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THE IN VITRO EFFECT OF 1α,25-DIHYDROXYVITAMIN D_3 ON INSULIN PRODUCTION BY NEONATAL RAT ISLETS

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The *in vitro* effect of 1α,25-dihydroxyvitamin D_3 on the function of beta cells of the endocrine pancreas was investigated. Neonatal islets maintained in serum-free medium, or medium supplemented with 0.5% fetal bovine serum achieved a 2.5-fold increase in medium insulin levels in response to 10^{-8}M 1α,25-dihydroxyvitamin D_3 ($P<0.001$). The effect of 1,25-dihydroxyvitamin D_3 required at least 96 h treatment to become evident and was similar at medium glucose concentrations of 10 and 20 mM. Cell-associated insulin was increased in 1α,25-dihydroxyvitamin D_3-treated cultures maintained in 0.5% serum. These data suggest that 1α,25-dihydroxyvitamin D_3 may have a direct effect in the beta cell.

The hormone 1,25-$(OH)_2D_3$ may affect the function of many tissues, or cells, not primarily involved in Ca^{2+} homeostasis (1). Receptors, or specific binding for the hormone have been demonstrated in a number of tissues other than those normally considered to be involved in Ca^{2+} homeostasis. These include the endocrine pancreas and the anterior pituitary (2); in the latter, 1,25-$(OH)_2D_3$ specifically increases hormone secretion in both normal and neoplastic pituitary cells *in vitro* (3,4). The presence of receptors for 1,25-$(OH)_2D_3$ in beta cells suggests that 1,25-$(OH)_2D_3$ may affect their function as well.

Vitamin D-deficient rats have impaired glucose tolerance and decreased insulin secretion (5). Several *ex vivo* studies have demonstrated impaired insulin secretion associated with vitamin D deficiency (6,7). However, vitamin D deficiency is associated with metabolic derangements which may impair insulin secretion, including hypocalcemia (8), and reduced food intake and body weight (9). Recent *ex vivo* studies have minimised possible effects of reduced body mass and hypocalcemia (10), but a direct effect of 1,25-$(OH)_2D_3$ in the beta cell has yet to be confirmed. We have investigated the direct effect of 1,25-$(OH)_2D_3$ in the beta cell *in vitro*. A neonatal islet cell culture system was utilised and the cell culture conditions were based on those used to demonstrate an effect of 1,25-$(OH)_2D_3$ on prolactin production by cultured GH_4C_1 cells (4).

ABBREVIATIONS: 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; FCS, fetal calf serum; CTFCS, charcoal-treated fetal calf serum.

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METHODS

Islet Cultures

Primary cultures of neonatal rat islets were prepared from 12-20 neonatal (less than 12 h old) Sprague-Dawley rats, using previously published methods (11,12). Hanks' balanced salt solution (C.S.L., Melbourne, Australia), pH 7.4, containing 10 mM glucose, 100 mg/l streptomycin and 10^5 U/l penicillin was used throughout the isolation procedure. Briefly, the pancreases were removed aseptically, cut into pieces less than 1 mm^3 and placed in 10 ml 0.25 % (wt/vol) collagenase (type V; Sigma, St. Louis, MO, USA) until the tissue began to break apart. Isolated islets were obtained by gentle trituration using a plastic transfer pipette. Equivalent numbers of islets (usually representing 1/3 to 1/2 of a pancreas per dish) were plated out in 6 cm dishes (Flow Laboratories, Sydney, Australia) in 3 ml of RPMI 1640 medium containing 10 % FCS and 15 mM HEPES. After 2 to 3 days, the medium and non-adherent islets were removed and 3 ml Ham's F12 medium (Gibco, Grand Is, NY, USA) with 1 % FCS was added. After a further 2 days, this medium was replaced with the experimental medium which was serum-free modified Ham's F12 medium (4). The glucose concentration of the medium was 10 mM and the Ca^{2+} concentration was 1.2 mM. The cultures were maintained in the serum-free conditions for up to 8 days, with medium changes being performed every 48 h. The medium which was removed was centrifuged at 750 x g, and the supernatant stored at -20 C prior to assay. In later experiments, the experimental medium was supplemented with charcoal-treated fetal calf serum (CTFCS) (13) as indicated.

