Induction of Calcium-binding Protein before 1,25-Dihydroxyvitamin D₃ Stimulation of Duodenal Calcium Uptake*

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Although chick intestinal calcium-binding protein has been intensively studied since it was discovered by Wasserman and Taylor (1) and Wasserman et al. (2), its exact physiological function remains unknown. The protein binds calcium with high affinity (2, 3) and is induced by vitamin D₃ and its more active metabolites (4, 5), suggesting that its function is related to vitamin D-dependent intestinal calcium transport. This suggested function, however, is not consistently indicated by studies of the time course of calcium-binding protein appearance relative to the onset of the calcium uptake response (1, 6-17). Some of these studies (1, 6, 8-13) have demonstrated that the induction of calcium-binding protein biosynthesis precedes or coincides with increases in calcium uptake and the protein is therefore available to participate in the uptake process. In contrast, other studies (7, 14-17) have shown that calcium-binding protein does not appear until after calcium uptake is increased, indicating that it is not involved in the uptake process. In order to establish whether or not calcium-binding protein functions in intestinal calcium uptake, these conflicting results must be resolved.

We describe here the use of an extremely sensitive technique for the detection of proteins induced by hormonal factors. Using these methods, the induction of calcium-binding protein prior to increased intestinal calcium uptake by 1,25-(OH)₂D₃ in chick intestinal organ cultures can be clearly demonstrated.

**EXPERIMENTAL PROCEDURES**

*Animals—Live chicken embryos (White Leghorns) were obtained from Sunnyside Hatcheries (Oregon, WI) at 18 days of incubation. Before use in duodenal organ culture experiments, the embryos were maintained overnight at 37°C in a humidified incubator.

*Chemicals—[4,5,3H]Leu (85 Ci/mmol), L-[U-14C]Leu (300 mCi/mmol), and CaCl₂ were obtained from Amersham Corp. in aqueous solutions containing 2% ethanol. Dr. M. Uskokovic of Hoffmann-LaRoche generously supplied 1,25-(OH)₂D₃, the purity and concentration of which were determined by its ultraviolet absorption spectrum (ε₂₂₄ nm = 18,200 M⁻¹ cm⁻²). Sephadex G-25 and DEAE-Sephadex (A-25) were obtained from Pharmacia Fine Chemicals (Upplands, Sweden), and Chelex 100 was obtained from Bio-Rad. All other reagents were of analytical grade.

*Media—Waymouth's 752/1 medium was obtained from Gibco (Grand Island, NY). Low leucine medium, containing 75.8 µmol leucine, was prepared according to the published formulation for Waymouth's 752/1 medium (18) by reducing the leucine concentration. The specific activity of leucine in low leucine medium prepared with [3H]Leu was 6.6 mCi/µmol, while that for low-leucine medium prepared with [14C]Leu was 0.33 mCl/µmol. Medium with 150 nM 1,25-(OH)₂D₃ was prepared by adding the hormone in ethanol. Equivalent volumes of ethanol alone were added to control medium. In all cases, the ethanol concentration in the medium never exceeded 0.1%. Penicillin (50 units/ml) and streptomycin (50 µg/ml), also obtained from Gibco, were added to all media before use.

*Duodenal Organ Cultures—Duodena excised from 19-day-old embryos were cultured for 24 h by the method of Corradi (10) as modified by Franceschi (19). All duodena were cultured in Waymouth's 752/1 medium for the first 18 h and in low leucine medium for the remaining 6 h, with changes every 2 h. One group of duodena was exposed to 150 nM 1,25-(OH)₂D₃ for the entire 24 h period, while three groups were exposed to this saturating concentration of hormone only for the last 2, 4, or 6 h. During the final 2 h of culturing, nine duodena (three from each of the last three groups) were incubated in medium containing [3H]Leu. A final group of duodena was never exposed to the hormone and served as a control group. Nine duodena from the control group were incubated in medium containing [14C]Leu during the final 2 h of culturing. At the end of the culture period, duodena labeled with [3H]Leu or [14C]Leu were individually

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rinsed with 4°C buffer (50 mm PO₄, 150 mm NaCl, pH 7.4), placed in separate vials, and frozen on dry ice. Nonradio labeled duodena were used for calcium uptake assays.

**Calcium Uptake Assay**—Total calcium uptake by each nonradio labeled duodenum was measured as previously described (19). Calcium uptake for each group of identically handled duodena was calculated as counts/min/mg of tissue/10 min ± S.E. and expressed as percentage of the calcium uptake determined for the control group.

