Effect of vitamin D deficiency on sarcoplasmic reticulum function and troponin C concentration of rabbit skeletal muscle

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Summary

1. Weanling rabbits were made rachitic either by a vitamin D-deficient diet or by parenteral administration of ethane 1-hydroxy-1,1-diphosphonate (EHDP) in amounts sufficient in other species to block the formation of 1,25-dihydroxycholecalciferol [1,25-(OH),D,].

2. The uptake of calcium into the isolated sarcoplasmic reticulum from mixed striated quadriceps muscle, and the amount of troponin C (the calcium-binding component of the troponin complex) in relation to other proteins from the same muscle, were measured.

3. In muscle from animals made rachitic by a dietary deficiency of vitamin D, the rate of uptake of calcium by the sarcoplasmic reticulum and the troponin C concentration were both significantly less (P < 0.02) than in control littermates. In EHDP-treated animals no significant differences from controls were found.

4. These results show that dietary deficiency of vitamin D in such animals can affect muscle physiology. Since no changes are found in animals made rachitic with EHDP, who presumably have a selective deficiency of 1,25-(OH),D, it is possible that the effect of vitamin D on muscle is mediated through metabolites other than 1,25-(OH),D, such as 25-hydroxycholecalciferol.

Key words: diphosphonate, muscle, sarcoplasmic reticulum, troponin C, vitamin D.

Abbreviations: 1,25-(OH),D, 1,25-dihydroxycholecalciferol; EHDP, ethane 1-hydroxy-1,1-diphosphonate.

Introduction

Weakness of proximal muscles may be a striking feature of osteomalacia (Smith & Stern, 1969; Schott & Wills, 1976), but its cause remains unknown. Most clinical evidence suggests that it results from a myopathy, but Mallette, Patten & Engel (1975) provide some evidence of a neuropathy. Although the weakness is most commonly associated with a deficiency of vitamin D, the evidence that such deficiency directly affects muscle function is meagre (Curry, Basten, Francis & Smith, 1974; Matthews, Heimberg, Ritz, Agostini, Fritzche & Hasselbach, 1977; Rodman & Baker, 1978). Birge (1978) emphasizes the importance of phosphate deficiency and Young, Brenton & Edwards (1978) consider that the predominant change is type II fibre atrophy.

It is logical in the experimental approach to this problem to examine separately both the sarcoplasmic reticulum, which controls the supply of calcium to the contractile elements, and the components of the contractile system itself (the actomyosin complex), in particular troponin C which confers calcium sensitivity on this complex.

This paper describes such an investigation in
rabbits made rachitic by receiving either a vitamin D-deficient diet or large doses of the diphosphonate EHDP (disodium ethane 1-hydroxy-1,1-diphosphonate), which appears to block the formation of the most active metabolite of vitamin D, vitamin D-deficient muscle (Curry et al., 1974) and also suggest a reduction of troponin C in such muscle. They also demonstrate that the effects on muscle of a vitamin D-deficient diet and of EHDP are different.

Materials and methods

Animals

Four-week-old male Dutch white rabbits (Hyline, Cheshire, U.K.) were used for all experiments. The control rabbits were always littermates.

Vitamin D-deficient rabbits. Rabbits were fed for 4 weeks on a vitamin D-deficient diet (kindly supplied by Dr David Fraser, Dunn Nutrition Unit, Cambridge) containing 0.33% Ca and 0.16% P, and were kept in the dark. Control rabbits were treated in the same way but were given vitamin D₃ in a daily oral dose of 10-13 i.u. in 0.1 ml of arachis oil.

EHDP-treated rabbits. Rabbits were given a daily subcutaneous injection of 40 mg of EHDP in sodium chloride solution (150 mmol/l): saline/kg body weight. Control animals received a daily injection of saline alone.

Methods

Rabbits were killed by cervical dislocation and blood was collected from the ear. Muscle was quickly dissected from the hind legs, placed in ice-cold saline and subcellular fractions were isolated as described. The tibiae were removed for histology. Plasma calcium was measured by atomic absorption spectrophotometry, and phosphorus by the method of Gomori (1942).

