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Changes in the activity of skeletal muscle calcium-activated neutral proteinase (*EC* 3 . 4 . 22 . 17) and its specific inhibitor in chickens grown at different rates in response to graded levels of dietary protein

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1. Two experiments are reported in which the effect of alteration in growth rate on the levels of avian skeletal muscle calcium-activated neutral proteinase (*EC* 3 . 4 . 22 . 17) (CANP or calpain) and its specific inhibitor (calpastatin), a system thought to be implicated in myofibrillar catabolism, was studied by means of manipulation of dietary protein concentration.

2. In Expt 1 broiler chicks were given free access to diets containing 105, 149, 197 and 212 g protein/kg for 20 d. In Expt 2 the four dietary treatments were 119, 141, 182 and 227 g protein/kg diet given for 16 d. Chick growth rate and total leg skeletal muscle weight significantly increased ($P < 0.001$) with increasing dietary protein concentration in both experiments. Total skeletal muscle protein increased with the level of dietary protein, the effect being significant ($P < 0.01$ and $P < 0.001$ in Expts 1 and 2 respectively).

3. Minced leg muscle was homogenized in low-salt buffers, and the extract chromatographed on DEAE-cellulose to separate proteinase and inhibitor activity. The partially purified CANP enzyme and inhibitor proteins were present at a concentration broadly consistent with literature reports, and their elution characteristics and Ca^{2+} concentration dependence were not varied by dietary protein concentration.

4. Both the muscle CANP and CANP inhibitor activities (units/kg muscle) exhibited upward trends with growth rate and increased muscle weight. However, these differences were not statistically significant ($P > 0.05$) and were not present at all when the results were expressed as units/g muscle protein.

The turnover of myofibrillar proteins in skeletal muscle occurring during growth can be regulated through changes in the rates of both protein synthesis and degradation. Whilst protein synthesis in muscle is almost certainly an important site of regulation, being particularly sensitive to dietary and hormonal changes (Waterlow & Stephen, 1968; Millward *et al.* 1976), it now appears that a substantial degree of modulation of muscle protein levels can be achieved through the control of the degradation process (Millward, 1985). Protein degradation can be manipulated by anabolic agents (Buttery & Sinnett-Smith, 1984) promoting an increase in growth rate.

The rate of myofibrillar protein turnover has been assessed from urinary excretion of N^{τ}-methylhistidine (MH) in the rat (Young *et al.* 1972; Nishizawa *et al.* 1977; Ward & Buttery, 1979) and poultry (Saunderson & Leslie, 1983; Saunderson & Bryan, 1985). Using MH excretion, Hayashi *et al.* (1985) reported rates of degradation of myofibrillar proteins in male layer and broiler chickens of 21, 42 and 63 d of age to be 6·1, 4·5 and 2·4 %/d (layer) and 5·0, 2·8 and 0·9 %/d (broiler) respectively, suggesting an inverse relation between live-weight gain and rate of muscle protein degradation.

The idea that myofibrillar protein degradation must involve disassembly and proteolysis of the insoluble myofibrillar array at physiological pH has led to the proposal that an intracellular calcium-activated neutral proteinase (*EC* 3 . 4 . 22 . 17) (CANP or calpain) may be at least partly responsible, since purified CANP from skeletal muscle causes partial degradation of myofibrils, attacking the Z-disk and several contractile proteins such as

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troponin-T, troponin-I, C-protein, tropomyosin and myosin (Dayton *et al.* 1975; Pemrick, 1981).

