Differentiation-dependent expression of calcitriol actions on absorptive processes in cultured chick intestine: modulation by triiodothyronine

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Abstract. Embryonic chick jejunum maintained in organ-culture exhibits a characteristic stage-specific pattern of responses to calcitriol and T₃. Whereas induction of luminal Na⁺/inorganic phosphate and Na⁺/D-glucose transport by calcitriol was only possible at an advanced state of differentiation prior to hatching on day 20, the steroid induced cellular calcium transport with high efficiency even in undifferentiated enterocytes in day 15 embryonic intestine. T₃ had no effect at all on calcium transport, but induced Na⁺/inorganic phosphate transport at all stages of epithelial maturation. In contrast, Na⁺/D-glucose transport was effectively induced by T₃ only in relatively immature intestinal epithelium. T₃, at a medium concentration of 10⁻⁶ mol/l, in a permissive fashion potentiated the effects of calcitriol (10⁻¹⁰, 10⁻¹² mol/l) on calcium transport as well as on Na⁺/inorganic phosphate and Na⁺/D-glucose transfer. Thereby, T₃ facilitated induction of transport activities by calcitriol against differentiation-related restraints. By facilitating the expression of genomic actions of calcitriol, T₃ may thus play an important role in the regulation of calcium and phosphate metabolism.

It has long been known that dysfunctions of the thyroid gland are frequently associated with disturbances of calcium and inorganic phosphate (Pᵢ) homeostasis. It is, therefore, obvious that thyroid hormones must play an important role in mineral metabolism (reviewed in 1), although the actions at the cellular level constituting their function as calcium and Pᵢ regulating hormones are not clarified at all. Within the wide spectrum of thyroid hormone effects, a number of actions on bone, the kidney and small intestine resemble those of the classic calcitropic hormone, calcitriol (1,25-dihydroxyvitamin D₃): Thus, thyroid hormones can stimulate bone resorption (2), enhance renal tubular reabsorption of Pᵢ (3), and increase Na⁺ gradient-driven uptake of Pᵢ by the small intestine (4). We want to emphasize that thyroid hormones may play an additional role in mineral metabolism owing to their ability to potentiate the genomic effects of calcitriol on calcium and Pᵢ absorption by the small intestine: Utilizing an organ culture system of embryonic chick jejunum (5), we were able to show that the various genomic effects of calcitriol on intestinal transport of calcium (6,8) and Pᵢ (9) are markedly enhanced by triiodothyronine (10,11). In addition, calcitriol-related absorption of D-glucose (12) can also be augmented by T₃ in a synergistic fashion (11). We reported previously that the expression of vitamin D₃ action on these absorptive functions of enterocytes depends on the state of embryonic development and thus on the degree of epithelial cell differentiation (13). We therefore sought to explore the possibility that the stage-specific expression of calcitriol actions on intestinal calcium uptake as well as on Na⁺/Pᵢ and Na⁺/D-glucose transport could be modulated by T₃.

Materials and Methods

Fertilized eggs were obtained from a local poultry farm and kept in an incubator with forced ventilation at 70% relative humidity and 38°C for 15-20 days. A single batch of eggs was used for each time-course experiment. Proximal segments of the jejunum (measuring 10-20 mm, de-
pending on the age of the embryo) were excised, slit open and cultured mucosa side-up on stainless steel grids in serum-free McCoy's 5A modified medium as described by Corradino (5). Culture time was 48 h.

Synthetic 1,25-dihydroxyvitamin D₃ (a generous gift from Hoffmann-La Roche, Basle) and T₃ (donated by Sanabo, Vienna, Austria) were added to cultures in ethanolic solution so that the final ethanol concentration did not exceed 0.15%. Ethanol only was added to control cultures. Calcitriol was tested in a concentration range between 10⁻¹⁰ and 10⁻⁷ mol/l in which the physiologic effects of hormone on intestinal absorptive processes are expressed in cultured embryonic chick small intestine (7,13). Because previous experiments had shown a dose-dependence of T₃ effects on calcitriol-related calcium and P₃ uptake by day 20 embryonic jejunum between 10⁻¹⁰ and 10⁻⁸ mol/l (4,10), T₃ was added to the culture medium, if appropriate, at its maximally effective concentration of 10⁻⁸ mol/l.

