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Modulatory role of 1,25 dihydroxyvitamin D_3 on pancreatic islet insulin release via the cyclic AMP pathway in the rat

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1 Previous studies have shown that vitamin D_3 deficiency impairs the insulin response to glucose via an alteration of signal transduction pathways, such as Ca^{2+} handling and the phosphoinositide pathway. In the present study the adenylyl cyclase pathway was examined in islets from 3 independent groups: normal rats, 4 weeks-vitamin D_3 deficient rats and one week-1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) treated rats.

2 We found that the very low rate of insulin release observed in vitamin D_3 deficient rats could be restored in vitamin D_3 deficient islets only with high concentrations of dioctanoyl-cyclic AMP (DO-cyclic AMP), whereas $1,25(OH)_2D_3$ improved the sensitivity of the islets to this exogenous cyclic AMP analogue.

3 The beneficial effect of $1,25(OH)_2D_3$ observed with or without DO-cyclic AMP was protein kinase A-dependent, since the addition of N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulphonamide (H-89), a specific inhibitor of cyclic AMP-dependent protein kinases, decreased the insulin release of treated rats back to the level seen in vitamin D_3 deficient islets.

4 The low rate of insulin release could not be consistently related to an alteration in cyclic AMP content of the islets. Indeed, low insulin response to a barium + theophylline stimulus observed in vitamin D_3 deficient islets was paradoxically associated with a supranormal cyclic AMP content in the islets.

5 This paradoxical increase in cyclic AMP observed in these conditions could not be attributed to a lower total phosphodiesterase (PDE) activity, although the portion of Ca^{2+}-calmodulin-independent PDE was predominant in islets from vitamin D_3 deficient rats.

6 On the other hand, the higher cyclic AMP content of vitamin D_3 deficient islets could be related to an increase in glucagon-induced cyclic AMP synthesis in relation to the hyperglucagonaemia previously observed in vitamin D_3 deficient rats. Since higher concentrations of exogenous glucagon and higher endogenous cyclic AMP concentrations were required *in vitro* to restore insulin release to normal values, the cyclic AMP-dependent pathways that usually potentiate insulin secretion appeared to be less efficient in relation to an alteration in the post cyclic AMP effector system.

7 $1,25(OH)_2D_3$ exerted a stimulating effect on insulin release via protein kinase A activation but reduced the supranormal cyclic AMP synthesis, thus exerting a differential modulatory influence on biochemical disturbances in islets induced by vitamin D_3 deficiency.

Keywords: Endocrine pancreas; β-cell; $1,25(OH)_2D_3$; cyclic AMP; glucagon; islet; insulin release; vitamin D_3 deficiency

Introduction

Vitamin D_3 is necessary for normal release of insulin and also for the maintenance of normal glucose tolerance in man (Boucher *et al*., 1985; Raghuramulu *et al*., 1993), besides its effect on calcium-phosphorus metabolism (Walters, 1992). In experimental animals, vitamin D_3 deficiency is known to induce a severe decrease in insulin secretion, that can be reversed by 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) treatment (Clark *et al*., 1980; Chertow *et al*., 1983; Kadowaki & Norman, 1985a; Billaudel *et al*., 1990). This positive action of $1,25(OH)_2D_3$ on the endocrine pancreas, a non classical target tissue (Walters, 1992), is mediated via $1,25(OH)_2D_3$ receptor (Clark *et al*., 1980; Stumpf *et al*., 1981; Ishida & Norman, 1988) as attested by the specificity of $1,25(OH)_2D_3$ as compared to other inactive vitamin D_3 metabolites (Kadowaki & Norman, 1995b; Faure *et al*., 1991). This positive effect of $1,25(OH)_2D_3$ on insulin release from β-cells could be mediated by an effect on one or several transduction pathways which are known to play an important role in excitation-secretion coupling (Henquin, 1985; Zawalich & Rasmussen, 1992). In a previous study we provided evidence for a disruptive effect of vitamin D_3 deficiency, which was corrected by $1,25(OH)_2D_3$ treatment, on calcium handling: both as regards to Ca^{2+} entry and Ca^{2+} mobilization from intracellular stores. This beneficial influence of $1,25(OH)_2D_3$ on insulin release was observed during glucose stimulation but not in basal conditions (Billaudel *et al*., 1988; 1990; 1993). Furthermore, we demonstrated, in parallel to the increased insulin response, a positive effect of $1,25(OH)_2D_3$ treatment on the β-cell phospholipid pathway in vitamin D_3 deficient rats. Indeed, during stimulation with acetylcholine, $1,25(OH)_2D_3$ enhanced both phosphoinositide hydrolysis and the rapid mobilization of Ca^{2+} stores, as well as Ca^{2+} entry through Ca^{2+} channels by protein kinase C activation (Billaudel *et al*., 1995). The hypothesis that $1,25(OH)_2D_3$ acts on the adenylyl cyclase signalling pathway within the islets of Langerhans is supported by the positive effect on insulin secretion observed in islets from vitamin D_3 deficient rats (Billaudel *et al*., 1993) during stimulation by barium + theophylline, which is known to increase adenosine 3′:5′-cyclic monophosphate (cyclic AMP) via activation of adenylyl cyclase and a concomitant inhibition of phosphodiesterase activity (PDE) (Malaisse, 1973a,b; Sener & Malaisse, 1979). This effect of $1,25(OH)_2D_3$ on the adenylyl cyclase system has been observed in other tissues, either as activation in duodenal cells (Long *et al*., 1986) and in muscle (De Boland & Boland, 1994), or as an inhibitory effect on hypophyseal cells (Sornes *et al*., 1994) and thyroid cells (Berg *et al*., 1994). For these reasons, we studied, in parallel, insulin secretion and the cyclic AMP pathway of islets from normal,

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vitamin D_3 deficient and vitamin D_3 deficient rats treated for one week with $1,25(OH)_2D_3$.