The theory of the involvement of CANP in myofibrillar protein degradation is supported by reports that the amount of CANP isolated from atrophying muscle tissue (where myofibril degradation is proceeding rapidly) is several-fold greater than the amount isolated from normal muscle tissue (Mayer *et al.* 1976; Dayton *et al.* 1979). Specifically, Kar & Pearson (1976) showed CANP activity to be 2·7 times higher in biopsies from muscle taken from humans possessing Duchenne muscular dystrophy compared with biopsies taken from non-dystrophic controls. Dayton *et al.* (1979) reported levels of CANP in the skeletal muscle of vitamin-E-deficient rabbits to be 3·5–4·0 times higher than control levels. Additionally, immunocytochemical localization of the 80 kDa subunit of CANP at the Z-disk of purified porcine myofibrils and in sectioned tissue (Dayton & Schollmeyer, 1981), further supports the theory that the proteinase plays a role in myofibrillar degradation, at least in pathological situations.

In contrast to this essentially catabolic activity, and based on a number of reported actions in non-muscle cells on protein kinases, hormone receptors and membrane proteins, Murachi (1983) has proposed a regulatory role for this enzyme in transforming Ca^{2+} signals into intracellular effects.

Several of the enzyme's functions, including an initiating action in myofibrillar degradation, could be related to the complex series of events underlying skeletal muscle growth. This has prompted us to examine whether the activity of this enzyme and its specific endogenous protein inhibitor calpastatin (Murachi *et al.* 1981; Lepley *et al.* 1985) might be related to changes in muscle weight or protein content in animals growing normally but at different rates. For this purpose, the chicken was selected as our experimental model because of its growth characteristics, and from preliminary studies which corroborated the results of Kawashima *et al.* (1984) of the existence of only one form of skeletal muscle CANP active at intermediate Ca^{2+} levels, whereas mammalian tissues contain two forms activated by micromolar and millimolar Ca^{2+} concentrations respectively (Inomata *et al.* 1983). The apparent coexistence in the same tissue of enzyme and inhibitor necessitates chromatographic separation of these activities before they can be quantified. Leg muscle was used in preference to breast muscle because work by Bryan *et al.* (1985), using broilers grown at different rates (fractional growth rates from 0·056/d to 0·126/d) had shown that leg muscle metabolism was more closely associated with growth rate than that of the breast muscle.

EXPERIMENTAL

Animals and management

Cob cockerels (1-d-old) from a commercial hatchery were housed in a continuously lighted room in wire-bottomed cages (three per cage) and allowed access to food and water *ad lib.* Birds were allowed to acclimatize to house conditions for 5 d and were given a wheat/soya-bean-based chick starter diet (see Boldizsár *et al.* 1973) before commencing the experimental diets. Chicks were assigned to experimental groups such that the mean weight of each group was similar, and randomly assigned to diets. The cages containing different diets were randomized within the animal house. Birds were weighed weekly. Mean daily gains were calculated from a least-squares regression of body-weight *v.* time.

At the end of the test period (20 d Expt 1, 16 d Expt 2) chicks were killed by cervical dislocation, exsanguinated and the thigh muscle from both hind-limbs quickly excised, weighed and frozen in liquid nitrogen. Samples were stored at $-15°$ until analysis could be performed, which took place over a period of up to 12 weeks. Expt 2 was essentially to confirm the observations of Expt 1.

Table 1. *Composition of experimental diets (g/kg)*

Expt 1				
Wheat	280	270	210	238
Maize	475	400	355	290
Barley	110	90	123	50
Maize oil	20	20	20	20
Fish meal (700 g/kg CP)	20	35	50	52
Extracted soya-bean meal (450g/kg CP)	45	135	192	300
Vitamins and minerals*	50	50	50	50
Determined CP	105	149	197	212
Expt 2				
Wheat	280	270	242	238
Maize	472	420	365	290
Barley	110	120	120	50
Maize oil	20	20	20	20
Fish meal (700 g/kg CP)	20	21	50	52
Extracted soya-bean meal (450 g/kg CP)	45	90	175	300
Vitamins and minerals*	50	50	50	50
Determined CP	119	141	182	227

CP, crude protein (nitrogen × 6·25).

