Ca²⁺-ATPase deficiency in a patient with an exertional muscle pain syndrome

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SUMMARY ³¹P Magnetic resonance spectroscopy studies were carried out in vivo on skeletal muscle of a patient with verapamil-responsive, chronic, progressive post-exertional muscle pain. A sister suffered from a similar complaint. The results showed that the muscle: (1) decreased its high energy phosphate content more rapidly than normal during exercise, indicating either increased utilisation or decreased production of ATP; (2) acidified more rapidly than normal during exercise suggesting an increased glycolytic rate; (3) continued in some studies to acidify markedly during the first minute after exercise, indicating that glycolysis remained active into the recovery period; (4) had phosphocreatine and ADP recovery rates consistent with normal rates of oxidative phosphorylation. On the basis of these results, it was proposed that the patient suffers from a defect in Ca²⁺ handling in the muscle. Subsequently, direct measurement of Ca²⁺-ATPase activity in the sarcoplasmic reticulum fraction from a muscle biopsy sample showed that the activity of this enzyme was reduced by about 90%.

There are several well-documented disorders of skeletal muscle in which a defect in a muscle enzyme can be demonstrated. In many of these disorders one of the steps in the energy-generating pathways is directly affected. Examples of such enzyme deficiencies in which ³¹P magnetic resonance spectroscopy (MRS) has demonstrated an altered energy status of the muscle at rest or as a result of exercise are glycogen phosphorylase,^{1,2} glycogen debrancher enzyme,³ phosphofructokinase,⁴⁻⁶ phosphoglycerate kinase,⁷ phosphoglycerate mutase,⁸ NADH-CoQ reductase⁹ and other steps in oxidative phosphorylation.¹⁰⁻¹²

Over-utilisation of energy might also result in a detectable change in cellular energetics. The most obvious need for ATP in muscle is for myofibrillar

contraction. However, there is also a large energy requirement for ion pumping. It has been estimated that one-third to one-half of the ATP used in contracting muscle is required for re-uptake of Ca²⁺ into sarcoplasmic reticulum via Ca²⁺-ATPase.¹³ Therefore, an alteration in the ability of the muscle cell to handle this ion might be reflected in the cell's energy status.

Two reports have been published describing patients with Ca²⁺-ATPase deficiency. These patients suffered from impaired skeletal muscle relaxation which was made worse by exercise. In the first report¹⁴ low Ca²⁺ uptake into the sarcoplasmic reticulum was described in a single patient. In the second, Karpati *et al*¹⁵ found low immunoreactive Ca²⁺-ATPase activity in sarcoplasmic reticulum from two patients (two close relatives of these patients also suffered from similar symptoms and were assumed to have the same metabolic abnormality).

In this paper we report on a patient with low Ca²⁺-ATPase activity in skeletal muscle who did not have the marked problem with muscle relaxation found in the previously reported cases; his main symptom was muscle pain following exercise. This was partially prevented by the Ca²⁺ antagonist verapamil (α -isopropyl- α [(N-methyl-N-homoveratryl)- γ -amino-propyl]-3,4-di-methoxyphenylacetone nitrile hydrochloride). ³¹P MRS studies of the patient's muscle

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revealed a pattern of abnormality in high energy phosphate metabolism not observed in patients with other types of metabolic abnormalities. The patient's sister suffers from similar symptoms and also showed an abnormal metabolic response to exercise. A preliminary report of these investigations has been presented.¹⁶

Case reports

Patient KR

Clinical history. This patient's condition had previously been extensively investigated in London at Guy's Hospital and at the National Hospital, Queen Square, before being referred to one of us (JW) in Newcastle upon Tyne where he was admitted for investigation in 1980. A preliminary report was published by Walton.¹⁷ It was noted that dantrolene sodium improved the patient's pain slightly but produced unacceptable muscle weakness. Naftidrofuryl oxalate greatly accentuated the pain and it was therefore decided to give verapamil which was rapidly effective. When first seen in Oxford in 1982 the patient was a physically active 37-year-old with a 10-year history of exercise-induced muscle pain. The symptoms were first noted in the calf muscle after walking, have gradually become much more pronounced and currently affect upper as well as lower limbs. He can no longer work as a marine engineer. The pain is minimal during exercise, increases in the resting period and slowly involves other muscles in the limb. Muscle stiffness and cramps following exercise have become increasingly common and can last for several days. Symptoms ease in hot weather and are worse in the winter.

