Incorporation of [^3H]Leucine into an Actin-Like Protein in Response to 1,25-Dihydroxycholecalciferol in Chick Intestinal Brush Borders

By PETER W. WILSON and D. ERIC M. LAWSON

*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council,
Milton Road, Cambridge CB4 1XJ, U.K.*

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1,25-Dihydroxycholecalciferol very rapidly stimulates the incorporation of [$4,5\text{-}^3\text{H}$]leucine into at least two proteins of the chick intestinal mucosal cells. The smaller of the two proteins has mol.wt. approx. 42000, very similar to actin. Other properties of this protein were investigated, including its solubility in salt solution and its behaviour on gel electrophoresis and isoelectric focusing, and in all cases the 1,25-dihydroxycholecalciferol-stimulated protein was indistinguishable from intestinal actin. It was also shown that the brush borders of mucosal cells contain both β - and γ -actin in approximately equal amounts and that both forms of the protein appear to be affected by the hormone. It is concluded therefore that one of the earliest actions of 1,25-dihydroxycholecalciferol is to stimulate the incorporation of leucine into β - and γ -actin of the mucosal cells or into two proteins very like them.

One of the most important actions of the steroid hormone 1,25-dihydroxycholecalciferol is to increase the proportion of dietary calcium absorbed by the small intestine of animals. The mechanism by which this is achieved is still far from clear, with most investigators being concerned so far with recognizing the components of the Ca^{2+} -transporting system. One component is almost certainly the protein known as Ca^{2+} -binding protein. As might be expected from what is known about the biochemical function of other steroid hormones, 1,25-dihydroxycholecalciferol controls the synthesis of Ca^{2+} -binding protein by regulating the formation of its mRNA (Spencer *et al.*, 1976a, 1978). The intracellular distribution of this steroid in the intestine and the effect of inhibitors of protein synthesis, such as actinomycin D and α -amanitin, are all consistent with a nuclear site of action of this hormone (Lawson & Emtage, 1974).

Recent studies on the comparison of the rate of formation of the mRNA for Ca^{2+} -binding protein, the rate of appearance of the protein in the tissue and the changes in Ca^{2+} absorption in vitamin D-deficient chicks in response to 125 ng of the hormone showed that other factors are involved in Ca^{2+} absorption and that these factors, unlike Ca^{2+} -binding protein, have a rapid turnover (Spencer *et al.*, 1976b). We have considered the possibility that such factors are proteins and have examined the synthesis of components of the intestinal microvilli to discover whether they were affected by 1,25-dihydroxycholecalciferol. The synthesis of at least two proteins was recognized to be stimulated by this steroid within 2–3 h of its administration to chicks (Wilson & Lawson, 1977).

The apparent mol.wts. of these proteins were 45000 and 84000, and the changes in the rate of incorporation of the radioactive leucine into them correlated very well with the changes in Ca^{2+} absorption after the hormone, indicating a role for these proteins in the transport of this ion. The protein whose synthesis is stimulated first by 1,25-dihydroxycholecalciferol has been investigated by electrophoretic and isoelectric-focusing techniques and appears to be the β and γ forms of actin present in the core fraction of the microvilli.

Experimental

Methods

Brush-border preparation. Flasks containing 0.5 g of everted chick jejunal slices (about 2 mm thick) were incubated in 10 ml of Krebs Improved Ringer 1 media (Dawson, 1969) for 2 h with shaking in O_2/CO_2 (19:1) atmosphere at 37°C. Slices from vitamin D-deficient chicks or such chicks dosed 3 h before death with 125 ng of 1,25-dihydroxycholecalciferol were incubated with [$\text{U-}^{14}\text{C}$]leucine (25 μCi) or [$4,5\text{-}^3\text{H}$]leucine (125 μCi) respectively. At the end of the incubation the slices were washed several times with 0.9% NaCl and used for tissue fractionation.

