10^{-12} M inactive as control, to 10^{-6} M. Values are means ± S.E.M.; number of experiments is in parentheses

	N (9)	**−D 10^{-12} M 1,25(OH)$_2$D$_3$** (10)	**−D 10^{-10} M 1,25(OH)$_2$D$_3$** (6)	**−D 10^{-8} M 1,25(OH)$_2$D$_3$** (7)	**−D 10^{-6} M 1,25(OH)$_2$D$_3$** (6)
Total islets labelled proteins (d.p.m.)	1 155 000 ± 86 000	530 800* ± 56 900	574 000 ± 64 000	1 064 000† ± 120 000	1 019 000† ± 82 000
Acid–alcohol-soluble proteins (d.p.m.)	244 400 ± 8700	117 800* ± 13 800	129 000 ± 8000	163 400† ± 8200	169 400† ± 12 100
[^{3}H]-tyrosine-labelled parts (d.p.m.)					
[^{3}H]-Ins (fraction 38)	37 100 ± 2200	13 300* ± 1900	15 500 ± 1300	27 600† ± 1800	28 200† ± 1900
[^{3}H]-PIns (fraction 30)	10 660 ± 460	3690* ± 820	4280 ± 540	6340† ± 840	5040 ± 400

*P<0·001 compared with islets from normal rats (N) (Student's t-test); †P<0·05 compared with (−D) with 10^{-12} M 1,25(OH)$_2$D$_3$ as control (ANOVA with Bonferroni/Dunn complementary tests).

inside the islets (Fig. 3A), and the amount of newly synthesised [^{3}H]tyrosine-labelled material, on the same islets (Fig. 3B). As verified by standards, fractions 38 and 30 were more specific to insulin and proinsulin-like material, respectively, but some other newly synthesised and heavier proteins were apparent just to the left of proinsulin (Fig. 3B). For these reasons, we studied insulin and proinsulin-like peaks, respectively, on fractions 38 and 30.

The analysis of the total immunoreactivity peak (fractions 38, Fig. 3A) showed that the insulin peak was strongly decreased by 4 weeks of vitamin D_3 deficiency (293·5 ± 26, n=10 compared with 507·9 ± 53·2 ng/ml, n=9 for normal islets; P<0·01). It was not significantly improved by a 6-h 1,25(OH)$_2$D$_3$ induction period, whatever the concentration of 1,25(OH)$_2$D$_3$: 10^{-10} M, 10^{-8} M or 10^{-6} M (respectively 348·8 ± 22·8, n=6; 355·3 ± 45·4, n=7; 346·0 ± 28·4 ng/ml, n=6). The total immunoreactive proinsulin-like peak (fraction 30, Fig. 3A) was not significantly modified by either vitamin D_3 deficiency (13·20 ± 1·76, n=10 compared with normal islets 13·00 ± 2·76 ng/ml, n=9) or the presence of 1,25(OH)$_2$D$_3$ in whatever concentration: 10^{-10} M, 10^{-8} M or 10^{-6} M (respectively 11·60 ± 3·28, n=6; 9·56 ± 1·80, n=7; 10·08 ± 2·00 ng/ml, n=6). However, this global immunoreactive insulin material was not very representative of the material newly synthesised in response to glucose. It corresponded to the large insulin stores present in islets, thus hindering the detection of the discrete variations induced by 1,25(OH)$_2$D$_3$ and glucose stimulation. Indeed, as is reported generally (Randle & Hales 1972), insulin release represented only about 10 percent/h/islet of the islet insulin content in perifusion and only 3 percent/h/islet in incubation in the present experiments, because of the feedback inhibition effect that usually occurs in a closed medium. These data made us aware of the need for a finer and more specific [^{3}H]labelling study of insulin newly synthesised in response to glucose stimulation at the end of 1,25(OH)$_2$D$_3$ induction.

The newly synthesised labelled insulin peak (Table 1, fraction 38) was strongly decreased by vitamin D_3 deficiency (P<0·001 compared with normal islets), and improved by 1,25(OH)$_2$D$_3$, with a maximal effect at 10^{-8} M and 10^{-6} M concentrations (P<0·05 compared with D_3-deficient control islets). In contrast, the [^{3}H]tyrosine-labelled proinsulin-like peak (fraction 30) was decreased by vitamin D_3 deficiency (P<0·001 compared with normal islets), and improved by 1,25(OH)$_2$D$_3$, with a maximal effect at 10^{-8} M (P<0·05 compared with D_3-deficient control islets). A comparison of the [^{3}H]tyrosine-labelled insulin/proinsulin peak ratios was made between each group of islets, as an index of the neoconversion of proinsulin to insulin (Fig. 4). This ratio was not significantly decreased by vitamin D_3 deficiency, but it showed a significant increase in the presence of 1,25(OH)$_2$D$_3$. The maximal effect was observed at 10^{-8} M and 10^{-6} M concentrations (P<0·05 compared with 10^{-12} M as control). This 1,25(OH)$_2$D$_3$-induced, dose-dependent increase in this ratio exceeded even that of normal islets, thus supporting the hypothesis of a 1,25(OH)$_2$D$_3$-induced acceleration of the conversion rate of the neosynthesised proinsulin to insulin during glucose stimulation.