Analytical Methods

Insulin production by the islets was assessed by measuring the amount of insulin secreted into the medium over each 48 h culture period. To assess insulin degradation over this period, [^{125}I]-insulin was added to the cultures. Cell-associated insulin was assessed after mobilising the cells with a rubber policeman in Ca^{2+}-Mg^{2+}-free phosphate-buffered saline containing 3 mM EDTA (pH 7.4). The cells in suspension were disrupted by sonication at 4 C for 45 sec, and stored at -20 C prior to assay.

The insulin content of the medium and cells was measured by radioimmunoassay, using a guinea pig anti-porcine insulin antibody (RD10; Wellcome, Sydney, Australia).

The total protein content of the sonicated cell suspension was determined according to the method of Bradford (14).

The data were analysed with Student's *t*-test for unpaired observations, and analysis of variance using a generalised linear model (15).

RESULTS

Cultures were maintained in the serum-free experimental medium for up to 8 days, and exposed to 10^{-10}-10^{-8}M 1,25-$(OH)_2D_3$ or vehicle. There was a 30% decrease in trichloroacetic acid-precipitable [^{125}I]-insulin over 48 h, with no difference between 1,25-$(OH)_2D_3$-treated and control cultures, indicating comparable low rates of insulin degradation. Therefore, the insulin content of the medium was considered to be an index of total insulin secretion over 48 h. Vehicle-treated cultures continued to produce insulin at a consistent rate over this period (fig.1). Cultures exposed to 10^{-8}M 1,25-$(OH)_2D_3$ had higher medium insulin levels overall ($P< 0.001$; 15), with a progressive increase in insulin levels over the initial 6 days of treatment. Significantly increased medium insulin levels were evident after the cultures had been exposed to 1,25-$(OH)_2D_3$ for 96 h or longer ($P< 0.05$): 1,25-$(OH)_2D_3$-treated cultures achieved a 125 % increase in medium insulin compared with controls during the third 48 h treatment period. Under these conditions, there was no significant increase in cell-associated insulin [without 1,25-$(OH)_2D_3$, 449 ± 230 ng/dish (n=4); with 1,25-$(OH)_2D_3$,