* Composition (per kg final diet): retinol (vitamin A) 2·25 mg, cholecalciferol (vitamin D) 0·038 mg, D,L-α-tocopherol (vitamin E) 10 mg, menadione (vitamin K) 3·00 mg, thiamin 3·00 mg, riboflavin 5·00 mg, pyridoxine 4·00 mg, pantothenic acid 10 mg, niacin 30 mg, folacin 0·15 mg, cobalamin 0·01 mg, biotin 0·13 mg, choline 1300 mg, calcium 10·00 g, phosphorus 5·00 g, potassium 4·00 g, sodium 1·50 g, chloride 1·50 g, magnesium 600 mg, manganese 60 mg, iron 80 mg, copper 6·00 mg, zinc 50 mg, iodine 0·50 mg, molybdenum 0·20 mg, selenium 0·15 mg, antioxidant (BHT) 125 mg, coccidiostat (Pancoxin) 500 mg.

Diets

Diets shown to contain four levels of crude protein (N × 6·25; CP) were used in each experiment; Expt 1: 105, 149, 197 and 212 g protein/kg diet; Expt 2: 119, 141, 182 and 227 g protein/kg diet. The compositions of the diets are given in Table 1.

Quantification of CANP and inhibitor

Unless otherwise indicated, all procedures were carried out at 4°. CANP and its specific inhibitor from skeletal muscle were isolated essentially by the method described by Inomata *et al.* (1983). Briefly, the thigh muscles from the birds in each cage were dissected, trimmed of external fat and connective tissue, weighed, minced and, using a Polytron homogenizer, homogenized (100 g wet weight) with 3 vol. ice-cold buffer containing 20 mM-Tris-hydrochloride, 5 mM-EDTA and 10 mM-2-mercaptoethanol, pH 7·5 (buffer A). The crude extract was centrifuged at 10000 ***g*** for 15 min, filtered through glass wool and mixed with 150 g DEAE-cellulose (Whatman DE-52) which had been equilibrated with buffer A.

The cellulose was packed into columns (30 × 330 mm) and eluted with linear gradients of 0–0·6 M-sodium chloride in buffer A (total volume 1·2 litres) monitored by conductivity measurements. CANP activity was assayed by measuring trichloroacetic acid-soluble peptides produced by casein proteolysis in the presence of 7 mM-Ca^{2+} and 10 mM-2-mercaptoethanol (Ishiura *et al.* 1982). Where appropriate the free Ca^{2+} concentration was varied between 0 and 10 mM. One unit of enzyme activity was defined as the amount of enzyme which produced an increase in absorption at 280 nm of 1·0 after incubation at 30° for 30 min. The assay was linearly dependent on enzyme concentration over all the range of activities measured, at least up to 0·3 units.

For the estimation of inhibitor activity, test samples were incubated with a fixed amount of CANP at 30° for 30 min and the decrease in proteolytic activity compared with controls.

One unit of inhibitor was defined as that amount of inhibitor that would inhibit 1 unit CANP under conditions of the standard assay following the lines suggested by Lepley (1983). The value was actually calculated from the straight line portion of the inhibition profile at 50% inhibition. Lepley (1983) calculated the quantity of inhibition needed to inhibit completely CANP from a regression line, ranging from 10 to 95% inhibition of CANP, which gave a correlation coefficient in excess of 0·99.

In preliminary experimentation, inhibitor-containing fractions and fractions immediately preceding the CANP peak from a DEAE-cellulose fractionation were pooled and subjected to hydrophobic chromatography on phenyl-sepharose in the presence of 0·3 M-NaCl in buffer A (Inomata *et al.* 1983). On this system CANP inhibitor passes through unbound, and bound CANP is eluted with an ethanediol gradient. No evidence was found for the low Ca^{2+} (micromolar)-requiring form of CANP in agreement with results for avian skeletal muscle reported by Kawashima *et al.* (1984).

Protein determination

CP determinations were performed in duplicate on samples of diet and freeze-dried pooled skeletal muscle samples by the Kjeldhal procedure (Association of Official Analytical Chemists, 1980). The extractable muscle protein, soluble in low-salt buffers, was measured using the microtannin turbidity method (Mejbaum-Katzenellenbosen & Dobryszycka, 1959), using dried bovine serum albumin as the standard.