Discussion

The hypothesis of an 1,25(OH)$_2$D$_3$-induced activation of insulin biosynthesis must be considered for several reasons. First, 1,25(OH)$_2$D$_3$ was able to increase the amount of

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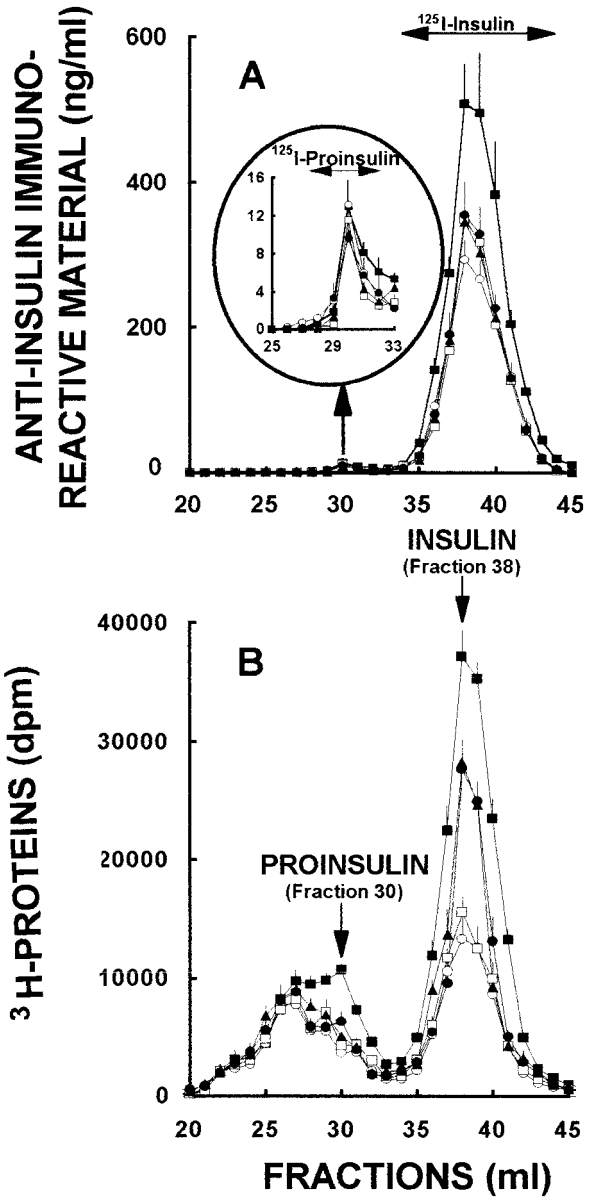

Figure 3 Influence of vitamin D_3 deficiency and 1,25(OH) $_2$D$_3$ *in vitro* on (A) the anti-insulin immunoreactive material (insulin peak (fraction 38) and proinsulin-like peak (fraction 30, insert) separated by chromatography), and on (B) labelled newly synthesised material from [^{3}H]tyrosine incorporation into 50 islets during a 2-h 16·7 mM glucose stimulation, after a 6-h incubation with or without 1,25(OH) $_2$D$_3$ induction. Islets from normal rats (■; n=9) and from vitamin D_3-deficient rats in the presence of various concentrations of 1,25(OH)$_2$D$_3$: 10^{-12} M inactive as control (○; n=10), 10^{-10} M (□; n=6), 10^{-8} M (●; n=7) or 10^{-6} M (▲; n=6). Values are means ± S.E.M. from n experiments.