P₃, D-glucose and Ca²⁺ transport was measured by determination of tissue uptake of radiotracers as described previously (5,9,13). Briefly, at the end of the culture period, 8 guts were transferred into a 25-ml Erlenmeyer flask containing 5.0 ml buffer. Ca²⁺ uptake was measured in a "low-sodium" mannitol buffer containing 0.25 mmol/l Ca²⁺ (5). Krebs-Henseleit bicarbonate buffer, either containing 1.2 mmol/l P₃ or P₃-free, was used for determination of P₃ or D-glucose uptake, respectively. Buffer solutions were saturated prior to experimentation with 95/5% O₂/CO₂ to achieve pH 7.4 (9). D-glucose absorption was evaluated from tissue uptake of the non-metabolizable analogue, α-methyl-D-glucoside (methyl-α-D-glucopyranoside) (14,15) at a buffer concentration of 1.0 mmol/l (13). Radioisotope concentration in each case was 0.5 mCi/l. The flasks were sealed with rubber stoppers and maintained for 30 min (Ca²⁺ and P₃ uptake measurements) or 10 min (D-glucose uptake) at 37°C in a shaking water bath. For determination of tracer concentration in adherent fluid volume, guts were immersed in incubation buffer and immediately removed from the flasks after sealing without incubation. Uptake was terminated by transferring the guts on filter paper in a rapid filtration apparatus, where they were rinsed thoroughly under continuous suction with 3 × 10 ml ice-cold 0.9% NaCl. The guts were then blotted lightly on tissue paper and weighed. Through this procedure any adherent fluid was effectively removed so that "zero time" uptake values were practically zero.

For liquid scintillation counting, guts were dissolved in 0.5 ml TS-1 tissue solubilizer (Zinsser Analytic, Frankfurt, FRG) by overnight shaking at 50°C; 50 μl glacial acetic acid and 5.0 ml Beckman Ready Value Liquid Scintillation Cocktail were then added. Radioactivity was determined with a Beckman LS-233 Liquid Scintillation System. ⁴⁶CaCl₂, H₃⁵PO₄ and methyl-(α-D-[¹⁴C]gluco)pyranoside were obtained from the Radiochemical Centre Amer- sham, England.

Data are presented as means ±SEM (N=8 guts per group). Distribution of results was symmetrical and Student's unpaired t-test was used for statistical evaluations. Significance of difference was assumed when p<0.05.

Results

The differential responsiveness of cultured chick small intestine to calcitriol between day 15-20 of embryonic development is illustrated by Fig. 1: The peak value of calcium uptake induced by 10⁻⁸ mol/l calcitriol was observed on day 15, when the epithelium is composed only of undifferentiated resorptive cells (16). The sterol-related increment then gradually declined until day 20. In contrast, in embryonic small intestine cultured between day 15-17, calcitriol was totally ineffective in inducing P₃ uptake, even at a 10-fold higher concentration than that used in the previous experiment. A significant stimulation of P₃ transport by calcitriol could only be observed on day 20 prior to hatching. Similarly, α-methyl-D-glucoside uptake in response to calcitriol increased from day 15 to 20 in parallel with advancing differentiation of the small intestinal epithelium.

A completely different pattern of responsiveness was obtained when gut segments at various stages of embryonic development were cultured in the presence of T₃ (1 × 10⁻⁸ mol/l): Whereas under this condition calcium accumulation, at any time point, was unchanged, an approximately 50% increase in P₃ uptake was observed at all stages of embryonic development. T₃-induced α-methyl-glucoside uptake was highest on day 15, but was practically undetectable at the final stage of maturation during day 17-20 (Fig. 1).

Since we had obtained evidence for a synergistic effect of T₃ on calcitriol actions in day 20 intestine (10,11), we subsequently compared the influence of T₃ on dose-response relations of calcitriol effects at two different time points of embryonic developement.