Brush borders were prepared by the method of Forstner *et al.* (1968) in 50 ml of 5 mM-EDTA buffer, pH 7.6/g of intestinal slices, and the final brush-border pellet was suspended in 2 ml of EDTA buffer. Examination of the preparation by phase-contrast microscopy showed it to consist of readily recognizable brush-border structures essentially free of nuclei,

which are possibly the major contaminants of this fraction. A sample of the ^{14}C -labelled brush-border fractions from vitamin D-deficient chicks was mixed with an equal volume of ^3H -labelled brush borders from 1,25-dihydroxycholecalciferol-treated chicks. The mixed-label brush-border pellet obtained by centrifugation was used for the experiments.

Polyacrylamide-gel electrophoresis. The mixed brush-border sample was dissociated before fractionation by electrophoresis by heating at 70°C for 30 min in a solution containing (per ml) 15 mg of dithiothreitol, 10 mg of sodium dodecyl sulphate, 12 mg of Tris and 0.1 ml of glycerol. The electrophoresis was carried out in a vertical slab gel apparatus (Raven Scientific Ltd., Haverhill, Essex, U.K.). The gel system was based on that described by Neville (1971), and consisted of an upper reservoir buffer, pH 8.64, (containing 0.04 M-boric acid, 0.041 M-Tris and 0.1% sodium dodecyl sulphate), an upper gel buffer, pH 6.1 [containing 0.0267 M- H_2SO_4 /0.0541 M-Tris (running pH 8.64)] and a lower gel buffer, pH 9.18 [containing 0.0308 M-HCl/0.4244 M-Tris (running pH 9.50)]. The stacker gels contained 3.0% acrylamide, 0.2% *NN'*-methylenebisacrylamide and 0.1% sodium dodecyl sulphate and the main gel was 11.0% acrylamide, 0.1% *NN'*-methylenebisacrylamide and 0.1% sodium dodecyl sulphate. The separation of the proteins was carried out at 150 V constant voltage for 3 h. Throughout this study a mixture of pure proteins was separated at the same time for comparison purposes and to establish molecular weights. The mixture consisted of lysozyme (mol.wt. 14300), tobacco-mosaic-virus coat protein (mol.wt. 17500), immunoglobulin light chain (mol.wt. 23500), galactose 6-phosphate dehydrogenase (mol.wt. 36000), creatine kinase (mol.wt. 40000), immunoglobulin heavy chain (mol.wt. 50000), bovine serum albumin (mol.wt. 68000), β -galactosidase (mol.wt. 130000) and myosin (mol.wt. 220000).

Isoelectric focusing. Isoelectric focusing was carried out on a $26\text{ cm} \times 12.5\text{ cm}$ horizontal plate by using a Multiphor tank and power supply (LKB Instruments, South Croydon, Surrey, U.K.). The plates were cast with 60 ml of a gel solution containing 30 g of urea, 16 ml of acrylamide stock solution (14.2% acrylamide and 0.81% *NN'*-methylenebisacrylamide), 12 ml of 10% Nonidet P40 and an Ampholine mixture of 1.2 ml of pH 4–6, 1.2 ml of pH 5–7 and 0.6 ml of pH 3.5–10 with 0.4 ml of 1.6 mg of flavine mononucleotide/100 ml (O'Farrell, 1975) and photopolymerized. The electrode solutions were 0.02 M-NaOH for the cathode and 0.01 M- H_3PO_4 for the anode. The mixed brush-border fraction was lysed in 9.5 M-urea/2% Nonidet P40/5% β -mercaptoethanol/2% Ampholines (1.6% of pH 5–7 and 0.4% of pH 3–10). The lysed samples were loaded towards the cathode of the gel on glass-fibre paper at a concentration of $40\text{ }\mu\text{l}/\text{cm}^2$, whereas for larger volumes

several papers were stacked on top of each other. The separation of the sample was carried out with the power supply set at 1000 V and a maximum load of 40 W for 3 h. The temperature of the system was maintained at 10°C and the sample papers were removed after 1 h.