Statistical analysis

One way analyses of variance were performed using the Genstat statistical package (Rothamstead Experimental Station, St. Albans, Herts). The criterion of significance was $P < 0{\cdot}05$ for all statistical analyses.

RESULTS

Separation of CANP and CANP inhibitor activities

Chicken leg muscle extracts on DEAE-cellulose chromatography gave a 280 nm absorption profile very similar to that described by Kawashima *et al.* (1984). From conductivity measurements (Expt 1 only) the mid-points of the CANP and inhibitor elution positions were between 0·25–0·28 M-NaCl and 0·02–0·04 M-NaCl respectively. Dietary regimen did not influence these elution characteristics, nor the Ca^{2+} dependence for half-maximal CANP activation which was found to be 0·57 mM (SEM 0·07; n 12), somewhat higher than that described by Suzuki & Ishiura (1983). It can be concluded that dietary protein did not lead to changes in the physical form of the enzyme and inhibitors present in this tissue.

Growth trial

Initially birds on all dietary treatments averaged 96 (SE 2·1) g in Expt 1 and 93 (SE 2·1) g in Expt 2. In Expt 1 (Table 2) growth rates of chickens varied from 9·8 to 26·2 g/d as the level of dietary protein increased. In Expt 2 chick growth rates varied from 12·3 to 32·2 g/d. The dietary protein concentrations used in Expt 2 produced a wider variation in growth rates compared with Expt 1. Changes in dietary protein concentration also concurred with increases in total leg skeletal muscle weights (Table 2). Similarly, muscle protein increased with increasing dietary protein concentration (Table 3). CANP activity (Table 4) was found to be in the range 772–1243 units/kg muscle. Ishiura *et al.* (1978) reported 1340 units/kg muscle in a crude extract when their units were based on a 30 min assay as in the present work.

As shown in Table 4, the effect of varying growth rate was without statistically significant

Table 2. *Growth rates (g/d) and total leg skeletal muscle weights (g) of chickens given different dietary protein concentrations at four levels*

(Values are means from four cages each with three birds, each cage taken as a replicate and are presented as mean values per bird)

					SED	Statistical significance of difference (12 df)
Expt 1						
Dietary protein concentration (g/kg)	105·5	149·0	196·9	213·3		
Growth rate (g/bird per d)	9·8	22·4	26·5	26·2	1·38	$P < 0·001$
Total leg muscle wet wt (g/bird)	32·8	62·0	63·9	69·7	4·97	$P < 0·001$
Expt 2						
Dietary protein concentration (g/kg)	119·2	140·7	182·3	227·4		
Growth rate (g/bird per d)	12·3	19·7	33·2	32·2	1·23	$P < 0·001$
Total leg muscle wet wt (g/bird)	30·7	43·5	67·3	68·7	3·02	$P < 0·001$

SED, standard error of difference.

Table 3. *Skeletal muscle protein content (g/kg muscle) of chickens given different dietary protein concentrations at four levels*

(Mean values are for four groups of three chicks each, each group taken as a replicate)

					SED	Statistical significance of difference (12 df)
Expt 1						
Dietary protein concentration (g/kg)	105·5	149·0	196·9	212·3		
Leg muscle protein wet wt (g/kg)	210	220	252	250	10·6	$P < 0·001$
Expt 2						
Dietary protein concentration (g/kg)	119·2	140·7	182·3	227·4		
Leg muscle protein wet wt (g/kg)	220	211	253	257	4·8	$P < 0·001$

SED, standard error of difference.

effect ($P > 0·05$) on skeletal muscle CANP (units/kg muscle). However, there was a consistent trend for higher CANP activity from birds fed on the higher protein diets in both experiments. When CANP activity was compared in terms of either units/g muscle protein or units/g extractable muscle protein (i.e. that amount solubilized in low-salt buffers), this upward trend was removed.