Methods

Experiments were performed in three independent groups of Wistar rats (CERJ, Le Genest-Saint-Isle, France): normal rats, 4 week-vitamin D_3 deficient rats and 1 week-$1,25(OH)_2D_3$ treated rats. Vitamin D_3 deficient rats were prepared as follows. New-born rats were kept with their mother in a dark room throughout the experiments. After weaning, from post natal day 21 and thereafter, they received a rachitogenic diet (US Biochemical Corporation Cleveland, OH, U.S.A.) devoid of vitamin D_3 but containing low calcium (0.50%, w/w) and low phosphate (0.30%, w/w). At 7 weeks of age (i.e. after 4 weeks of vitamin D_3 depletion) we obtained a group of vitamin D_3 deficient rats which were intended for comparisons with normal rats. Some of the vitamin D_3 deficient rats received, during the final week, a $1,25(OH)_2D_3$ treatment (intraperitoneal injection: 1 μg kg^{-1} day^{-1} in ethanol vehicle and NaCl 0.9% (v/v) over a 7 day period). All animal experiments were carried out in accordance with the guidelines laid down by the Ministère de l'Agriculture et du Développement Rural.

Experiments were performed in pancreatic islets isolated by collagenase pretreatment (Lacy & Kostianovsky, 1967). Islets from the three groups of rats were run, in parallel, in the same experimental conditions. Groups of 20 islets were incubated in microvials for 1 h at 37°C in Krebs-Ringer bicarbonate (0.5% albumin) medium (KRBA) in the presence of various stimuli or inhibitors.

DO-cyclic AMP was used to mimic the effects of exogenous cyclic AMP with a greater activity, because it easily permeates cell membranes and is not deactivated by cyclic nucleotide phosphodiesterases (Nakamura *et al*., 1979). Since it is a non-hydrolysable compound, it exerts, as does endogenous cyclic AMP, a prolonged action on β-cell insulin secretion mediated by protein kinase A activation. This potentiating effect of DO-cyclic AMP on insulin response to glucose (8.3 mmol l^{-1}) stimulation was studied in groups of 20 islets during a 1 h incubation. DO-cyclic AMP was dissolved in methanol and then diluted in KRBA medium containing 1 mmol l^{-1} Ca^{2+}.

In a further experiment, a combination of 2 mmol l^{-1} barium + 1.4 mmol l^{-1} theophylline was used to stimulate the islets insulin release in the absence of extracellular Ca^{2+} and glucose. Experiments were performed in 20 islets incubated for 1 h with or without the stimulus in the KRBA medium. Barium, a calcium agonist, can stimulate adenylyl cyclase (Sener & Malaisse, 1979), whereas theophylline, a phosphodiesterase inhibitor (Allen *et al*., 1973; Beavo & Reifsnyder, 1990) increases cyclic AMP content by an inhibitory effect on its enzymatic degradation system (Cheung, 1970). Both Ba^{2+} and theophylline can massively displace Ca^{2+} from its intracellular stores (Malaisse, 1973a,b; Brisson & Malaisse, 1973).

Another experiment to test the hypothesis that the mechanism of action of $1,25(OH)_2D_3$ is *post* cyclic AMP was performed when the maximal effect of DO-cyclic AMP (10^{-4} mol l^{-1} DO-cyclic AMP + 8.3 mmol l^{-1} glucose) was apparent for each of the three groups of animals, in the absence and presence of H-89 (10^{-5} mol l^{-1}), a selective and potent inhibitor of cyclic AMP-dependent protein kinases (Chijiwa *et al*., 1990). H-89 was dissolved in dimethyl sulphoxide and then diluted in KRBA.

At the end of all the incubations the supernatant was kept for the determination of insulin release by radioimmunoassay according to Herbert *et al*. (1965), with rat insulin as a standard (Novo Laboratories, Paris, France and Copenhagen, Denmark). Islets were stored for sonication before measurement of cyclic AMP content as an aliquot treated with 10% trichloracetic acid to precipitate proteins. After centrifugation, the supernatant was washed 5 times with diethyl ether and then evaporated under vacuum. The dry extract was dissolved in the assay buffer for cyclic AMP determination by radioimmunoassay (kit n° 1117 Immunotech, Marseille, France). The cyclic AMP extraction rate (>90%) was verified by the use of ^{125}I-labelled cyclic AMP run in parallel with the assays.