Gel treatment. Before staining, isoelectric-focusing gels were fixed overnight in 10% (w/v) trichloroacetic acid and then washed for several hours in acetic acid/propan-2-ol/water (2:5:13, by vol.). Staining of the polyacrylamide slab gel and the fixed isoelectric-focusing gels was carried out for 2 h in 0.05% Coomassie Blue R in acetic acid/propan-2-ol/water (2:5:13, by vol.) and then for 16 h in 0.005% Coomassie Blue R in the same solvent. For destaining, the gels were immersed in acetic acid/propan-2-ol/water (1:1:8, by vol.) for 8 h and finally cleared in 10% (v/v) acetic acid. The gels were then photographed and sliced according to the position of the bands. The stained bands, or 2 mm slices where no bands were seen, were cut out and treated with 1 ml of 90% NCS tissue solubilizer at 60°C for 2 h in closed vials. After cooling, 0.5 ml of NCS tissue solubilizer and 10 ml of scintillator containing 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.4% 2,5-diphenyloxazole in toluene was added to the digested gel and counted in a Packard Tri-Carb scintillation spectrometer model 3375. Quenching corrections were calculated by means of automatic external standardization and correlation curves for combined ^{14}C and ^3H . The ratio of ^3H to ^{14}C was then calculated for each band. Expression of the radioactivity distributed throughout the gel as a $^3\text{H}/^{14}\text{C}$ ratio indicated by an increased ratio any change in protein synthesis due to 1,25-dihydroxycholecalciferol. In these experiments spurious variations in ratio could be greatly decreased when care was taken to measure radioactivity with maximum accuracy (i.e. with an accumulation of 10000 counts at least). Changes in ratio were only regarded as significant when they exceeded the variation in the ratio measurements throughout the gel, i.e. the standard deviation of the ratio values was calculated and only those greater than the mean by two standard deviations were further investigated.

Actin preparation. Acetone-dried powders were prepared from chicken leg muscle and gizzard by the method of Seraydarian *et al.* (1967), and the actin was extracted as described by Spudich & Watt (1971) up to the first polymerization stage. The pellets were either dissociated for fractionation by polyacrylamide-gel electrophoresis or lysed by the method of O'Farrell (1975) for analysis by isoelectric focusing.

Acetone-dried powders of intestinal brush borders were prepared by treating them three times with acetone (1 mg in 1 ml). The resulting powder was dried and extracted with $80\text{ }\mu\text{l}$ of a low-ionic-strength ATP-containing solution (Spudich & Watt, 1971).

After centrifugation, the resulting soluble fraction and residual insoluble powder were treated with either the dissociating or lysis solution described above.

Materials

1,25-Dihydroxycholecalciferol was generously given by Dr. N. Pollitt of Roche Products, Welwyn Garden City, Herts., U.K.; Nonidet P40 was obtained from BDH Chemicals, Poole, Dorset, U.K.; NCS tissue solubilizer was obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K. Ampholines were obtained from LKB Instruments, South Croydon, Surrey, U.K. L-[4,5- ^3H]Leucine and L-[U- ^{14}C]leucine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Intestinal brush-border proteins solubilized by treatment with sodium dodecyl sulphate and dithiothreitol can be resolved by slab-gel electrophoresis into almost 50 components (Fig. 1). Careful comparison of the brush-border proteins in the vitamin D-deficient birds with those in birds dosed with 1,25-dihydroxycholecalciferol has not revealed any proteins whose presence is entirely dependent on this hormone. However, it is not yet possible to measure the amount of these proteins in the intestinal brush borders, so it is possible that the effect of the hormone is to cause an increased turnover of these membrane components. To investigate this possibility we have observed the changes in the biosynthesis of microvillar proteins under the influence of 1,25-dihydroxycholecalciferol by the double-labelling technique. At early time intervals after treatment of deficient birds with 1,25-dihydroxycholecalciferol there is an increase in the rate of synthesis of at least one protein (Fig. 1). This is indicated by the increase in the $^3\text{H}/^{14}\text{C}$ ratio in one part of the gel relative to the remaining parts. In eight different experiments an increase of $60 \pm 22.2\%$ (s.d.) in the $^3\text{H}/^{14}\text{C}$ ratio was observed in this area A (Fig. 1a).

The apparent molecular weight of the protein in this area was assessed within the accuracy of the thickness of the gel slice and from a series of standard proteins of known size as 42000–45000 (see Fig. 1). Furthermore, co-migrating in the same area of the gel as this material is a protein present in high concentration in the intestinal brush-border fraction and now known to be actin (Tilney & Mooseker, 1971). The molecular weight of actin irrespective of source is approx. 42000 (Clarke & Spudich, 1977).