On examination the patient was found to have well-developed muscles with no wasting or weakness. No muscle fasciculation was noted and eye movement and sensation were normal. There was no muscle tenderness. In 1986 a mild grip myotonia was observed after maximal isometric contraction on a rubber bulb. Immersing the forearm in cold water caused the patient severe discomfort.

Clinical investigations Routine blood count, haemoglobin, blood urea, total serum protein, albumin, bilirubin, alkaline phosphatase and aspartate amino transferase were all normal. Electromyographic studies in 1981 and 1983 gave results which were within normal limits. In 1985 some motor unit potentials were found to be of long duration and maximal voluntary effort evoked only a moderate number of single motor units. The electrocardiogram was normal. Histological examination of a muscle biopsy specimen from quadriceps obtained in January 1987 revealed a mild fibre atrophy; myosin ATPase staining showed the atrophic fibres to be of both types I and II. There were no architectural abnormalities or inclusions. Fat and glycogen contents were within normal limits. NADH dehydrogenase, succinate dehydrogenase and cytochrome oxidase activities were found to be normal. When examined by electron microscopy the appearance of the muscle was unremarkable except for a possible increase in the number of membranous bodies within the sarcoplasmic reticulum vesicles. A muscle biopsy had also been obtained in 1980 and the concentration of carnitine and the activities of the following enzymes found to

Taylor, Brosnan, Arnold, Bore, Styles, Walton, Radda

be normal in the muscle (Prof JB Harris and Dr RJT Pennington, Newcastle-upon-Tyne): AMP deaminase, carnitine palmityl transferase, carnitine acetyl transferase, adenylate kinase, creatine kinase.¹⁷ Mitochondrial function was normal (Dr John Morgan-Hughes, National Hospital).

Drugs which have been tried without effect are phenytoin, carbamazepine, diazepam, calcium gluconate, naproxen, piroxicam, diclofenac sodium, β -adrenergic blocking agents, procaine amide and ibuprofen. Unless otherwise indicated, KR was taking verapamil (120 mg q.i.d.) at the time of the MRS investigations.

Patient AW

Clinical history This patient is the 46 year old sister of KR. Sixteen years ago she noticed difficulties in climbing stairs. Pain was present in both calves after walking or exercising. The condition has progressed and she now has pain in all muscles. This is present at rest and is much worse after activity. Walking more than 200 yards is very difficult. The pain is associated with muscular stiffness. It is exacerbated by cold and relieved by a hot bath. There is no muscle wasting or tenderness or clear limb weakness. All reflexes are present symmetrically and the plantars flexor. There is no abnormality of sensation or gait. She has had an appendicectomy and a hysterectomy. Verapamil had originally provided some relief, but was discontinued due to side effects (palpitations).

Clinical investigations Routine haematology and clinical laboratory biochemistry results were normal. Serum creatine kinase activity was 100 IU/l and there was no myoglobinuria. A muscle biopsy had been performed in 1978 by Dr J Morgan-Hughes at the National Hospital for Nervous Diseases, Queen Square, London. Muscle fibre diameters were in the normal range. There was a normal distribution of fibre types with the standard myosin ATPase reactions. Activities of succinate dehydrogenase, phosphorylase, phosphofructokinase and adenylate deaminase were all reported to be normal. An EEG performed in 1985 was normal.

Methods

³¹P MRS

Studies were performed as previously described¹⁸ using a Fourier transform spectrometer (Oxford Research Systems, Oxford, England) interfaced with a 1.9 Tesla, 25 cm bore, super-conducting magnet (Oxford Magnet Technology, Oxford, England). Pulsing conditions (40 μ s pulse length, 2.0 s interpulse delay) were chosen to optimise the ³¹P signal obtained with the 2.5 cm diameter surface coil. Spectra were collected over times ranging from 0.5–4 min depending on the rapidity of the biochemical changes taking place in the muscle and signal-to-noise required. A 4 min spectrum collected from resting muscle provided baseline values for each study. Quantification of the spectra was performed as previously detailed.^{11,18} In short, ratios of the peak areas from phosphocreatine (PCr), Pi, β -ATP and phosphomonoesters were determined and corrected for saturation. Myoplasmic concentrations of the metabolites were calculated assuming that the area of the β -ATP peak in the spectrum from resting muscle represented 8.2 mmol ATP/l intracellular H₂O. The pH was determined from the chemical shift difference of the Pi and PCr peaks. The concentration of free ADP, which is

too low to observe directly by ³¹P MRS, was calculated from the creatine kinase equilibrium:

$$K_{eq} = [\Sigma ATP][\text{creatine}]/[\Sigma ADP][H^+][PCr]$$

where Σ refers to the sums of all free and metal-complexed species and K_{eq} was taken as $1.66 \times 10^9 \text{ M}^{-1}$.¹⁹