The cyclic AMP degradation, mediated via various phosphodiesterases (PDE), was studied within the islets of Langerhans. Since some of them are calcium-calmodulin dependent, whereas others are calcium-calmodulin independent (Valverde & Malaisse, 1984), the total and calcium-calmodulin dependent phosphodiesterase activities were measured within islets via the hydrolysis of labelled cyclic AMP, in the absence or in the presence respectively, of EGTA. Phosphodiesterase activity was determined following the method of Thompson & Appelman (1971), applied to islets according to Sugden *et al*. (1979). Briefly, groups of 100 isolated islets in 500 μl buffer (20 mmol l^{-1} Tris, 2 mmol l^{-1} magnesium acetate, 250 mmol l^{-1} sucrose; 2 mmol l^{-1} benzamidine; 2000 iu ml^{-1} iniprol) were homogenized via transit through a Millipore filter (Millipore Corporation, Bedford, MA, U.S.A.). Aliquots of 50 μl (i.e. 10 islets equivalents) were kept for protein determination (Biorad, Richmond, CA, U.S.A.) according to Bradford (1976). Phosphodiesterase was determined either in the presence of 1 mmol l^{-1} EGTA (ethylene glycol-bis (β-amino-ethyl ether) N,N,N′,N′-tetra-acetic acid, Sigma) to inhibit Ca^{2+}-calmodulin dependent PDE, or its absence but with 10 μmol l^{-1} $CaCl_2$ and 18 nmol l^{-1} calmodulin to activate calcium-calmodulin dependent PDE to measure the total PDE activity. These 50 μl aliquots, corresponding to 10 islets, provided the optimal concentration for maximal sensitivity of the total PDE activity assay, according to Sugden *et al*. (1979). PDE activity was determined by hydrolysis of cyclic AMP in the presence of labelled cyclic AMP ([2,8-^{3}H]-cyclic AMP; 1.85 Tbq mmol l^{-1}, Amersham France SA, Les Ullis, France) as a marker following a two step hydrolysis: first, cyclic AMP hydrolysis in 5′-AMP (30 min., 30°C incubation), stopped with a 'PDE stop solution' (100 mmol l^{-1} Tris; 50 mmol l^{-1} EDTA, 30 mmol l^{-1} theophylline, 10 mmol l^{-1} cyclic AMP) and a second hydrolysis of 5′-AMP to adenosine (20 min incubation with 10 mg ml^{-1} *Crotalus atrox* snake venom nucleotidase, 100 mmol l^{-1} Tris, pH 8), stopped with 'SV stop solution' (0.1 mmol l^{-1} adenosine; 15 mmol l^{-1} EDTA; pH 7.5). Adenosine was separated from other nucleotides by elution through a ion exchange resin column (AG1-X2, 200-400 mesh, Bio-Rad, Richmond, CA, U.S.A.), rinsed with 20 mmol l^{-1} ammonium acetate, pH 4. The labelled product was counted in a β spectrophotometer (Prias, PL, Packard). After islets protein content determination (Bradford 1976), results are expressed as fmol cyclic AMP degradation min^{-1} μg^{-1} protein.

To study the cyclic AMP synthesis induced by glucagon, exogenous glucagon was used at various concentrations ranging from 0 to 10^{-6} mol l^{-1}. Glucagon was added to the Krebs Ringer bicarbonate incubation medium (which contained albumin), in the presence of a non stimulating concentration of glucose (5.5 mmol l^{-1}). Theophylline (1.4 mmol l^{-1}) was added to inhibit cyclic AMP degradation, and 2000 iu ml^{-1} iniprol, a protease inhibitor was added to avoid glucagon degradation in the incubation medium (Rohner-Jeanrenaud & Jeanrenaud, 1984; Faure *et al*., 1988).

Materials

Collagenase and adenosine were obtained from Boehringer Co., Mannheim, Germany. Synthesized crystalline $1,25(OH)_2D_3$ was obtained from Hoffman-La-Roche (Basel, Switzerland). Iniprol was purchased from Choay-Sanofi (Gentilly, France) and glucagon from Novo Nordisk Pharmaceutique (Boulogne, France). Dioctanoyl-cyclic AMP (DO-cyclic AMP) and N-[2-(p-bromocinnamylamino) ethyl] - 5 - isoquinolinesulphonamide (H-89) were from Calbiochem-Novabiochem Corporation (La Jolla, CA, U.S.A.). Barium (chlorine), theophylline, benzamide, EGTA, EDTA and *Crotalus Atrox* snake venom nucleotidase were purchased from Sigma-Aldrich Chimie (St.

Quentin Fallavier, France). All other chemicals were of analytical grade (Merck, Darmstadt, Germany).

Statistical analysis

This was performed by use of unpaired Student's *t* test and values are expressed as means ± s.e.means; *n* represents the number of assays (from five to six rats) in at least three experiments. Correlation analysis (coefficient) was determined by least squares linear regression applied to concentrations of stimuli expressed as logarithmic values.

Results

Effect of exogenous enhancement of islets cyclic AMP induced by dioctanoyl-cyclic AMP (DO-cyclic AMP), a cyclic AMP analogue, on insulin release

As seen in Figure 1, the insulin response to 8.3 mmol l^{-1} glucose was significantly potentiated by DO-cyclic AMP ($P<0.05$ from 10^{-7} mol l^{-1} DO-cyclic AMP and upwards)