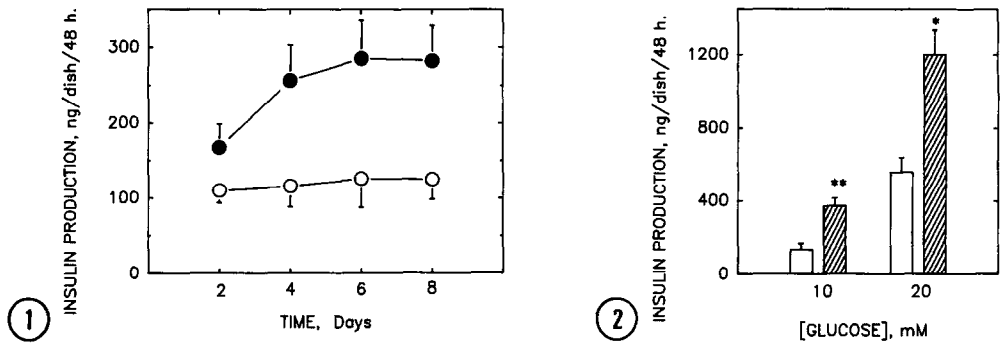


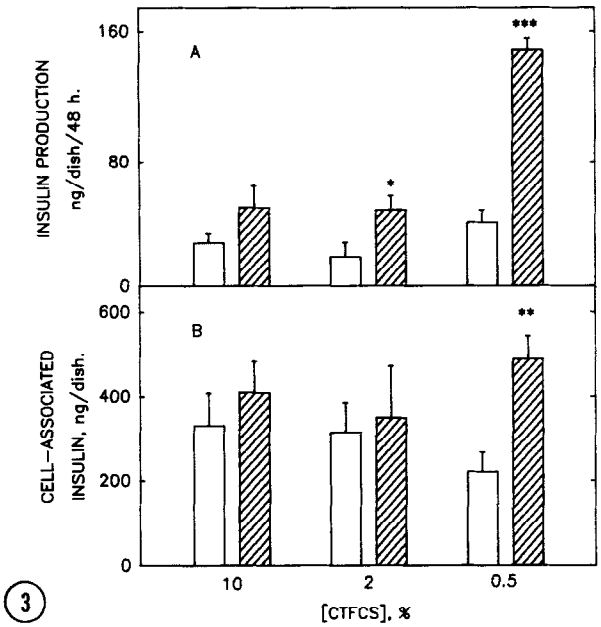
Fig. 1. THE EFFECT OF 1,25-$(OH)_2D_3$ ON INSULIN ACCUMULATION IN THE MEDIUM. Cultures were prepared as in the Methods section and maintained in serum-free experimental medium in the presence of 10^{-8}M 1,25-$(OH)_2D_3$ or ethanol-containing vehicle. Shown are the amounts of insulin which accumulated in the medium during each 48 h treatment period with (●) or without (○) 1,25-$(OH)_2D_3$. Each point represents the mean ± S.E.M. of 5 replicate cultures. (* = P< 0.05 compared with vehicle-treated cultures at the same time point).

Fig. 2. THE EFFECT OF GLUCOSE AND 1,25-$(OH)_2D_3$ ON INSULIN ACCUMULATION. Cultures were maintained in the serum-free experimental medium containing 10 mM or 20 mM glucose, plus 10^{-8} 1,25-$(OH)_2D_3$ or vehicle for 8 days. Shown are the amounts of insulin which accumulated in the medium during the third 48 h incubation period in the presence (hatched bars) or absence (open bars) of 1,25-$(OH)_2D_3$. Each point represents the mean ± S.E.M. of 4-5 replicate cultures. (** = P< 0.01; * = P< 0.05 compared with vehicle-treated cultures maintained at the same glucose concentration).

582 ± 199 ng/dish (n=5)]. There was no difference in total protein content between cultures (without 1,25-$(OH)_2D_3$, 87 ± 2 µg/dish; with 1,25-$(OH)_2D_3$, 90 ± 3 µg/dish). Treatment with 10^{-10}M and 10^{-9}M 1,25-$(OH)_2D_3$ for up to 8 days did not increase insulin levels in the cells or medium (data not shown).

Vehicle-treated cultures maintained at a glucose concentration of 20 mM had a 3- to 4-fold higher insulin level than similarly treated cultures maintained in 10 mM glucose throughout (P< 0.01; fig. 2). Treatment with 1,25-$(OH)_2D_3$ increased medium insulin accumulation at both glucose concentrations: in the third 48 h of treatment there was a 167 % and a 108 % increase in insulin levels observed at glucose concentrations of 10 mM (P<0.01) and 20 mM (P< 0.05), respectively. Similar results were obtained in the fourth 48 h treatment period (data not shown). There was no significant difference in cell-associated insulin induced by 1,25-$(OH)_2D_3$ at either glucose concentration [20 mM glucose with 1,25-$(OH)_2D_3$, 685 ± 208 ng/dish; without 1,25-$(OH)_2D_3$, 556 ± 63 ng/dish (n=4); 10 mM glucose with 1,25-$(OH)_2D_3$, 923 ± 157 ng/dish, without 1,25-$(OH)_2D_3$ 658 ± 164 ng/dish (n=5)]. There was no effect of 1,25-$(OH)_2D_3$ on the total protein content of the cultures, but there was an increase in total protein content of cultures maintained at the higher glucose concentration [20 mM glucose, 312 ± 33 µg dish; 10 mM glucose, 234 ± 14 µg/dish; P<0.05 (n=9)].