The response of flexor digitorum superficialis muscle to aerobic, dynamic exercise was investigated as detailed by Arnold *et al.*¹¹ The subject compressed a rubber bulb to a pre-set pressure of 100 mm Hg at a rate of 22 min⁻¹ while four spectra were collected and stored (5 min exercise time). The pressure in the bulb was then increased to 300 mmHg and the exercise was continued during the collection of two more spectra (2.5 min). Spectra were collected throughout 12 min of recovery.

Studies were also carried out in the magnet under ischaemic conditions. A blood pressure cuff placed around the upper arm was inflated above systolic arterial pressure and the subject worked to exhaustion by squeezing a rubber bulb to a pre-set pressure of 300 mmHg. A spectrum was collected, the cuff was deflated and a series of spectra was obtained during 26 min of recovery. At the midpoint of each spectral accumulation a blood sample was drawn from an indwelling catheter placed in the antecubital vein of the exercising forearm. Each sample was immediately deproteinised with 4% (final concentration) ice-cold perchloric acid and the supernatant solutions were analysed for lactate and pyruvate.²⁰

A second ischaemic exercise protocol was used to estimate the amount of ATP used in muscle contraction. The subject was placed in the magnet with a sphygmomanometer cuff around the upper arm. A baseline spectrum was collected from the resting muscle. The cuff was inflated and the subject performed 4–5 bouts of exercise. Each bout consisted of eight contractions in which a rubber bulb was compressed to a pre-set pressure of 100 mmHg. After each group of eight contractions a spectrum was collected. The exercise and data collection were then repeated until the subject was unable to continue because of discomfort. The cuff was then deflated. Metabolite concentrations and pH were determined from each spectrum. The ATP utilisation was calculated as follows:²¹

$$\text{ATP used} = \Delta[\text{ATP}] + 1.5 \times \Delta[\text{lactate}] - \Delta[\text{PCr}]$$

where Δ refers to the change in concentration between the resting and exercising muscle. $\Delta[\text{lactate}]$ was calculated from:

$$\Delta[H^+] = \Delta[\text{lactate}] + \Delta[\text{PCr}] + \Delta[\text{Pi}^{2-}]$$

assuming a buffering capacity of 43 mmol/l of intracellular H₂O (29 mmol/kg wet wt¹⁹). Because blood blow was occluded, all ATP would be produced by glycolysis, and pH would be a true measure of glycolytic activity because all pyruvate formed would be converted to lactate + H⁺. Glycolytic production of ATP from glycogen yields 3 mol of ATP per mol of glycosyl units, or 1.5 mol of ATP per mol of lactate formed. The Pi²⁻ term of the equation allows for the additional buffering provided by the increase in [Pi²⁻] during exercise. The results are variable from subject to subject and therefore, the patient served as his own control. Results were compared from investigations performed when he was on and off verapamil.

In some investigations an assessment of work was obtained from a purpose-built transducer which measured the pressure within the bulb, with a correction for any isometric work performed.²¹

Ca²⁺-ATPase activity of sarcoplasmic reticulum

An open muscle biopsy was performed on KR under a local anaesthetic. The sample of 2.85 g wet wt was taken from the quadriceps. All purification procedures²² were carried out at 4°C. The muscle was chilled, washed several times in sucrose medium (0.25 M sucrose, 5 mM HEPES, pH 7.5) and minced with a tissue grinder. The minced tissue was suspended in a 4 fold volume of sucrose medium and further homogenised with an ultra-turrax. The suspension was centrifuged for 10 min at 1,600 g after which the supernatant was filtered through 4 layers of cheesecloth. The filtrate was centrifuged for 20 min at 10,000 g and the resulting supernatant again filtered through cheesecloth. The centrifugation was repeated and the final supernatant was centrifuged at 150,000 g for 35 min. The precipitate was resuspended in 0.6 M KCl (pH 7.5) with gentle stirring and centrifuged at 150,000 g for 35 min. The suspension in KCl and centrifugation at 150,000 g were repeated. The final pellet was resuspended in a small volume of sucrose medium.