Calcium transport

On day 15 of embryonic development, T₃ (10⁻⁸ mol/l) had no effect on induction of calcium transport by calcitriol. However, in guts cultured on day 20, the thyroid hormone most notably changed the dose-response relation of the sterol's effect on calcium uptake (Fig. 2).
When $10^{-8}$ mol/l T$_3$ was added in combination with graded calcitriol concentrations, which alone did not produce any effect on P$_i$ uptake in cultures of day 15-17 embryonic small intestinal segments (see Fig. 1), the thyroid hormone apparently induced a response to the sterol (Fig. 3). In explants cultured on day 20 of embryonic development, again a synergistic effect of the two hormones could be observed. However, this was seen only at relatively low calcitriol concentrations, whereas with concentrations of the sterol increasing to the maximally effective level, cooperativity between the thyroid hormone and calcitriol gradually disappeared.

**D-glucose transport**

With respect to the interaction of the two hormones on induction of Na$^+$-dependent D-glucose transport (as measured by uptake of α-methyl-D-glucoside), a synergistic effect became apparent, particularly in undifferentiated small intestinal epithelium.

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**Fig. 1.**
Differential sensitivity of cultured embryonic chick small intestine to various calcitriol and T$_3$ actions. Upper panel: calcium uptake. Hormone concentration in culture medium: •: nil; □: $10^{-8}$ mol/l calcitriol; ■: $10^{-7}$ mol/l T$_3$. Middle panel: Na$^+$/P$_i$ transport. Hormone concentration in culture medium: •: nil; □: $10^{-7}$ mol/l calcitriol; ■: $10^{-8}$ mol/l T$_3$. Lower panel: Na$^+$/D-glucose transport (measured by uptake of α-methyl-D-glucoside). Hormone concentration in culture medium: •: nil; □: $10^{-8}$ mol/l calcitriol; ■: $10^{-7}$ mol/l T$_3$. Culture time 48 h. Data (means ± SEM from N=8 guts) from a single experiment (out of at least three) are shown. Asterisks indicate statistically significant (p at least <0.05) difference from hormone-free control group.

**Fig. 2.**
Influence of T$_3$ on calcitriol-dependent calcium transport in embryonic chick small intestine on day 15 (upper panel) and on day 20 of embryonic development (lower panel). Culture time 48 h. T$_3$ concentration in culture medium: □: none; ■: $1×10^{-8}$ mol/l. Each data point represents the mean ±SEM from two separate experiments (N=12 guts). Asterisks indicate statistically significant (p at least <0.05) difference from T$_3$-free control group.
lium (Fig. 4): Sensitivity to the steroid hormone was enhanced by T₃ (1×10⁻⁸ mol/l) at all calcitriol concentrations in guts cultured on day 15, whereas this effect was restricted to low sterol concentrations on day 20 of embryonic development.

Discussion

There is ample evidence that calcitriol-related calcium uptake by organ cultured embryonic chick small intestine (5-8) actually reflects cellular transport mediated through calbindin-28k, the vitamin D-dependent calcium binding protein. Calbindin has been shown to be an integral element of the calcitriol-dependent transcellular calcium transfer process (17). The observed effects of calcitriol on calcium transport therefore are likely to result from receptor-mediated changes in the activity of the calbindin gene. However, the present study shows that, although the vitamin D receptor is present already in undifferentiated day 15 embryonic intestine (18), calcitriol-related calcium transport gradually declines with advancing epithelial maturation (Fig. 1). This may imply that expression of the specific cytoplasmic receptor of the steroid hormone does not necessarily lead to the full expression of its target cell actions. This assumption gains support if one takes into account that induction of Na⁺ gradient-driven transport processes by the sterol, particularly of Na⁺/Pᵢ, is severely restricted in day 15 embryonic small intestine, despite

![Fig. 3.](image)

**Fig. 3.** Influence of T₃ on calcitriol-dependent Pᵢ transport in embryonic chick small intestine on day 17 (upper panel) and on day 20 of embryonic development (lower panel). Culture time 48 h. T₃ concentration in culture medium: □: none; ■: 1×10⁻⁸ mol/l. Individual Pᵢ uptake values were corrected for T₃-related increments. Each data point represents the mean ±SEM from two separate experiments (N ≥12 guts). Asterisks indicate statistically significant (p at least <0.05) difference from T₃-free control group.