These results would seem to indicate that the apparent elevation in CANP activity in the higher growth rate birds could be attributed, in part, to increased muscle protein levels. Inhibitor activity was only determined quantitatively in Expt 2 and found to be present at 290–473 units/kg muscle (Table 4). Ishiura *et al.* (1982) reported 500 units/kg muscle. It is not clear at present whether our lower inhibitor activity reflects a difference between the leg muscle used here and the more usual breast muscle, or slight differences in assay procedure. Inhibitor activity in Expt 2 (Table 4) increased slightly with increased dietary protein concentration. However this was not statistically significant ($P > 0·05$). This slight

Table 4. *Activity of calcium-activated neutral proteinase (CANP) (EC 3 · 4 . 22 . 17) and the specific inhibitor (units/kg muscle and units/g muscle protein) of chickens at different rates of growth*

(Values are means of four cages of three birds each, each cage taken as a replicate)

					SED	Statistical significance of difference (12 df)
Expt 1						
Growth rate (g/bird per d)	9·8	22·4	26·5	26·2	1·38	$P < 0·001$
CANP (units*/kg muscle)	823	772	1243	1192	280·1	NS
CANP (units/g extractable muscle protein)	43·4	34·3	41·0	29·1	15·87	NS
Expt 2						
Growth rate (g/bird per d)	12·3	19·7	33·2	32·2	1·23	$P < 0·001$
CANP (units/kg muscle)	785	776	1040	904	172·4	NS
CANP (units/g extractable muscle protein)	33·9	32·8	35·2	29·3	7·50	NS
CANP inhibitor (units/kg muscle)	361	290	312	473	85·1	NS
CANP inhibitor (units/g extractable muscle protein)	15·3	12·7	10·4	15·5	3·34	NS

NS, not significant ($P > 0·05$); SED, standard error of difference
*Units: see pp. 143–144.

upward trend again disappeared when inhibitor activity was expressed as either units/g muscle protein or units/g extractable muscle protein.

There was no significant correlation between the CANP:CANP inhibitor ratio ($P > 0·05$) and growth rate.

DISCUSSION

Various authors have attempted to relate muscle CANP activity to protein turnover and in particular myofibrillar protein breakdown in this tissue. For example Brooks *et al.* (1983) using a starvation/refeeding schedule in rats and assay of a crude CANP fraction concluded that this enzyme could play an initiating role in contractile protein catabolism. On the other hand Millward (1985) has reviewed several studies where Ca^{2+}-mediated effects on muscle protein catabolism seem to operate through a lysosomal or non-proteolytic mechanism. In general such studies have concentrated on the enzyme's supposed catabolic role in muscle turnover and growth, whereas Murachi (1983) has stressed an alternative regulatory function, possibly related to enzyme activation, or hormone receptor processing and translocation (Murayama *et al.* 1984) so that the CANP enzyme could conceivably influence muscle growth rate without necessarily being directly involved in myofibrillar proteolysis.

Few studies have attempted to measure both CANP and CANP inhibitor activities under normal conditions of growth, albeit at different rates, induced by nutritional means.

The work described in the present paper suggests that chicken skeletal muscle CANP and its specific inhibitor are not modified with respect to isoelectric point or Ca^{2+} requirement in muscle undergoing growth at rates varying by four-fold. Furthermore, when expressed in terms of units/g muscle protein or units/g extractable protein, there was no significant correlation between growth rate and changes in the enzyme and inhibitor activities quantified after a single chromatographic step.

The possibility remains that the interaction between CANP, CANP inhibitor, Ca^{2+} and muscle proteins is modified by subcellular proximity to membranes or cytoskeletal elements. Recent studies indicate that membrane lipids such as phosphatidyl inositol can regulate CANP binding to membranes and the Ca^{2+} requirement of the enzyme (Imajoh *et al.* 1986). Regulation at this level would be unlikely to be detected in the present experimentation.

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