It has been observed in normal pituitary cells that an effect of 1,25-$(OH)_2D_3$ on hormone secretion was not evident in cultures maintained in serum-free conditions (d'Emden & Wark, *unpublished observations*; 16) whereas responses were observed in the presence of CTFCS (3). Therefore, the effect of supplementation of the experimental medium with CTFCS was assessed. Cultures were maintained in medium supplemented with 10 %, 2 %



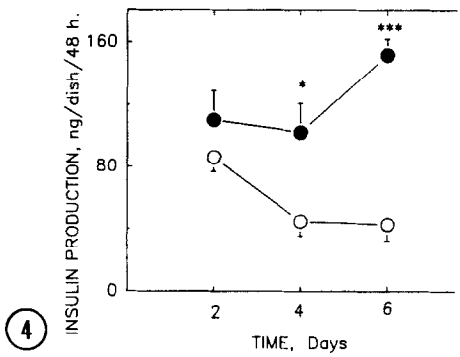


Fig. 3. THE EFFECTS OF SERUM ON INSULIN ACCUMULATION
Cultures were prepared as described in the Methods section, and maintained in experimental medium supplemented with 0.5 %, 2 % and 10 % CTFCS. After 2 days, this medium was removed, fresh medium containing the same concentration of CTFCS was added, and treatment for 6 days with 10^{-8}M 1,25-$(OH)_2D_3$ (hatched bars) or vehicle (open bars) commenced. The medium and treatment were replaced every 2 days. Fig 3A shows medium insulin levels after the final 48 h incubation period. Fig. 3B shows cell-associated insulin at the completion of the experiment. (*** = P< 0.001, ** = P< 0.01, * = P< 0.05 compared with vehicle-treated cultures exposed to the same concentration of CTFCS).

Fig. 4. TIME COURSE OF THE 1,25-$(OH)_2D_3$ EFFECT ON INSULIN ACCUMULATION.
Cultures were maintained as described in fig. 3. Shown are the amounts of insulin which accumulated in the medium every 48 h over the 6 day treatment period with 1,25-$(OH)_2D_3$ (●) or vehicle (○) in cultures maintained in medium supplemented with 0.5 % CTFCS. (*** = P< 0.001; * = P< 0.05 compared with vehicle-treated cultures at the same time point).

or 0.5 % CTFCS for eight days. Treatment with 1,25-$(OH)_2D_3$ increased the medium insulin content by 112 % (P< 0.5) and 267 % (P< 0.001) in cultures maintained in medium supplemented with 2 % and 0.5 % CTFCS respectively, but was without effect in cultures maintained in 10 % CTFCS. Vehicle-treated cultures maintained in 0.5 % CTFCS had higher insulin levels during the third 48 h treatment period compared with cultures maintained in 2 % or 10 % CTFCS (P< 0.01; fig. 3A). Cultures maintained in 0.5 % CTFCS had increased cell-associated insulin levels following treatment with 1,25-$(OH)_2D_3$ compared with vehicle-treated cultures (P< 0.01; fig. 3B). There was no difference in protein content between 1,25-$(OH)_2D_3$-treated and vehicle-treated cultures (data not shown). Cultures treated with 1,25-$(OH)_2D_3$ in 0.5% CTFCS had greater insulin levels at all time points over the six-day treatment period; the greatest effect was evident during the final 48 h treatment period (P< 0.001; fig. 4).

The effects of glucose and 1,25-$(OH)_2D_3$ were reassessed in cultures maintained in medium supplemented with 0.5 % CTFCS. There was an increase in the medium insulin level in cultures maintained in the presence of 20 mM glucose, compared with 10 mM

glucose, and treatment with 1,25-$(OH)_2D_3$ increased insulin accumulation at both glucose concentrations [10 mM glucose with 1,25-$(OH)_2D_3$, 363 ± 49 ng/dish, without 1,25-$(OH)_2D_3$, 244 ± 22 ng/dish ($P = 0.01$; n=5); 20 mM glucose with 1,25-$(OH)_2D_3$, 1,740 ± 102 ng/dish, without 1,25-$(OH)_2D_3$, 1,240 ± 140 ng/dish ($P< 0.05$; n=4)].