**Extraction of Soluble Proteins from Radiolabeled Duodena**—The 18 duodena labeled with either [³H]Leu or [¹⁴C]Leu were grouped into 6 pairs. Each of three [³H]labeled duodena cultured with 1,25(OH)₂D₃ for 2 h was paired with one of three [¹⁴C]labeled duodena which had been identically handled but not exposed to the hormone. Similarly, each of the six [³H]labeled duodena exposed to 1,25(OH)₂D₃ for 4 or 6 h was paired with an identically handled [¹⁴C]labeled duodenum not exposed to the hormone. During the extraction of the soluble proteins, the duodena of each pair were processed simultaneously, but separately.

Each radiolabeled duodenum was transferred to a Potter-Elvehjem homogenizer and individually homogenized in 1.5 ml of buffer (5 mm Tris, pH 7.4) with six strokes of a motor-driven pestle. The homogenates were centrifuged at 100,000 g for 6 h at 4°C. The 18 supernatants containing the extracted soluble proteins were recovered.

**Uptake and Incorporation of Radiolabeled Leucine**—Tissue uptake of radiolabeled leucine was monitored during the final 2 h of culturing. At 30-min intervals, triplicate samples of culture medium were analyzed by liquid scintillation spectrometry, and the remaining portion of concentrates containing [³H]-labeled duodena were processed simultaneously, but separately.

Each gel containing a mixture of nonradiolabeled protein and resolved by two-dimensional polyacrylamide gel electrophoresis (22). All gels (120 × 140 × 1 mm) were standardized internally during electrophoresis in the second dimension. Two-dimensional gels were stained with Coomassie blue (22), impregnated with 2,5-diphenyloxazole (23), and dried under vacuum. Each gel containing a mixture of H- and [³H]labeled proteins was exposed sequentially to preflashed Kodak X-Omat AR film for 72 h at −70°C and to Kodak No Screen film for 35 h at 22°C (24).

**Fluorography and Autoradiography**—Two-dimensional gels were stained with Coomassie blue (22), impregnated with 2,5-diphenyloxazole (23), and dried under vacuum. Each gel containing a mixture of H- and [³H]labeled proteins was exposed sequentially to preflashed Kodak X-Omat AR film for 72 h at −70°C and to Kodak No Screen film for 35 h at 22°C (24). Gels containing only [³H]labeled proteins were exposed to X-Omat AR film for 48 h at −70°C but not to No Screen film.

**Calcium Binding Protein—Duodena excised from 4-week-old White Leghorn chicks (Summerside Hatcheries, Oregon, WI) were slit open lengthwise and rinsed with 4°C buffer (14 mm Tris, 150 mm NaCl, 5 mm KCl, 1 mm 2-mercaptoethanol, pH 7.4). The mucosa was scraped free of the underlying muscle at 4°C with glass slides, washed with buffer, and homogenized in an equal volume of buffer with a Polytron (Brinkmann Instruments). The supernatant remaining after high speed centrifugation of the homogenate (100,000 × g for 2 h at 4°C) was desalted by passage through a Sephadex G-25 column (2.5 × 85 mm) equilibrated with Tris buffer containing 50 mm NaCl. Fractions containing protein as determined by the Bradford method (21) were pooled, heated to 60°C for 20 min, and chilled to 4°C. The proteins were precipitated from solution by heat treatment and recovered as the supernatant solution following ultracentrifugation (100,000 × g for 45 min at 4°C). The recovered supernatant solution was applied without concentration to a column (1.5 × 90 cm) of DEAE-Sephadex A-25 equilibrated at 4°C with Tris buffer containing 50 mm NaCl. Proteins were eluted from the column with 200 ml of Tris buffer containing 50 mm NaCl followed by a 506-mm NaCl gradient (50 to 300 mm) and 200 ml of Tris buffer containing 300 mm NaCl. The flow rate was 8 ml/cm² and 10.5-ml fractions were collected. All fractions were analyzed for calcium-binding activity by Chelex ion exchange assay (2), NaCl concentration using a conductivity meter (London Company, Cleveland, OH), and protein content by the Bradford method (21).