Isolation of sarcoplasmic reticulum vesicles. The method was based on that of Martinosi & Ferretos (1964), as modified by Curry (1976). All procedures were done at 0–4°C. Quadriceps muscle dissected free of connective tissue and major blood vessels was chopped with scissors and homogenized in 5 vol. of KCl (0-1 mol/l)/histidine (5 mmol/l) buffer, pH 7.0, with an Ultraturrax homogenizer, and then in a ground-glass homogenizer. The mixture was centrifuged at 1000 g for 20 min (rₑᵥ, 8 cm) and the supernatant further centrifuged at 14 000 g for 20 min (rₑᵥ, 8 cm) to sediment the mitochondrial fraction. The microsomal fraction was separated from the resultant supernatant by centrifugation at 70 000 g (rₑᵥ, 8 cm) for 1 h. The sediment containing this fraction was resuspended in KCl (0.6 mol/l)/histidine (5 mmol/l), pH 7-0, and incubated for 1 h at 0°C to solubilize residual contamination with contractile proteins (Curry, 1976).

Finally the suspension was centrifuged at 70 000 g for a further 1 h; the pellet was resuspended in a small volume of KCl (0.1 mol/l)/histidine (5 mmol/l), pH 7-0, and used as the sarcoplasmic reticulum vesicle fraction. By electron microscopy this fraction was shown to contain vesicles without mitochondrial contamination. The cytochrome c oxidase activity was very low. Two fractions were isolated from each rabbit, i.e. one from the muscles of each hind leg.

Measurement of calcium uptake by sarcoplasmic reticulum vesicles. The rate of calcium uptake by the sarcoplasmic reticulum was measured on the day of isolation by indirect spectrophotometry (Fairhurst & Jenden, 1966). The incubation medium contained histidine (20 mmol/l), KCl (30 mmol/l), potassium oxalate (5 mmol/l), MgCl₂ (5 mmol/l) and ATP (5 mmol/l) at pH 7-0. Sarcoplasmic reticulum suspension (20–100 μg) was incubated at 30°C for 3 min to establish a steady baseline. The reaction was started by adding 50 μl of CaCl₂ solution to give a final concentration of 100 μmol of Ca²⁺/l in the cuvette. The increase in absorbance due to the precipitation of calcium oxalate in the sarcoplasmic reticulum vesicles was recorded at 375 nm. The initial rate of change in absorbance was taken to be the rate of calcium uptake by the sarcoplasmic reticulum. All samples were measured at least four times.

Preparation and estimation of troponin C. After the rabbit was killed the quadriceps muscle was quickly dissected and placed in ice-cold saline. The muscle was either used immediately or stored at -20°C before use. Blood vessels, fat and connective tissue were removed and the muscle was chopped with scissors. Muscle (1 g) was dispersed with an Ultraturrax homogenizer in 6 vol. of urea (8 mol/l)/Tris (20 mmol/l)/mercaptoethanol (50 mmol/l)/EGTA (5 mmol/l), pH 7-8. The resulting solution was centrifuged at 70 000 g (rₑᵥ, 8 cm) for 30 min at 0°C to yield a supernatant soluble
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preparation of muscle protein containing troponin C. Its amount was measured by polyacrylamide-gel electrophoresis (Head & Perry, 1974). The absorbance at 280 nm was adjusted to about 3. An equal volume of bromophenol blue in glycerol/water (1:1, v/v) was then added as a marker and samples of 20–70 µl were applied to polyacrylamide tube gels [8% polyacrylamide containing urea (6 mol/l)/Tris (20 mmol/l)/EGTA (5 mmol/l), pH 8.6]; electrophoresis was carried out for about 4 h at 1·5 mA/tube.

After electrophoresis, the gels were stained for at least 3 h with 0.15% Coomassie blue in methanol/water/acetic acid (7:12:1, by vol.) and destained in the same solvent. Troponin C (Fig. 1) was identified as the front-running band by its behaviour in the presence of calcium; when samples were prepared and electrophoresed in the presence of CaCl₂ (1 mmol/l) instead of EGTA (5 mmol/l), the pattern was different and troponin C was bound to troponin I; a clear front-running band was therefore no longer seen on the gel. The gels were scanned at 570 nm in a linear transporter attachment of a Gilford spectrophotometer. The troponin C absorbance peaks were cut from the paper trace and weighed; the amount of troponin C was expressed as mg of paper/unit of total absorbance (a measure of total soluble muscle protein). Two samples were prepared from the pooled muscles of both hind legs and four gels run for each sample.