DISCUSSION

The *in vitro* model system we used to assess the effect of 1,25-$(OH)_2D_3$ on the function of islet cultures was prepared from neonatal rat pancreas. The neonatal islet in culture has served as a useful model for studies of signal transduction and insulin secretion (11,12). The experimental medium had a basal glucose concentration of 10 mM, which would be anticipated to chronically stimulate insulin secretion (12). Islet cultures were able to be successfully maintained for nearly 2 weeks, and control cultures had consistent medium insulin levels over the final 8 days in culture (fig. 1). With increased medium glucose concentrations, appropriate increases in medium insulin levels and total protein content were observed; the increased protein content presumably reflected increasing growth of the islets exposed to the higher glucose concentration. Overall, in the experiments reported here, treatment with 1,25-$(OH)_2D_3$ increased the medium insulin content 2.5-fold ($P< 0.001$; 15). As the *in vitro* model system avoided other metabolic effects of 1,25-$(OH)_2D_3$ which are observed *in vivo*, the present data suggest that 1,25-$(OH)_2D_3$ directly enhanced insulin secretion by beta cells in the presence of a consistently elevated medium glucose concentration. The time course of the 1,25-$(OH)_2D_3$ effect in the islets was similar to that observed in GH_4C_1 cells, in which 48 h exposure to 1,25-$(OH)_2D_3$ was required before prolactin production was augmented (17). This may partially explain the failure of others to observe an effect of 1,25-$(OH)_2D_3$ on insulin secretion *in vitro*, since islets were exposed to 1,25-$(OH)_2D_3$ for durations of 24 h or less in other reported studies (9,18). The present data support previous reports of an effect of 1,25-$(OH)_2D_3$ on insulin secretion *ex vivo* (6,7,9,10).

The significance of the data reported here remains to be determined. Supraphysiological concentrations of 1,25-$(OH)_2D_3$ were required in the presence of low, or absent serum conditions in which there would be minimal binding of 1,25$(OH)_2D_3$ to serum binding proteins. Binding to serum proteins may partially explain the decreased effect of 1,25-$(OH)_2D_3$ at higher CTFCS concentrations, although the presence of inhibitory factors in serum could also explain these observations. Further assessment of the effect of 1,25-$(OH)_2D_3$ in the islet will be required. In particular, it will be necessary to define the effect of 1,25-$(OH)_2D_3$ exposure on the acute secretory response of islets, from mature as well as from neonatal rats, to glucose and other secretagogues.

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REFERENCES

1. Reichel H, Koeffler HP & Norman AW. (1989) N. Engl. J. Med. 320: 980-991.
2. Stumpf WE, Sar M & Reid FA. (1979) Science 206: 1188-1190.

3. Wark JD & Gurtler V. (1988) J. Endocrinol. 117:293-298.
4. d'Emden MC & Wark JD. (1987) Endocrinology 121:1192-1194.
5. Cade C & Norman AW. (1986) Endocrinology 119:84-90.
6. Norman AW, Frankel BJ, Heldt AH & Grodsky GM. (1980) Science 209:823-825.
7. Tanaka Y, Seino Y, Ishida M, Yamaoka K, Satamura K, Yabuuchi H, Seino Y & Imura H. (1986) Endocrinology 118:1971-1976.
8. Lundquist I, Fanska R & Grodsky GM. (1976) Endocrinology 99:1304-1312.
9. Chertow BS, Sivitz WJ, Bevanetsky NG et al. (1983) Endocrinology 113:1511-1518.
10. Kadowaki S & Norman AW (1984) J. Clin. Invest. 73:759-766.
11. Hellestrom C, Lewis NJ, Borg H, Johnson R & Freinkel N. (1979) Diabetes 28:769-776.
12. Dunlop M & Larkins RG. (1984) Biochem. Biophys. Res. Comm. 118:601-608.
13. Drouin J, Lagace L & Labrie F. (1976) Endocrinology 99:1477-81.
14. Bradford MM (1976) Anal. Biochem. 72:248-254.
15. Baker RJ & Nelder JA. (1978) In: *GLIM Manual (Release 3)*. Oxford: Numerical Algorithm Group.
16. Tornquist K & Lamberg-Allardt C. (1987) Acta Endocrinol. 114: 357-361.
17. Wark JD & Tashjian AH Jr. (1982) Endocrinology 111:1755-1757.
18. Chan JCM & Rogers KS. (1986) Experimentia 42: 1253-1254.