**RESULTS**

**Time of Onset of the Calcium Uptake Response to 1,25(OH)₂D₃**—Three groups of eight duodena were cultured for 24 h and exposed to medium containing a saturating concentration of 1,25(OH)₂D₃ at various times before the end of the culture period. As shown in Fig. 1A, calcium uptake for duodena exposed to 1,25(OH)₂D₃ for 2 h was not increased over that for an identically handled control group. In contrast, uptake by duodena exposed to the hormone for 6 or 24 h was increased (p < 0.05) over that of the control group, the response observed at 24 h being more than double that observed at 6 h. The results of this experiment indicated that the calcium uptake response was first detectable between 2 and 6 h of continuous exposure to the hormone.

**Fig. 1. Time of onset of the calcium uptake response.** Groups of eight duodena were cultured for 24 h and introduced to medium containing 150 nM 1,25(OH)₂D₃ at various times before the end of the culture period. Calcium uptake (see "Experimental Procedures") was calculated as ⁴⁰Ca counts/min/mg of tissue/10 min ± S.E. (n = 8) and expressed as a percentage of the uptake determined for a control group. Uptake values at 6 and 24 h were significantly different from control values (p < 0.05). The results of two experiments are shown (A and B).
In a second experiment, the time of onset of the calcium uptake response to 1,25-(OH)₂D₃ was more precisely determined. Three groups of eight duodena were cultured for 24 h under the same experimental conditions and were continuously exposed to medium containing 1,25-(OH)₂D₃ for the final 2, 4, or 6 h, respectively. As shown in Fig. 1B, calcium uptake by duodena exposed to the hormone for either 2 or 4 h was unchanged relative to that of a control group. Calcium uptake by duodena exposed to 1,25-(OH)₂D₃ for 6 h was significantly increased (p < 0.05) and comparable to that observed in the previous experiment, demonstrating that this hormonal response was first detectable between 4 and 6 h.

**Uptake and Incorporation of Radiolabeled Leucine**—Uptake of radiolabeled leucine by cultured duodena is displayed in Fig. 2. Uptake of [³H]Leu by duodena exposed to 1,25-(OH)₂D₃ was linear with time and essentially identical with the uptake of [¹⁴C]Leu by duodena not exposed to the hormone. Incorporation of radiolabeled leucine into the soluble proteins is summarized in Table I.

**Fluorographs and Autoradiographs**—Shown in Fig. 3 is a fluorograph produced from one of the gels containing both ³H- and ¹⁴C-labeled proteins. This fluorograph, like all others in this experiment, was prepared with Kodak X-Omat AR film which detects ³H and ¹⁴C equally well when the isotopes are present in a 10:1 dpm ratio (25). The area immediately surrounding the circled protein at the lower right is redisplayed in Fig. 4A. Shown in Fig. 4B is the matching area of an autoradiograph produced from the same gel. This autoradiograph, like all others in this experiment, was prepared with Kodak No Screen film which detects ¹⁴C but not ³H. The protein identified by the arrow was detected by fluorography but not autoradiography, indicating that it was present only in the ³H-labeled tissue. As the ³H-labeled proteins contained in this gel were extracted from a duodenum exposed to 1,25-(OH)₂D₃, for 6 h, the area of the fluorograph shown in Fig. 3 containing the circled protein is redisplayed in Fig. 4A.
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Fig. 5. Detection of a hormonally responsive protein in a duodenum exposed to 1,25-(OH)₂D₃ for 4 h. Matching areas of a fluorograph and autoradiograph produced from the same gel are shown in A and B, respectively. This gel contained ³H-labeled proteins from a duodenum treated with 1,25-(OH)₂D₃ for 4 h and ¹⁴C-labeled proteins from a control duodenum. The marked protein was detected by fluorography but not by autoradiography, indicating that it was present only in the 1,25-(OH)₂D₃-treated duodenum. This protein is the same as that identified in Fig. 4.

(OH)₂D₃ for 6 h and the ¹⁴C-labeled proteins were obtained from an identically handled duodenum not exposed to the hormone, this protein appeared to be hormonally induced by the time that an increase in calcium uptake was first observed. The protein appeared consistently in all three gels containing ³H-labeled proteins from duodena exposed to hormone for 6 h. To conserve space, only 1 of the 3 gel pairs is presented.

Displayed in Fig. 5 are matching areas of a fluorograph and autoradiograph prepared from another gel containing both ³H- and ¹⁴C-labeled proteins. The protein identified in Fig. 4 was again detected by fluorography but not by autoradiogra-

Fig. 7. Position of calcium-binding protein on a two-dimensional gel. One μg of vitamin D-dependent calcium-binding protein (CaBP), purified from the duodena of 4-week-old chicks, is readily apparent in this gel, which was stained with Coomassie blue and dried. Molecular weight standards, apparent as horizontal lines, are as follows: myosin (220,000; extreme top), phosphorylase a (94,000), catalase (60,000), and actin (43,000).