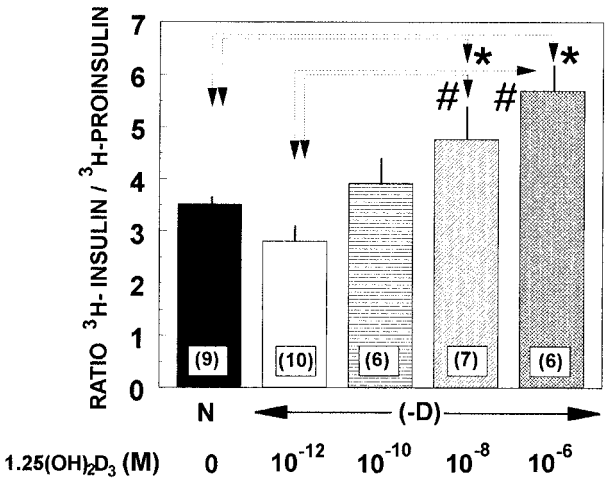

Figure 4 Influence of vitamin D_3 deficiency and 1,25(OH)$_2$D$_3$ *in vitro* on the ratio of [^{3}H]tyrosine-labelled insulin/proinsulin-like peaks as an index of the neoconversion of proinsulin to insulin. N, islets from normal rats; (−D), islets from vitamin D_3-deficient rats in the presence of concentrations of 1,25(OH)$_2$D$_3$ from 10^{-12} M inactive as control, to 10^{-6} M. Values are means ± S.E.M. from n experiments. *P<0·05 compared with N; #P<0·05 compared with (−D) 10^{-12} M 1,25(OH)$_2$D$_3$ as controls (ANOVA with Bonferroni/Dunn complementary test).

preproinsulin mRNA in islets from vitamin D_3-deprived rats either 8 h or 24 h after intraperitoneal injection (Ozono *et al.* 1990). Secondly, the beneficial effect of 1,25(OH)$_2$D$_3$ on insulin release was observed only in islets in which β cells were stimulated by glucose, but not under basal conditions (Taylor *et al.* 1988, Billaudel *et al.* 1990). Similarly, other authors have observed stimulatory effects of 1,25(OH)$_2$D$_3$ exclusively in pair-fed vitamin D_3-deficient rats, but not in fasted rats (Ozono *et al.* 1990), suggesting a better adaptation of the β cell to glucose, its preferential stimulus, which is able to activate insulin exocytosis and insulin biosynthesis. Finally, 1,25(OH)$_2$D$_3$ requires a delay to activate the β cell insulin response to glucose: this varies from 3 h to 20 h *in vivo* (Ishida *et al.* 1983, Kadowaki & Norman 1985, Cade & Norman 1987, Ozono *et al.* 1990) and after a 6-h induction *in vitro* (Billaudel *et al.* 1990) or after a 48-h culture (Taylor *et al.* 1988), but not directly *in vitro* from 0 to 4 h (Chertow *et al.* 1983, Taylor *et al.* 1988, Billaudel *et al.* 1990). To our knowledge, there exist few data on the influence of vitamin D_3 deficiency or on the effect of 1,25(OH)$_2$D$_3$ on insulin biosynthesis. The divergence in existing results is probably due to the differing experimental conditions used: no effect on insulin biosynthesis *in vitro* in 48-h cultured islets from mice (Taylor *et al.* 1988), but an *in vivo* activation of preproinsulin mRNA in islets from fed rats (Ozono *et al.* 1990).

In our present experimental conditions, islets were preincubated in the presence of an amino acid mixture during a 6-h 1,25(OH)$_2$D$_3$ induction and during the incubation or perifusion experiments, to favour insulin biosynthesis. We showed that, even in the presence of a supplementation of amino acid *in vitro*, islets from vitamin

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D_3-deficient rats presented an altered insulin response to glucose, whereas their basal secretion was not significantly affected. In contrast, we confirmed that, even in the presence of amino acids, a 6-h 1,25(OH)$_2$D$_3$ induction could selectively improve the release of insulin from islets during glucose stimulation. Both phases of insulin response were facilitated: the first rapid phase, believed to reflect insulin exocytosis, and the second, longer lasting phase that is more dependent upon insulin biosynthesis (Portha 1991). It was indeed this second phase that was found to be the more reduced by vitamin D_3 deficiency in another study (Chertow *et al.* 1983). This second phase was the first to be increased by 3 days of vitamin D_3 repletion *in vivo*, whereas both phases of insulin release were increased after a longer time (6 days) of treatment (Billaudel *et al.* 1988). *In vitro*, we previously found that both phases of insulin response to glucose were activated after 6 h of 1,25(OH)$_2$D$_3$ induction, but neither of them at shorter times (<6 h) (Billaudel *et al.* 1990). In the present experimental conditions, the presence of amino acids and the 6-h 1,25(OH)$_2$D$_3$ induction period may have induced the biosynthesis of numerous proteins implicated either in exocytosis mechanisms or in the preparation of the β cell for insulin biosynthesis activation. Indeed, the ability of 1,25(OH)$_2$D$_3$ to induce an activation of insulin secretion in response to glucose was blocked by cycloheximide, supporting the hypothesis that the synthesis of new proteins was required for these mechanisms.