Statistics. The significance of the results was analysed by Student's t-test for paired data where appropriate. All results are expressed as mean ± 1 SEM.

Results

Evidence for vitamin D deficiency

Plasma phosphorus and calcium. The mean plasma calcium concentration for vitamin D-deficient rabbits (D– rabbits) was 3.16 ± 0.10 mmol/l (mean ± SEM, n = 11) and for the control (D+ rabbits) 3.71 ± 0.05 mmol/l (12) (P < 0.001). In the D– rabbits the plasma phosphorus concentrations were lower but not significantly different from that of the D+ rabbits: 2.24 ± 0.21 mmol/l and 2.53 ± 0.10 respectively (n = 11). The plasma calcium and phosphorus in the EHDP-treated group did not differ from their littermates (Ca: 3.59 ± 0.09 and 3.50 ± 0.10 mmol/l respectively, n = 18; P: 2.45 ± 0.10 and 2.66 ± 0.11 mmol/l respectively, n = 16).

Histology. The tibiae of both the D-deficient rabbits and EHDP-treated rabbits showed clear evidence of rickets with widening of the growth plate and an increase in width of the maturing and hypertrophic cartilage cell regions.

Growth. Over the 4 weeks required to produce rickets the vitamin D-deficient rabbits gained less weight than their littermates (nine pairs of D+ rabbits gained 300 ± 43 g and D– rabbits gained 197 ± 42 g) and appeared weaker; during 10 days of EHDP treatment, the average weight gain for
Vitamin D-deficient rabbits. The rate of change in absorbance (10³ ΔA min⁻¹ mg⁻¹ of protein) as an indicator of the rates of calcium uptake varied between individuals, but repeated measurement on the muscles of the same animal gave similar results and the SE of the measurements on each individual is shown in the Figures. The initial rate of change of absorbance by the sarcoplasmic reticulum of D- rabbits was consistently less than that of control littermates (Fig. 2). The mean difference of 140 ± 44 (5) between pairs of D+ and D- littermates was significant (P < 0.025).

EHDP-treated rabbits. In 11 pairs of rabbits treated with EHDP the rate of calcium uptake by the sarcoplasmic reticulum was not different from the untreated littermates. The mean difference in rate (10³ ΔA min⁻¹ mg⁻¹ of sarcoplasmic reticulum protein) between the pairs was 5 ± 25 (11), which is not significant.

Vitamin D₃-replenished rabbits. Rabbits that had been on a vitamin D-deficient diet for 3 weeks were then given vitamin D (10–13 i.u. of vitamin D₃ daily) for between 7 and 12 days. At the end of this time the rate of absorbance change by sarcoplasmic reticulum from these rabbits was compared with that from rabbits who had continued on the vitamin D-deficient diet. The results were variable (Fig. 3); in five cases the rate of calcium uptake was higher by the sarcoplasmic reticulum from replenished than from depleted animals and in four it was unchanged or lower. The histology of the vitamin D-deficient rabbits which had been replenished with vitamin D was also variable, as were the values for plasma calcium. There was no relationship between the rate of calcium uptake by the sarcoplasmic reticulum and the plasma calcium.

Addition of vitamin D and its metabolites in vitro to sarcoplasmic reticulum from vitamin D-deficient rabbits. The following had no effect when added in vitro to sarcoplasmic reticulum from vitamin D-deficient rabbits: 10–400 ng of vitamin D₃, 500 pg–200 ng of 25-hydroxycholecalciferol, 1 pg–200 ng of 1,25-(OH)₂D₃, 100 pg–200 ng of 1,24,25-(OH)₃D₃, or 50 pg–1 µg of 24,25-(OH)₂D₃.