Fig. 8. Marking the position of calcium-binding protein in a two-dimensional double-labeled gel. One μg of calcium-binding protein (CaBP) was mixed with ³H-labeled proteins (90 μg) extracted from a duodenum treated with 1,25-(OH)₂D₃ for 6 h. This protein mixture, containing 6.0 × 10⁶ dpm, was resolved by two-dimensional electrophoresis (22), and the resulting gel (shown) was stained with Coomassie blue. The same molecular weight standards seen in Fig. 7 were also present in this gel, but they appear much fainter since the gel was still wet. By reference to these internal standards and the standardized gel (Fig. 5), the position of calcium-binding protein was easily identified. A small hole was punched in the wet gel at this position.
calcium-binding protein. The gel shown in Fig. 8 was im-
pregnated with 2,5-diphenyloxazole (22), dried, and exposed to Kodak
X-Omat AR film for 48 h at -70 °C. The area of the resulting
fluorograph in which the hormonally responsive protein was located
(Figs. 3 and 4) is shown here. This protein can be seen surrounding
the punched hole which marked the exact position of calcium binding-
protein, indicating that it was present only in the 3H-labeled
tissue. As 3H-labeled proteins contained in this gel were ex-
tracted from a duodenum exposed to 1,25-(OH)2D3 for only 4 h,
this protein appeared to be hormonally induced before an
increase in calcium uptake was observed. The protein was
easily detected in all three gels containing 14C-labeled proteins from
duodena exposed to hormone for 4 h. It was also detect-
able in the fluorographs prepared from all three gels contain-
ing 3H-labeled proteins from duodena exposed to hormone for
only 2 h, one of which is shown in Fig. 6.

To examine the electrophoretic mobility of this hormonally
responsive protein relative to calcium-binding protein, non-
radiolabeled calcium-binding protein was purified from 4-
week-old chicks and subjected to two-dimensional electropho-
resis. Fig. 7 shows the resulting gel stained with Coomassie
blue. Calcium-binding protein can be seen at the lower right
relative to molecular weight standards that appear as hori-
3ontal lines. An identically prepared gel also stained with
Coomassie blue containing both nonradioiabeled calcium-
binding protein and 14C-labeled proteins extracted from a
duodenum exposed to 1,25-(OH)2D3 for 6 h is shown in Fig. 8.
Calcium-binding protein, easily identified by reference to the
standardized gel (Fig. 7), was marked by punching a small
hole in the gel. A fluorograph was then prepared from the
same gel, which contained a hole at the position occupied by
calcium-binding protein. The area of the fluorograph in which
the unidentified hormonally responsive protein was located is
shown in Fig. 9. This protein can be seen at the site of the
punched hole, demonstrating that it co-migrated exactly with
calcium-binding protein.

**DISCUSSION**

The experiments presented above demonstrate that cul-
tured duodena from 19-day-old chick embryos rapidly synthe-
size a protein in response to 1,25-(OH)2D3. This protein was
consistently detected in duodena exposed to 1,25-(OH)2D3 for
only 2 h and was more apparent in duodena exposed to
hormone for longer periods of time. Its synthesis was examined
in relation to the calcium uptake response. Under the cultur-
ing conditions used, the embryonic duodena did not show
increased calcium uptake until after 4 h of continuous ex-
sposure to a saturating concentration of 1,25-(OH)2D3. By 6 h of
exposure to the hormone, calcium uptake was increased by
approximately 30% over control values. By 24 h, calcium
uptake was further increased to more than 70% above control
values. These results are consistent with a previous report
from our laboratory concerning calcium uptake by cultured
duodena from 19-day-old chick embryos (19) which showed
that increases in calcium uptake were not detectable before
4.5 h of exposure to a saturating concentration of hormone,
but increases of approximately 30 and 60% occurred by 6 and
24 h, respectively. Since the protein identified in the present
study was detected in duodena exposed to 1,25-(OH)2D3 for
only 2 h, its synthesis preceded the onset of the calcium
uptake response by at least 2 h.