Recent work on the organization of the protein structure of intestinal microvilli has shown that they consist of a core of actin filaments enclosed in a plasma membrane (Mooseker & Tilney, 1975). The distribution of this 1,25-dihydroxycholecalciferol-dependent

Fig. 1. Intestinal slices from vitamin D-deficient chicks before and after dosing with 125 ng of 1,25-dihydroxycholecalciferol incubated with [U- ^{14}C]leucine and [4,5- ^3H]leucine respectively

A purified brush-border fraction was prepared and solubilized as described in the text. Samples of the two preparations were mixed and fractionated by gel electrophoresis. Proteins were stained with Coomassie Blue R and the stained gels sliced according to the protein bands and treated as described under 'Methods'. (a) Whole brush-border preparation; (b) brush-border proteins insoluble in 2% Triton X-100; (c) brush-border proteins soluble in 2% Triton X-100.

protein between the membrane and core fractions has been assessed by solubilizing the brush-border fraction labelled with [^3H]- and [^{14}C]leucine in 2% Triton X-100/5 mM-EDTA. The core fraction was obtained as a pellet by centrifugation of the resulting solution and this was then dissolved and the component proteins were fractionated by slab gel electrophoresis. The soluble membrane proteins were similarly separated. α -Actin prepared from chick skeletal muscle was used as a standard in the electrophoretic separations. After staining the gels were sliced and counted as described under 'Methods'. The original brush-border preparation had 8% of its radioactivity in the actin area, whereas after treatment with Triton X-100 the radioactivity in this area of the gels was 4 and 18% of the total in the membrane and core fractions respectively. In terms of the radioactivity in the actin area of the brush-border fraction

about 60% of it was found in the core fraction and had a significantly raised $^3\text{H}/^{14}\text{C}$ ratio (Fig. 1*b*; in two experiments the ratio was raised 2-fold compared with the ratio in the rest of the gel). In contrast, the recovered radioactivity in the membrane fraction had only a slightly increased $^3\text{H}/^{14}\text{C}$ ratio (Fig. 1*c*; raised by an average of 12% in two experiments).

Further evidence for the identification of the 1,25-dihydroxycholecalciferol-dependent protein as actin was sought by comparison of the chemical properties of the two proteins and their behaviour on isoelectric focusing. Actin can be extracted from tissues in a soluble form by treatment first with acetone followed by extraction of the residue with a low-ionic-strength solution containing ATP. An acetone-dried powder was made of the chick brush borders labelled with [^3H]- and [^{14}C]-leucine as usual. It was treated with the dilute salt solution and the soluble proteins were separated by gel electrophoresis. The material insoluble in the low-ionic-strength solution was also resolved on the slab-gel-electrophoretic system. The radioactivity in the acetone-dried powder remaining after extraction with the dilute salt solution contained about 40% of an actin-like protein, but

this radioactivity did not have an increase in $^3\text{H}/^{14}\text{C}$ ratio, compared with the radioactivity in the rest of the gel. In contrast, the 60% of the radioactivity soluble in the dilute salt solution did have a $^3\text{H}/^{14}\text{C}$ ratio which was 3-fold higher than the ratio in the remainder of the gel.

The actin family of proteins present in a wide range of cell types can be analysed by isoelectric focusing into at least three components, α , β and γ forms (Garrels & Gibson, 1976). Fractionation by isoelectric focusing of the proteins of the brush border soluble in dilute salt solution gave two bands in the actin area (Fig. 2*a*), one of which co-migrated with γ -actin obtained from gizzard (Rubenstein & Spudich, 1977) and the other protein was in the position of β -actin (Fig. 2*b*). Consequently it appears that intestine contains β - and γ -actin in approximately equal proportions, as judged from the intensity of the stain taken up by these two bands. In agreement with the results of the gel electrophoresis of the proteins from the acetone-dried powder, there was no increase in the $^3\text{H}/^{14}\text{C}$ ratio in either the β - or γ -actin area in the acetone-dried-powder residue. However, in the proteins soluble in the dilute salt solution the $^3\text{H}/^{14}\text{C}$ ratio was increased by 48.7 and 38.5% compared with the ratio in the remainder of the gel for the β - and γ -actin proteins respectively.