Measurement of troponin C

Vitamin D-deficient rabbits. In each pair the amount of troponin C from the muscle of the D-
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FIG. 4. Amount of troponin C in relation to total extracted muscle protein in D+ and D− littermate rabbits. The lines join the value for the D+ (control) animal to its D− littermate. Repeated measurements have been made on the muscles of each animal; the values are expressed as means ± 1 SEM. For explanation of units and significance of difference between groups see the text.

rabbit was lower than in its individual D+ control (Fig. 4). The average difference, expressed in the units defined in the Materials and Methods section, of 0.32 ± 0.06 (7) was significant (P < 0.01). As with the measurement of calcium uptake by sarcoplasmic reticulum there was considerable individual variation, but repeated estimates of troponin on muscle from the same animals gave consistent results.

EHDP-treated rabbits. In five pairs of EHDP-treated rabbits and controls, the average difference from the muscles of D+ and D− rabbits of 0.16 ± 0.12 was not significant.

Discussion

Calcium is of prime importance for muscle contraction (Huxley, 1974; Perry, 1974). Since vitamin D, via its metabolites, influences calcium movements in its known target organs, and since myopathy occurs in osteomalacia (Stanbury, 1965; Smith & Stern, 1967; Schott & Wills, 1976), it is reasonable to investigate the possible effect of vitamin D on muscle. We have found that the function of the sarcoplasmic reticulum and the amount of troponin C is abnormal in animals made deficient by diet but not those treated with EHDP.

Calcium uptake by the sarcoplasmic reticulum

The sarcoplasmic reticulum contains many calcium-binding proteins and an ATPase. By concentrating calcium it deprives actin and myosin of this ion and acts as a 'relaxing factor' (Tada, Yamamoto & Tonomura, 1978).

Our findings suggest that the reduced rate of calcium uptake by the sarcoplasmic reticulum results directly from lack of vitamin D, but other possibilities exist. Thus this reduction could be secondary to growth retardation, or to a decrease in the intracellular concentration of phosphate, both of which might follow the vitamin D-deficient diet. However, in the EHDP-treated animals growth is retarded but calcium uptake by the sarcoplasmic reticulum is normal; and Rodman & Baker (1978) have demonstrated delayed relaxation in a vitamin D-deficient soleus muscle preparation in situ in the rat irrespective of changes in plasma or dietary phosphate.

Our results confirm those obtained with a Milli-pore filtration method and 45Ca (Curry et al., 1974), and provide information on sarcoplasmic reticulum function in EHDP-induced rickets. In rats large doses of EHDP appear to block the formation of 1,25-(OH)2D3 (Hill et al., 1973; Trechsel, Schenk, Bonjour, Russell & Fleisch, 1977) and produce a defect in mineralization which resembles rickets. Despite clear evidence of defective mineralization in the EHDP-treated animals, the rate of calcium uptake by the sarcoplasmic reticulum derived from their muscles appears to be normal. This raises the possibility that 1,25-(OH)2D3 deficiency is not the cause of the defective uptake of calcium by the sarcoplasmic reticulum from vitamin D-deficient animals.

The repletion experiments in vivo do not provide any clear answer. In about half the rabbits replenished with vitamin D (without any other changes in diet), the calcium uptake was more rapid than in the vitamin D-depleted animals. There was also no evidence that addition in vitro of assorted vitamin D metabolites to the sarcoplasmic reticulum had any effect on calcium uptake. Matthews et al. (1977) also found that 1,25-(OH)2D3, given in vitro, had no effect on the defective calcium transport by sarcoplasmic reticulum from uraemic rabbits, although it was effective in vivo.

Amount of troponin C

Troponin C is one of three subunits of the troponin complex (Perry, 1974) to which it binds
in the presence of calcium. This binding appears to alter the position of troponyosin exposing the myosin binding sites on actin (Cohen, 1975). Without troponin C the contraction of the isolated actomyosin complex is no longer influenced by calcium. Troponin C can be readily distinguished from other contractile proteins on an acrylamide gel by its behaviour with and without calcium but it is difficult to make a direct quantitative comparison with these components because of its relatively small amount. We have therefore adopted an indirect method, which compares the amount of troponin C with total absorbance, a measure of the amount of soluble muscle protein which includes contractile elements and sarcoplasmic proteins.