Conversely, certain of our results support the hypothesis that vitamin D_3 deficiency exerts its deleterious influence on islets of Langerhans via a progressive and long-term alteration of the processes of synthesis of the proteins necessary for the β cell insulin response to glucose. (1) The global total protein content of islets was not significantly affected by 4 weeks of vitamin D_3 deficiency, in agreement with results of previous experiments performed later, at week 5 (Labriji-Mestaghanmi *et al.* 1988), whereas, during glucose stimulation, newly synthesised islet proteins (labelled by [^{3}H]tyrosine incorporation) were already decreased by 4 weeks of vitamin D_3 deficiency. (2) The total insulin content of islets from 4-week vitamin D_3-deficient rats was already decreased at 4 weeks (as was also the case at 5 weeks), but not at 3 weeks of vitamin D_3 deficiency (Labriji-Mestaghanmi *et al.* 1988). (3) After 4 weeks of vitamin D_3 deficiency, a parallel decrease was observed in the labelled alcohol–acid-soluble small proteins containing the *de novo* synthesised insulin and proinsulin-like material. (4) A more specific chromatographic study of islets confirmed that 4-week vitamin D_3 deficiency decreased the biosynthesis of insulin (total immunoreactive insulin or its labelled part alone, newly synthesised during glucose stimulation).

However, a period of only 6 h of 1,25(OH)$_2$D$_3$ induction, applied to islets from vitamin D_3 deficient rats, improved the insulin biosynthesis of islets, even though it did not completely restore (within 6 h) the deleterious influence of 4 weeks of vitamin D_3 deficiency. The maximal effect was obtained in the presence of a 10^{-8} M concentration of 1,25(OH)$_2$D$_3$, which is precisely the concentration used most commonly by numerous authors. Indeed, 1,25(OH)$_2$D$_3$ increased the amount of newly synthesised proteins that were labelled during glucose stimulation. This was the case both for global proteins of the islets and for the alcohol–acid-soluble part containing new insulin and proinsulin material. This is the first demonstration of a beneficial influence of a 6-h 1,25(OH)$_2$D$_3$ induction on the *de novo* biosynthesis of labelled insulin and proinsulin-like material separated by column chromatography.

Finally, the present results provide evidence in support of the idea that 1,25(OH)$_2$D$_3$ exerted its beneficial effect on β cells by a particular activation of insulin turnover, accelerating the neoconversion of proinsulin to insulin. This hypothesis was supported by the complementary study of the [^{3}H]tyrosine-labelled insulin/proinsulin ratio (proinsulin including split proinsulins), in which 1,25(OH)$_2$D$_3$ increased the ratio in a dose-dependent manner, showing that the newly synthesised insulin appeared more rapidly, with a conversion rate exceeding that of islets from normal rats. Such an acceleration of the neoconversion of proinsulin to insulin has previously been observed in our laboratory, with another steroid, 17-β-oestradiol (Faure *et al.* 1986).

In conclusion, the present data demonstrated that 1,25(OH)$_2$D$_3$ could activate the *de novo* biosynthesis of insulin in islets from vitamin D_3-deficient rats during the intense stimulation of the islets by a glucose stimulus, and could, in particular, increase the rate of conversion of proinsulin to insulin. We could not establish whether this acceleration of the conversion of proinsulin to insulin was a direct or an indirect effect, as this step was previously reported to be calcium-dependent (Steiner *et al.* 1996). All the present experiments were performed *in vitro* in the presence of 0·5 M extracellular calcium in the medium. Other authors reported that calcium can induce the transcription of certain genes in other tissues (Jackson & Bancroft 1988, Sheng *et al.* 1988). However, in recent studies we showed that, *in vitro*, calcium deprivation of islets from vitamin D_3-deficient rats rendered then more competent in mobilising intracellular calcium, for a better adaptation of the insulin response to glucose (Faure-Dussert *et al.* 1997). Further experiments are needed to study other proteins with larger molecular weights using the electrophoresis method. Indeed, some authors have reported that 1,25(OH)$_2$D$_3$ can increase translocation of protein kinase C to the nucleus and the modulation of gene expression in the kidney (Simboli-Campbell *et al.* 1992). This observation is in accord with our previous work in which we showed that 1,25(OH)$_2$D$_3$ can activate the protein kinase C and inositol triphosphate pathways in islets from vitamin D_3-deficient rats with a shorter delay (<45 min) (Billaudel *et al.* 1993, 1995), thus preceding its

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effect on calcium handling at 4 h and on the insulin response to glucose after a 6-h 1,25(OH)$_2D_3$ induction (Billaudel *et al.* 1990, 1993).

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