![Fig. 4.](image)

**Fig. 4.** Influence of T₃ on calcitriol-dependent α-methyl-D-glucoside transport in embryonic chick small intestine on day 15 (upper panel) and on day 20 of embryonic development (lower panel). Culture time 48 h. T₃ concentration in culture medium: □: none; ■: 1×10⁻⁸ mol/l. Individual uptake values were corrected for T₃-related increments, if appropriate. Each data point represents the mean ±SEM from two separate experiments (N ≥12 guts). Asterisks indicate statistically significant (p at least <0.05) difference from T₃-free control group.
the presence of the specific calcitriol receptor. There is evidence, therefore, that in enterocytes, in addition to the vitamin D receptor, various stage-specific factors, which possibly act also as transcriptional regulators, control the expression of individual calcitriol actions.

With respect to the effect of T3 on calcitriol-related calcium transport, our data suggest that the thyroid hormone facilitates the expression of genomic action of calcitriol. It should be noted that the interaction between the two hormones is apparent when induction of calcium transport by the sterol is hindered by stage-specific restraints (Fig. 2). At present it is not clear at which stage in the multistep process of activation and control of gene expression the interaction of calcitriol and thyroid hormone takes place. Although it cannot be excluded that T3 increases the binding affinity of the vitamin D receptor or augments its synthesis, an interaction of the two hormones at the DNA level has to be considered a probability. A logical basis for this assumption is provided by the high degree of sequence homology between the respective nuclear hormone receptors (19). Steroid hormone-sensitive genes contain apparently clusters of overlapping "hormone response elements" in their 5' flanking region, and can be activated, therefore, in a cooperative fashion by receptor-mediated binding of one hormone molecule in combination with another member of the steroid/thyroid hormone family (20). This is certainly valid also for the calbindin genes since, e.g. the mammalian calbindin-9k gene is a calcitriol-sensitive gene which contains also a thyroid hormone responsive element in its 5' flanking region (21). We have reasons to assume that the T3 effects observed in the present study result from a receptor-mediated activation of genes by the hormone. In previous studies utilizing only day 20 embryonic small intestine (4,10), we observed a concentration dependence of the T3 effect on calcitriol-related calcium and P1 uptake between $10^{-10}$ to $10^{-8}$ mol/l, which most probably reflects a receptor-mediated action of the hormone (discussed in 4,10).

Unlike calcium transport, the Na+/P1 and Na+/D-glucose transport mechanisms exhibit sensitivity not only calcitriol but also to T3. Our data provide evidence that the induction of transport processes by T3 too is subject to regulation by stage-specific factors. This is particularly obvious in case of Na+/D-glucose transport, where an inverse relationship between the degree of epithelial differentiation and the extent of the T3 response can be observed (Fig. 1).

When cultures were treated with a combination of T3 and calcitriol a more than additive effect of the two hormones on Na+/P1 and Na+/D-glucose transport was observed (Figs. 3-4). Again, this cooperativity could overcome the restraints on gene expression by calcitriol at certain stages of epithelial differentiation (Figs. 3-4).

It is tempting to speculate that an interaction of the two hormones at the respective hormone-sensitive elements of the Na+/P1 and Na+/D-glucose transporter genes is underlying the synergistic effect of T3 and calcitriol on Na+/P1 and Na+/D-glucose transport. The presence of such regulatory sites can be inferred from the sensitivity of the two Na+ gradient-driven transport systems to both calcitriol and T3. Although the cDNA coding for the intestinal Na+/D-glucose transporter has been sequenced (22), nothing is known about regulatory elements in the 5' flanking region of the gene. To the best of our knowledge, the analysis of the Na+/P1 transporter gene has not yet been accomplished.

With respect to the potentiating action of T3 on calcitriol responses, we believe that the observed effects of T3 reflect possible actions of the hormone in vivo. It must be noted that the minimum concentration of T3, at which an increase in calcitriol-related calcium and P1 uptake was observed, is $10^{-10}$ mol/l (4,10). This is not too far above the circulating level of "free" T3 in embryonic chicks (23). It should be noted too that the potentiating effect of thyroid hormone on calcitriol actions is not limited to the small intestine, but can also be observed in another target organ of the two hormones, namely in bone (24). Therefore, we would like to put forward the notion that thyroid hormone is required for optimal expression of calcitriol actions in its classic target tissues. If so, this would stipulate a specific role of thyroid hormones in the regulation of calcium and phosphate metabolism in vivo.

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