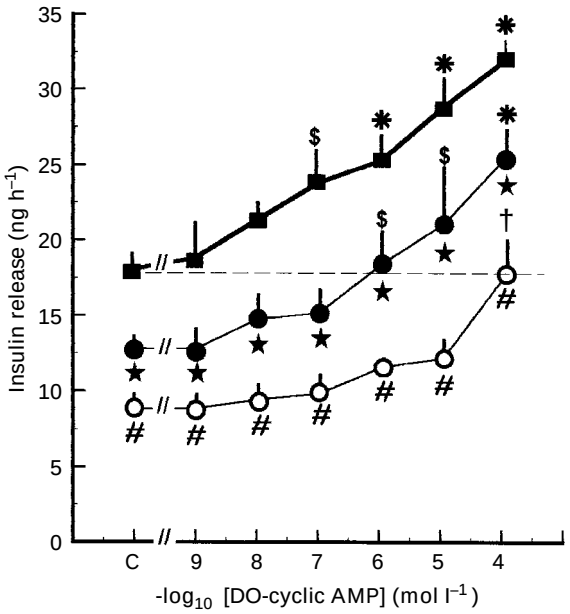

Figure 1 The effect of DO-cyclic AMP (cyclic AMP mimetic) on insulin release in response to 8.3 mmol l^{-1} glucose in islets from normal rats (N, (■), $n=12$), vitamin D_3 deficient (−D, (○), $n=14$) and one week $1,25(OH)_2D_3$-treated rats (+D, (●), $n=8$). Islet incubations were performed with DO-cyclic AMP or with its vehicle as control (C). Values shown are means and vertical lines indicate s.e.mean. #$P<0.001$, −D versus N for respective concentrations. ★$P<0.05$, +D versus −D for respective concentrations. \$$P<0.05$, †$P<0.01$, *$P<0.001$, vs own control assay without DO-cyclic AMP.

and this effect was dose-dependent ($r=0.994$, $y=2.565\times+41.61$, $n=72$) from 10^{-9} to 10^{-4} mol l^{-1} DO-cyclic AMP for islets from normal rats. The insulin response of islets from vitamin D_3-deficient rats was very low ($P<0.001$ versus normal) for each DO-cyclic AMP concentration studied. This insulin secretion could be significantly potentiated by DO-cyclic AMP only at a concentration of 10^{-4} mol l^{-1} ($P<0.01$) when compared to the control response in the absence of DO-cyclic AMP. The $1,25(OH)_2D_3$ treatment improved the DO-cyclic AMP potentiation of the insulin response to glucose ($P<0.05$ vs vitamin D_3 deficient islets) for all DO-cyclic AMP concentrations tested. This beneficial effect exerted by $1,25(OH)_2D_3$ on the DO-cyclic AMP potentiating effect on insulin response to glucose was significant ($P<0.05$), compared to the control response in the absence of DO-cyclic AMP, from a concentration of 10^{-6} mol l^{-1} upwards. It was of interest to compare these results with the response of islets from normal rats, obtained without any potentiation by exogenous cyclic AMP, that is to say in the absence of DO-cyclic AMP as a control assay. A similar value to controls could only be reached in vitamin D_3 deficient islets at the highest DO-cyclic AMP concentration used, 10^{-4} mol l^{-1}, revealing the very poor efficiency of DO-cyclic AMP in vitamin D_3 deficient islets, whereas the islets from $1,25(OH)_2D_3$-treated rats required a much lower DO-cyclic AMP concentration (between 10^{-7} and 10^{-6} mol l^{-1}), suggesting that $1,25(OH)_2D_3$ facilitated or rendered more efficient the potentiating effect of cyclic AMP on the insulin response to glucose.

Activation of insulin release by an enhancement of endogenous cyclic AMP induced by 2 mmol l^{-1} barium + 1.4 mmol l^{-1} theophylline

Table 1 shows that barium + theophylline, in the absence of Ca^{2+} and glucose, enhance both islet cyclic AMP content and insulin secretion from islets of each of the three groups of rats, as compared to the respective basal conditions ($P<0.001$). The deleterious effect of a 4 week-vitamin D_3 deficiency on insulin secretion, observed during this stimulus but not in basal conditions, was confirmed ($P<0.001$, versus normal rats). Surprisingly, this decrease in insulin release was associated with a net increase in cyclic AMP content of islets from vitamin D_3 deficient rats ($P<0.001$). However, in basal conditions, the islets cyclic AMP content was not statistically different between control and vitamin D_3 deficient rats.

In further experiment a time-course study was performed from week 2 to week 4 of vitamin D_3 deficiency. It showed that this increase in cyclic AMP content of vitamin D_3 deficient rat islets, observed at week 4 could be detected from the third week of vitamin D_3 deprivation (1376 ± 242, $n=16$ versus 559 ± 76 fmol/20 islets, $n=7$, $P<0.01$) but not on week 2 (674 ± 156, $n=6$ versus 656 ± 148 fmol/20 islets, $n=6$). The islets insulin response to barium + theophylline, measured in parallel, was also decreased after 3 weeks of vitamin D_3 deficiency (34.7 ± 3.6, $n=16$ versus 51.3 ± 8.3 ng/20 islets, $n=7$, $P<0.01$) but not after 2 weeks (23.8 ± 3.9, $n=6$ versus

Table 1 Effect of barium (2 mmol l^{-1}) + theophylline (1.4 mmol l^{-1}) versus basal conditions, on parallel measurements of insulin release, cyclic AMP content and insulin content of twenty islets from normal rats (N), vitamin D_3 deficient (−D) or one week $1,25(OH)_2D_3$ treated rats (+D)

	Basal conditions			*Barium + theophylline*		
	N ($n=24$)	*−D* ($n=25$)	*+D* ($n=16$)	*N* ($n=24$)	*−D* ($n=28$)	*+D* ($n=17$)
Insulin release (ng h^{-1})	15.3 ± 3.3	11.2 ± 1.7	12.5 ± 1.7	80.0 ± 5.2†	53.4 ± 3.0†,*	81.7 ± 7.2†,\$
Cyclic AMP content (fmol)	396 ± 34	450 ± 38	233 ± 35\$	1708 ± 165†	2553 ± 188†,*	1550 ± 238†,\$
Insulin content (ng)	942 ± 71	1081 ± 51	1055 ± 95	938 ± 57	1045 ± 58	1094 ± 74

Incubations were performed in the absence of extracellular calcium and glucose. *$P<0.001$ vs N rats during stimulation; \$$P<0.001$ vs (−D) rats during stimulation. †$P<0.001$ vs respective basal values.