Discussion

In these investigations we were able to show by isoelectric focusing that intestinal mucosal brush borders contain both β - and γ -actin in approximately similar proportions. α -Actin was not detectable in this fraction. The properties of actin, which are also shown by the 1,25-dihydroxycholecalciferol-stimulated protein, include a common molecular weight, a similar distribution within the brush-border fraction (both proteins are in the core fraction of the microvilli) and a similar solubility in dilute salt solutions. In addition, these two proteins cannot be separated by either gel electrophoresis or isoelectric focusing. The conclusion from these studies therefore is that one of the earliest effects of 1,25-dihydroxycholecalciferol is to increase the synthesis of actin or a protein extremely like actin. It should be appreciated, however, that our findings could be explained on the basis of an increased incorporation of newly synthesized actin into the microvilli rather than by synthesis of this protein *de novo*. Although actinomycin D lowers this effect of 1,25-dihydroxycholecalciferol (Wilson & Lawson, 1977), confirmation with other approaches is needed to show that this hormone increases actin synthesis. A further point deserving investigation concerns the uniformity of the actin within the brush border. We repeatedly observed in this study that the newly synthesized protein was more readily soluble than was existing actin.

Fig. 2. Isoelectric focusing of intestinal brush-border proteins

Intestinal brush borders labelled with ^3H and ^{14}C as described in the text were treated with acetone and the residues extracted with dilute salt solution containing ATP. The extracted actin-like proteins were then separated by isoelectric focusing (a). (b) Actin preparation from chick gizzard; (c) actin preparation from chick skeletal muscle.

The role of 1,25-dihydroxycholecalciferol in maintaining the transport of Ca^{2+} across the mucosal cell remains obscure. The presence of actin in the cytoskeleton of cells implies a structural role for this molecule and it has usually been implicated in any movement that the cells undergo. It is difficult to relate such a role to any presently conceivable mechanism of action of Ca^{2+} absorption. Thus the intimate connection of actin with the cell membrane may mean that changes in this protein occur whenever the functional activity of the cell membrane increases. However, much has still to be learnt about the role of actin in non-muscle cells and on the implications of its membrane attachment, and without this information it would be premature to regard these changes in the biosynthesis of actin as an indirect effect of this hormone.

References

- Clarke, M. & Spudich, J. A. (1977) *Annu. Rev. Biochem.* **46**, 791–822
- Dawson, R. M. C. (1969) in *Data for Biochemical Research* (Dawson, R. M. C., Elliot, C. D., Elliot, W. H. & Jones, K. M., eds.), 2nd edn., 476–508, Clarendon Press, Oxford
- Forstner, G. G., Sabesin, S. M. & Isselbacher, K. J. (1968) *Biochem. J.* **106**, 381–390
- Garrels, J. I. & Gibson, W. (1976) *Cell* **9**, 793–805
- Lawson, D. E. M. & Emtage, J. S. (1974) *Vitam. Horm. (N. Y.)* **32**, 277–298
- Mooseker, M. S. & Tilney, L. G. (1975) *J. Cell Biol.* **67**, 725–743
- Neville, D. M. (1971) *J. Biol. Chem.* **246**, 6328–6334
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Rubenstein, P. A. & Spudich, J. A. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 120–123
- Seraydarian, K., Briskey, E. J. & Mommaerts, W. F. H. M. (1967) *Biochim. Biophys. Acta* **133**, 399–411
- Spencer, R., Charman, M., Emtage, J. S. & Lawson, D. E. M. (1976a) *Eur. J. Biochem.* **71**, 399–409
- Spencer, R., Charman, M., Wilson, P. W. & Lawson, D. E. M. (1976b) *Nature (London)* **263**, 161–163
- Spencer, R., Charman, M., Wilson, P. W. & Lawson, D. E. M. (1978) *Biochem. J.* **170**, 93–101
- Spudich, J. A. & Watt, S. (1971) *J. Biol. Chem.* **246**, 4866–4871
- Tilney, L. G. & Mooseker, M. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2611–2615
- Wilson, P. W. & Lawson, D. E. M. (1977) *Biochim. Biophys. Acta* **497**, 805–811