Since this measurement is comparative it suggests the reduction in troponin C is selective and is therefore more likely to be related to vitamin D lack than to the relatively poor growth of these vitamin D-deficient animals. Further this reduction is not seen with the EHDP-treated animals. However, the possibility that the reduction in troponin C relative to muscle proteins is produced by an increase in these proteins cannot be excluded.

The fact that a protein binds calcium should not necessarily suggest that its amount can be affected by vitamin D lack; but it is of particular interest that troponin C and the calcium-binding protein of the intestinal cell, whose formation is known to be controlled by vitamin D, are homologous (Kretsingher, 1979).

Relation to type II fibre atrophy

The main histological change in the muscle in osteomalacia is atrophy, particularly affecting type II fibres (Dastur, Gagrat, Wadia, Desai & Bharucha, 1975; Young et al., 1978). This common finding may be related to disuse and wasting of muscle, and is not unexpected in the elderly. In younger people where mobility is maintained, muscle wasting is not prominent and proximal weakness may respond within a few days to vitamin D. This suggests that vitamin D lack may produce reversible biochemical alterations in addition to type II fibre atrophy. Publicover, Duncan & Smith (1978) have described experiments which connect biochemical with structural changes in muscle. They have shown that the irreversible release of calcium by sarcoplasmic reticulum leads to myofilament degeneration within about 40 min. It is possible therefore that the defect in sarcoplasmic reticulum function associated with vitamin D deficiency might lead to an increased intracellular concentration of calcium, with eventual loss of muscle tissue, the effects of which could be worsened by a deficiency of troponin C.

Clinical aspects

Muscle weakness in osteomalacia may occur either in association with hypophosphataemia, or with a lack of vitamin D (or a disturbance of its metabolism). The myopathy of hypophosphataemia is probably not related to changes in vitamin D. It occurs in adult-onset hypophosphataemia and in phosphate deficiency induced by excessive ingestion of aluminium hydroxide (Dent & Winter, 1974), but not in infantile-onset vitamin D-resistant rickets, and it is postulated that in the latter disorder intracellular phosphate must be normal. This would explain why plasma 1,25-(OH)₂D₃ concentration is not increased in vitamin D-resistant rickets (Haussler & McCaın, 1977; Scriven, Reade, DeLuca & Hamstra, 1978). If muscle is a target organ of vitamin D, which is the responsible metabolite? That it need not be 1,25-(OH)₂D₃ is suggested by Birge & Haddad (1975), who found that 25-hydroxycholecalciferol stimulated protein synthesis of diaphragmatic muscle, and Eastwood, Stamp, de Wardener, Bordier & Arnaud (1977), who noted that 25-hydroxycholecalciferol improved the myopathy of renal osteodystrophy. Further, both Birge & Haddad (1975) and Lawson, Charmán, Wilson & Edelstein (1976) have reported the presence of a binding protein for 25-hydroxycholecalciferol in muscle. However, our results do not explain the apparently beneficial effects of 1,25-(OH)₂D₃ administration in vivo, either on the ability of isolated sarcoplasmic reticulum from uraemic rabbits to concentrate calcium (Matthews et al., 1977) or on the myopathy in patients with renal osteodystrophy (Henderson, Russell, Ledingham, Smith, Oliver, Walton, Small, Preston, Warner & Norman, 1974). More importantly they do not explain the severe muscle weakness, which is a feature of vitamin D-dependent rickets, thought to be due to a specific defect in the renal 1-hydroxylase.

Finally, the significance of two recent reports on the improvement of myopathy brought about by 1α-hydroxylated vitamin D metabolites in disorders other than osteomalacia is difficult to assess. Thus Sorensen, Lund, Saltin, Lund, Andersen, Hjorth, Melsen & Mosekilde (1979) describe an increase in indirectly measured muscle strength and in oxidative enzymes in muscle biopsies from patients with ‘bone loss of ageing’ (in which there is an
increase in amounts of osteoid but normal serum 25-hydroxycholecalciferol concentrations) treated with 1α-hydroxycholecalciferol. Heyburn & Peacock (1979) also imply that 1α-hydroxycholecalciferol or 1,25-(OH)2D3 can improve muscle strength in conditions such as osteoporosis and thyrotoxicosis, and in patients on corticosteroid therapy.

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