26.3 ± 3.8 ng/10 islets, $n = 6$). In contrast in basal conditions, the cyclic AMP content of vitamin D_3 deficient rats islets was not significantly modified at 3 weeks of vitamin D_3 deficiency (243 ± 21, $n = 12$, versus 183 ± 23 fmol/20 islets, $n = 9$) or 2 weeks (183 ± 20, $n = 6$, versus 175 ± 15 fmol/20 islets, $n = 6$). Neither was the basal insulin release altered after 3 weeks of vitamin D_3 deficiency (5.9 ± 1.4, $n = 12$ versus 9.3 ± 1.2 ng/20 islets, $n = 9$), or after 2 weeks (6.6 ± 0.5, $n = 6$ versus 7.9 ± 1.1 ng/20 islets, $n = 6$). On the other hand, $1,25(OH)_2D_3$ applied to 4 weeks vitamin D_3 deficient rats reversed the effects on both the insulin response to barium + theophylline and the cyclic AMP content of the islets, as seen in Table 1. The $1,25(OH)_2D_3$ treatment enhanced insulin secretion ($P < 0.001$) and decreased the islet cyclic AMP content to normal values ($P < 0.001$). Once more, these effects observed during the barium + theophylline stimulation were not seen in basal conditions, except for the cyclic AMP content which was decreased, and could not be attributed to variations in individual islets as the insulin contents of the islets was homogeneous (Table 1).

The mean insulin release/cyclic AMP content ratio from the same islets (week 4) was calculated to give a measure of cyclic AMP efficiency on insulin release. It was 0.035 in normal rats but was decreased to 0.021 in vitamin D_3 deficient rats and then was increased to 0.053 by $1,25(OH)_2D_3$.

Inhibition of the cyclic AMP effect by H-89, a selective AMP-dependent protein kinase inhibitor

The potentiating influence of DO-cyclic AMP on the insulin response to 8.3 mmol l^{-1} glucose was confirmed within each of the three groups and H-89 was shown to reduce insulin secretion within the three groups of animals ($P < 0.001$) (Table 2). However, the deleterious effect of vitamin D_3 deficiency on insulin secretion persisted ($P < 0.001$ versus normal), providing evidence that vitamin D deficiency alters insulin secretion via mechanisms other than the cyclic AMP pathway. In the $1,25(OH)_2D_3$-treated rat islets group, H-89 not only decreased the insulin release back to the level seen in the vitamin D_3 deficient group, but also close to that seen in the vitamin D_3 deficient group in the absence of DO-cyclic AMP activation and below the $1,25(OH)_2D_3$ treated rat islets without DO-cyclic AMP ($P < 0.05$), suggesting that $1,25(OH)_2D_3$ was no longer efficient in improving insulin secretion when cyclic AMP-dependent protein kinases were inhibited.

Study of the paradoxical increase in cyclic AMP observed in islets from vitamin D_3 deficient rats

The supranormal increase of cyclic AMP levels in vitamin D_3 deficient islets seen during barium + theophylline stimulation may be due not only to the loss of cyclic AMP efficiency but also to a disturbance of phosphodiesterase activity, or to a supranormal cyclic AMP synthesis induced by glucagon. Phosphodiesterase activity and glucagon-stimulated cyclic AMP synthesis were, thus, measured.

As shown in Table 3, total PDE activity was not significantly different between normal, vitamin D_3 deficient and $1,25(OH)_2D_3$ treated rat islets. However the relative proportions of calcium-calmodulin dependent or independent fractions were somewhat modified. The results showed that the calcium-calmodulin independent phosphodiesterase fraction was increased in vitamin D_3 deficient islets ($P < 0.05$) whereas is was reduced towards normal with the $1,25(OH)_2D_3$ treatment. Indeed, it was 63.3% of total PDE activity in normal islets, increased to 96.4% in vitamin D_3 deficient islets and reduced to 81.6% in the $1,25(OH)_2D_3$-treated group.

When islets were stimulated by glucagon, (Figure 2), their insulin secretion and cyclic AMP content increased in a dose-dependent manner in normal rats (respectively: $r = 0.999$, $y = 2.025 \times + 33.2$, $n = 18$ and $r = 0.996$, $y = 82.25 \times + 1165$, $n = 28$ from 10^{-10} to 10^{-6} mol l^{-1} glucagon). The insulin secretion in response to glucagon was much lower in vitamin D_3 deficient rats than in normal rats, with all glucagon concentrations studied. Moreover, vitamin D_3 deficient islets were less sensitive to glucagon than normal islets, as a 10^{-6} instead of 10^{-8} mol l^{-1} glucagon was needed to activate significantly the insulin release ($P < 0.01$ and $P < 0.05$, as compared to their own control without glucagon). However, $1,25(OH)_2D_3$ administered to vitamin D_3 deficient rats, improved the insulin response of the islets to glucagon, an effect which was statistically significant ($P < 0.05$ versus vitamin D_3 deficient islets) from 10^{-8} mol l^{-1} glucagon upwards ($P < 0.001$).