The 1,25-(OH)2D3-responsive protein was detected by dou-
ble label autoradiography (25). In this technique, double-
labeled gels were exposed sequentially to X-Omat AR and No
Screen films. During exposure to the X-Omat AR films, both
isotopes contributed equally to the production of photo-
graphic images, 3H by fluorography and 14C by a combination
of fluorography and direct autoradiography. In contrast, the
No Screen films were completely insensitive to fluorography
under the conditions used and recorded images in response to
14C by direct autoradiography alone. Images recorded on any
pair of films exposed to the same gel were completely super-
imposable, thereby allowing direct comparison of identical
image areas. Given that the exposures of the films were
appropriately selected, consistent detection of a protein image
on X-Omat AR films without its detection at the same location
on matching No Screen films demonstrated that 1) this pro-
tein was present in the gels only in 3H-labeled form, and 2) it
was induced in response to 1,25-(OH)2D3. As the identified
protein was invariably detected only on X-Omat AR films, it
followed that this protein was hormonally induced.

Exposures of the films were carefully selected so that the
faintest images produced on one film were also produced on
the other, with a few exceptions.2 However, the X-Omat AR
films were always more highly exposed than the No Screen
films (Figs. 4 and 5). This difference in exposures, which could
be misconstrued as the cause for differential detection of a few
proteins, was required since both isotopes contributed to
image formation on the X-Omat AR films but only one formed
the image recorded on the No Screen films.

Since the protein identified in this report was induced by
1,25-(OH)2D3, a strong possibility existed that it was the
vitamin D3-dependent intestinal calcium-binding protein (2).
Two observations further supported this possibility. 1) The
identified protein was extracted from duodenal homogenates
as a soluble protein, and 2) it migrated during two-dimensional
electrophoresis as a protein having a molecular weight of
27,000 and an apparent pl of 4.2. The electrophoretic mobility
of this protein was therefore examined relative to calcium-
binding protein purified from 4-week-old chicks, with the
result that both proteins co-migrated identically during two-
dimensional electrophoresis. This result led to the further
conclusion that the protein is indeed calcium-binding protein.

The experiments presented above, when considered as a
whole, support the conclusion that calcium-binding protein is
synthesized in duodena from 19-day-old chick embryos within
2 h of exposure to 1,25-(OH)2D3 and at least 2 h before the
onset of the calcium uptake response. This conclusion is in
agreement with two other studies using embryonic chick
duodena which demonstrated that calcium-binding protein
was induced in 1,25-(OH)2D3-treated duodena without stim-
ulation of calcium uptake (10, 11). This conclusion, however,

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2 These exceptions, aside from the protein in question, are presently
being studied in greater detail and will be discussed in a future
publication.
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is at variance with other studies of 1,25-(OH)_{2}D_{3} action using 3- or 4-week-old chicks. In two of these studies (14, 17), in vitro synthesis of calcium-binding protein by isolated duodenal polysomes was detected by immunoprecipitation at all times when calcium transport was significantly increased, beginning at 2 h; but calcium-binding protein synthesis was not detected before increases in calcium transport. These and other studies (15, 16) also demonstrated that calcium transport was increased by 1,25-(OH)_{2}D_{3} several hours before calcium-binding protein was detected in duodenal homogenates by immunoelectrophoresis, immunodiffusion, or the Chelex ion exchange assay.

To reconcile the conclusion of the present study with the results from studies of 4-week-old chicks, we postulate that smaller amounts of calcium-binding protein can be detected by double label autoradiography than by immunological or ion exchange assays. The absolute lower limit of detection for immunoelectrophoresis, immunoprecipitation, and immunodiffusion is approximately 1 µg of calcium-binding protein/g of mucosa, wet weight (13, 26, 27), while that of the Chelex ion exchange assay is considerably higher (6). The absolute lower limit of detection for calcium-binding protein by double label autoradiography is currently under investigation in our laboratory. Preliminary data indicate that it is at least 1 order of magnitude below that for the immunosays mentioned under the conditions selected for the present experiments. It is conceivable, therefore, that calcium-binding protein is synthesized in the duodena of 4-week-old chicks before 1,25-(OH)_{2}D_{3} stimulation of calcium transport in quantities too small to be detected by immunological or ion exchange techniques.

Although the synthesis of trace amounts of calcium-binding protein prior to stimulation of calcium uptake is, at present, of unknown physiological significance, it is clear from these experiments that the induction of calcium-binding protein in the embryonic duodenum is not a secondary response to 1,25-(OH)_{2}D_{3} stimulation of calcium uptake. The detection of calcium-binding protein biosynthesis after only 2 h of exposure to 1,25-(OH)_{2}D_{3} suggests instead that its induction is a primary hormonal response which may yet be directly related to the calcium transport response.

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REFERENCES

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