In contrast to the low insulin secretion of islets from vitamin D_3 deficient rats, the islets cyclic AMP content measured in parallel during the glucagon stimulation showed a large increase in the vitamin D_3 deficient group versus normal rats ($P < 0.05$ for all tested concentrations tested from 10^{-10} to 10^{-6} mol l^{-1}). This increase was reversed to normal levels by $1,25(OH)_2D_3$ treatment ($P < 0.05$ versus vitamin D_3 deficient islets) for each concentration tested from 10^{-10} to 10^{-6} mol l^{-1} glucagon.

Table 3 Total phosphodiesterase activity and Ca^{2+} −calmodulin independent phosphodiesterase activity in islets from normal rats (N), vitamin D_3 deficient (−D) or one week $1,25(OH)_2D_3$ treated rats (+D)

	N ($n = 5$)	*−D* ($n = 8$)	*+D* ($n = 7$)
Total activity	39.5 ± 4.8	41.5 ± 4.2	37.0 ± 2.8
Ca^{2+}-calmodulin independent activity	25.0 ± 4.6	40 ± 3*	30.2 ± 4.1

Total activity was measured within islets via hydrolysis of labelled cyclic AMP in the presence of calcium (10 μmol l^{-1}) and calmodulin (18 nmol l^{-1}) whereas Ca^{2+} -calmodulin independent phosphodiesterase activity was determined in the presence of EGTA (1 mmol l^{-1}) and in the absence of calmodulin in the incubation medium. *$P < 0.05$ vs N rats. Phosphodiesterase activity is expressed as fmol cyclic AMP degradation min^{-1} μg^{-1} protein.

Table 2 Effect of DO-cyclic AMP and H-89 on insulin release in response to glucose and on cyclic AMP content of the islets from normal rats (N), vitamin D_3 deficient (−D) or one week $1,25(OH)_2D_3$ treated rats (+D), during 1 h incubations

	Insulin release (ng h^{-1})		
	N ($n = 11$)	*−D* ($n = 24$)	*+D* ($n = 18$)
Glucose (8.3 mmol l^{-1})	19.1 ± 1.1	10.8 ± 0.6‡	14.5 ± 1.0†
Glucose (8.3 mmol l^{-1}) + DO-cyclic AMP (10^{-4} mol l^{-1})	28.0 ± 2.6***	15.9 ± 1.2‡,**	20.1 ± 1.8***,††
Glucose (8.3 mmol l^{-1}) + DO-cyclic AMP (10^{-4} mol l^{-1}) + H-89 (10^{-5} mol l^{-1})	18.4 ± 0.5#	10.8 ± 1.1‡,#	10.5 ± 1.4*,#

‡$P < 0.001$ vs normal rats; †$P < 0.05$ and ††$P < 0.01$ vs (−D) rats; #$P < 0.001$ vs respective conditions without H-89; *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$ vs 8.3 mmol l^{-1} glucose alone).

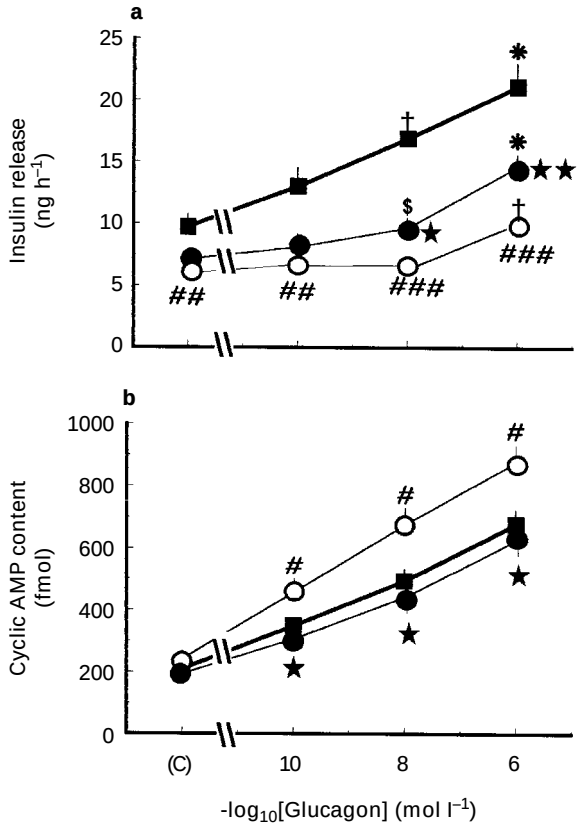

Figure 2 Glucagon dose-effect curve for (a) insulin release and (b) cyclic AMP content of the same ten islets from normal rats ((■,N), vitamin D_3 deficient rats (○, −D) and one week 1,25$(OH)_2D_3$-treated rats. (●, +D). Incubations of islets were performed in the presence of a non-stimulating concentration of glucose (5.5 mmol l^{-1}), but with 1.4 mmol l^{-1} theophylline and 2000 iu ml^{-1} iniprol, with glucagon or its vehicle as control (C). Values are means and vertical lines show s.e.mean, $n=6$ assay for each point. #$P<0.05$, ##$P<0.01$, ###$P<0.001$, −D versus N for respective concentrations. ★$P<0.05$, ★★$P<0.01$, +D versus −D for respective concentrations. \$$P<0.05$, †$P<0.01$, *$P<0.001$: vs own control assay without glucagon.

Discussion

The regulation of insulin release involves several mechanisms including both secretagogue types: initiators, such as glucose or calcium, which are independently able to increase insulin secretion (Ashcroft & Aschroft, 1992) and potentiators, such as acetylcholine (Sharp *et al*., 1974), β-adrenoceptor agonists, glucagon (Sharp, 1979; Malaisse & Malaisse-Lagae, 1984) and cyclic AMP (Henquin, 1985), which are ineffective alone but which potentiate insulin secretion in the presence of glucose (Portha, 1991). We have previously shown that vitamin D_3 deficiency impairs insulin release via several different mechanisms: either in response to initiators such as glucose or calcium (Billaudel *et al*., 1988; Labriji-Mestaghanmi *et al*., 1988) or in response to potentiators, such as acetylcholine (Billaudel *et al*., 1995). However, the basal insulin secretion was not significantly affected. The administration of 1,25$(OH)_2D_3$, *in vivo* or *in vitro*, is able to improve deficient insulin release (Billaudel *et al*., 1989) by different mechanisms including Ca^{2+} entry by calcium channels (Billaudel *et al*., 1990; Faure *et al*., 1991) or Ca^{2+} mobilization from intracellular stores (Billaudel *et al*., 1993) mediated by a stimulating effect on both the glucose metabolic pathway and the phosphoinositide pathway, including the production of inositol trisphosphates and the activation of protein kinase C (Billaudel *et al*., 1995). However, few studies concerning the adenylyl cyclase pathway have been undertaken. Thus, in the present study we examined the respective influences of vitamin D_3 deficiency and 1,25$(OH)_2D_3$ treatment on the signal-transducing systems via cyclic AMP and cyclic AMP-dependent-protein kinase (PKA) necessary for protein phosphorylation implicated in insulin exocytosis mechanisms.

Insulin release and the cyclic AMP pathway during vitamin D_3 deficiency

The acute activation of the cyclic AMP-PKA pathway usually stimulates insulin secretion from islets (Henquin, 1985; Schuit & Pipeleers, 1985). More precisely, cyclic AMP potentiates the insulin response to glucose, since a metabolic supply is required for cyclic AMP-induced insulin release (Henquin, 1985). In the present study, islets from 4 week vitamin D_3 deficient rats appeared to be less sensitive to cyclic AMP. Indeed, we did not succeed in reversing the impairment of insulin release of the vitamin D_3 deficient islets, except with very high concentrations of several stimuli of the adenylyl cyclase signalling pathway: either with exogenous glucagon that is known to increase the cyclic AMP synthesis (Pipeleers *et al*., 1985), with DO cyclic AMP, a cyclic AMP mimetic (Nakamura *et al*., 1979) or by inhibition of cyclic AMP hydrolysis with theophylline (Cheung, 1970). The present results support the hypothesis of a loss of cyclic AMP efficiency by a disturbance of *post* cyclic AMP mechanisms. This hypothesis was supported by the observation of a supranormal cyclic AMP content, found for the first time, in vitamin D_3 deficient islets, in spite of a low insulin secretion measured in parallel to cyclic AMP in the same islets, and confirmed by the decrease in the insulin release/cyclic AMP content ratio. This disturbance induced by vitamin D_3 deficiency was only observed when islets were activated, it was not apparent in basal conditions, suggesting a disturbance of the islets' ability to response to stimuli.

Relationship between the cyclic AMP increase and vitamin D_3 deficiency in rat islets

The supranormal increase in islet cyclic AMP levels observed after stimulation either by glucagon or by barium + theophylline, appeared to be related to the progressive disturbances induced by vitamin D_3 deficiency, since it was observable from the third week but not the second week of deficiency in the time-course study. Likewise, we have previously shown that the altered insulin response occurs from the third week of vitamin D_3 deficiency (Labriji-Mestaghanmi *et al*., 1988). This increase in cyclic AMP levels may not be the consequence of an inhibition of cyclic AMP degradation, as the total phosphodiesterase (PDE) activity within the islets was not modified. However, there was a decrease in Ca^{2+}-calmodulin-dependent PDE, which was compensated for by an increase in Ca^{2+}-calmodulin-independent PDE. This compensatory effect is compatible with the altered calcium metabolism previously observed in islets from vitamin D_3 deficient rats (Billaudel *et al*., 1993), which also occurs from the third week of vitamin D_3 deficiency (Billaudel *et al*., 1988; Labriji-Mestaghanmi *et al*., 1988).

Within the islets of Langerhans, there is an important interplay between α and β cells. Indeed, α-cell glucagon enhances insulin secretion from β-cells, acting as a cyclic AMP signalling pathway activator (Pipeleers *et al*., 1985) mediated by its membrane receptor, coupled to adenylyl cyclase by a G protein (Schuit & Pipeleers, 1985; Prentki & Matschinsky, 1987). The rise of cyclic AMP levels observed within vitamin D_3 deficient islets is more probably related to a glucagon-induced increase in cyclic AMP synthesis within β cells which represent 80% of islet cells. Indeed, we showed that the addition of exogenous glucagon increased the cyclic AMP content of vitamin D_3 deficient islets in a dose-dependent manner and with an enhanced sensitivity to glucagon stimulation, since cyclic AMP levels were higher in vitamin D_3 deficient than in normal islets. However, in spite of elevated cyclic AMP levels, the ability of glucagon to

stimulate insulin release was decreased, since higher concentrations of glucagon were required for a significant insulin response in the vitamin D_3 deficient islets. The hypothesis of a *post* cyclic AMP defect may be compatible with the surprising supranormal glucagonaemia of vitamin D_3 deficient rats previously found in our experimental conditions (in the range of 0.98×10^{-7} mol l^{-1} versus normal rats values 0.62×10^{-7} mol l^{-1}; Bourlon *et al*., 1996). We have shown that this hyperglucagonaemia is the result of an enhanced glucagon release from vitamin D_3 deficient islets (Bourlon *et al*., 1996). Moreover, we have previously found that hyperfunctioning of the α cell of the vitamin D_3 deficient islets could also be observed from the 3rd week of vitamin D_3 deficiency. Thus the supranormal functioning of the α cells may reflect a compensatory mechanism to activate the β-cell cyclic AMP pathway as a consequence of a *post* cyclic AMP defection in the effector system occurring from the 3rd week of vitamin D_3 deficiency. However, these data do not exclude the possibility that insulin release is inhibited consecutive to an excess of cyclic AMP via a reduction in the activity of the phospholipid pathway observed with different conditions by other authors (Zawalich & Zawalich, 1990; 1996), compatible with the alteration in the phospholipid pathway observed within islets from vitamin D_3 deficient rats in previous experiments (Billaudel *et al*., 1995).

Effect of 1,25(OH)$_2$D$_3$ on the islets cyclic AMP-PKA signalling pathway

In contrast to the disturbances induced by vitamin D_3 deficiency, $1,25(OH)_2D_3$ treatment improved the ability of cyclic AMP to induce insulin release, since cyclic AMP levels were decreased towards normal values and the islets insulin release was improved, leading to an increase of the insulin release/cyclic AMP content ratio. This beneficial effect of $1,25(OH)_2D_3$ occurred in islets stimulated by either exogenous glucagon or DO-cyclic AMP. The barium + theophylline stimulus that exerts a double action on both intracellular Ca^{2+} mobilization and the cyclic AMP-PKA pathway, in the absence of extracellular calcium or glucose, exhibited the best (quantitatively) restoration of insulin release after $1,25(OH)_2D_3$ treatment, drawing attention to the importance of intracellular calcium. The stimulant effect exerted by $1,25(OH)_2D_3$ on the insulin response to stimuli such as glucagon, DO-cyclic AMP or barium + theophylline, was mediated (at least in part) via the cyclic AMP effector system. It may be more particularly mediated via an action of this vitamin on protein kinase A, or a *post* protein kinase A mechanism, since the positive effect of $1,25(OH)_2D_3$ on the insulin response to DO-cyclic AMP disappeared in the presence of H-89, a cyclic AMP-dependent protein kinase inhibitor. The possibility that $1,25(OH)_2D_3$ activates the PKA pathway within islets needs to be investigated further. A similar effect has been described in thyroid cells with $1,25(OH)_2D_3$ (Berg *et al*., 1994) and also with retinoic acid in psoriatic cells (Tournier *et al*., 1995).

The influence exerted by $1,25(OH)_2D_3$ on the supranormal cyclic AMP content of islets may be a consequence not only of its beneficial effect on the *post* cyclic AMP effector system but also of its modulatory effect on glucagon secretion. Indeed experiments have shown that $1,25(OH)_2D_3$ is able to regulate the hyperfunctioning of α-cells from vitamin D_3 deficient rats, reducing glucagonaemia and glucagon secretion towards normal values (Bourlon *et al*., 1996). Such a negative regulatory influence of $1,25(OH)_2D_3$ on the cyclic AMP generating pathway, although non classical, has been observed in other tissues such as thyroid cells (Berg *et al*., 1993), GH_4C_1 pituitary cells (Sornes *et al*., 1994) and UMR 106 osteosarcoma cells (Mortensen *et al*., 1995).

Moreover, the decrease in cyclic AMP induced by $1,25(OH)_2D_3$ could not be attributed to an effect on cyclic AMP degradation, since total phosphodiesterase activity was not modified. However, it was interesting to note that $1,25(OH)_2D_3$ increased the proportion of Ca^{2+}-calmodulin dependent phosphodiesterase, a probable compensatory influence in relation to the positive effect exerted by this vitamin on Ca^{2+} handling (Faure *et al*., 1991; Billaudel *et al*., 1993).

In summary, we showed that the reduction of insulin release observed during vitamin D_3 deficiency, could be in part attributable to an alteration of a *post* cyclic AMP effector system. The present data also provide evidence in support of the hypothesis of a positive post translational influence of $1,25(OH)_2D_3$ on the cyclic AMP effector system, since protein kinase A activation, which is necessary for protein phosphorylation or synthesis, is implicated in insulin exocytosis. Thus, $1,25(OH)_2D_3$ exerts its modulatory effect on the different transduction signalling pathways within islets: Ca^{2+}, inositol triphosphates, diacylglycerol-protein kinase C, (as shown previously), and also cyclic AMP-PKA in order to restore insulin release disturbed by vitamin D_3 deficiency. Further experiments are needed to determine the direct effects of $1,25(OH)_2D_3$ and those which may only be the consequence of altered calcium homeostasis observed during vitamin D_3 deficiency.

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