Fractionation was carried out on a linear sucrose gradient (25–40% w/w) buffered with 2 mM HEPES (pH 7.5). Centrifugation was performed for 20 h at 135,000 g. Aliquots of 1 ml each were removed and assayed for Ca²⁺-ATPase activity. Mixed skeletal muscle from male Wistar rats was prepared in parallel with the patient's muscle because no control human muscle was available. Subsequently, muscle from normal volunteer individuals undergoing hip replacement surgery was obtained and sarcoplasmic reticulum vesicles purified by the procedure outlined.

An optical assay coupled with pyruvate kinase and lactate dehydrogenase was used for determination of ATPase activity at 25°C.²³ For total activity the assay mixture contained 25 mM HEPES, 0.1 M KCl, 5 mM MgCl₂, 50 μM CaCl₂, 1 mM phosphoenolpyruvate, 0.6 mM NADH, 7.5 U pyruvate kinase and 5 U lactate dehydrogenase. Cuvettes were incubated for 5 min after addition of sarcoplasmic reticulum. The reaction was started by the addition of ATP (final concentration 6 mM). For measurement of Mg²⁺-dependent (basal) ATPase activity CaCl₂ was omitted from the incubation medium and 1 mM EGTA included. Ca²⁺-ATPase activity was taken as the difference between total and basal ATPase activities. Protein was determined using a Biorad kit.

In order to exclude erroneous results due to contamination of the sarcoplasmic fractions with fragments of mitochondria or plasma membranes, ATPase activities were determined in the presence of 0.25 mM ouabain and 5.0 mM sodium azide or 0.015 mg oligomycin. Mitochondrial contamination was also checked by determining succinate dehydrogenase activity.

Informed consent was obtained from all normal volunteers and patients. Control subjects were normal men and women age 20–80 yr on ordinary mixed diets. The mean age of each control group was between 30 and 40 yr. No athletes were used as control subjects. Results are presented as the mean, 1 SD. Statistical significance was evaluated using Student's *t* test.

Results

Resting muscle

The pH_i and ratios of signal intensities from intracellular ATP, PCr and P_i were normal in AW and in KR when he was taking verapamil (fig 1). This suggests that the intracellular concentrations of these metabolites were also normal (table 1). However, the muscle P_i concentration and pH increased significantly in KR's muscle when he was not taking verapamil.

Aerobic dynamic exercise

A striking feature observed in the exercising muscle of KR was the large decrease in pH_i (fig 1, 2a). This became significantly different from the pH of control subjects after 2 min of exercise and reached 6.20, SD 0.25 at the end of the 7.5 min exercise protocol. Normal men reached a pH of 6.77, SD 0.23. One investigation was carried out on AW. She was not able to complete the exercise protocol, but as shown in fig 2a her muscle pH decreased during 5 min of exercise to 6.56 (5 normal women, 6.84, SD 0.07).

When the rate of contraction was increased from 22 min^{-1} to 40 min^{-1} the abnormal decrease in pH with exercise was more striking: KR's intracellular pH decreased to 5.96 within 2 min of the start of exercise while the corresponding values for the 3 control subjects were 6.64, 7.02 and 6.99.

The decrease in [PCr] was also greater than in control subjects (fig 1, 2b). The response to exercise of the free [ADP] is shown in fig 2c. In controls, [ADP] rose to a steady state which was achieved at about 2 min, and the concentration increased when the work load was increased. In both patients, [ADP] increased to a maximum by 2 min and then decreased steadily during the remaining exercise period. Both $[H^+]$ and [PCr] are used in the [ADP] calculation, so this result suggests that the relationship between $[H^+]$ and high energy phosphates is abnormal in the patients' muscles.

Fig 1 ^{31}P MRS spectra of finger flexor muscle of a normal control (a & c) and patient KR (b & d). Spectra a and b are from resting muscle and were collected over a 4 min period (128 accumulations). Spectra c and d were obtained during the 6th min (32 accumulations) of the aerobic exercise described in Methods. Peak assignments: (1) phosphomonoesters (2) P_i , (3) phosphocreatine, (4) γ phosphate of ATP, (5) α phosphate of ATP plus NAD(H), (6) β phosphate of ATP. The intracellular pH values determined from these spectra are: (a) 7.02, (b) 7.05, (c) 6.86, (d) 6.05.

At the end of exercise the phosphomonoester peak in KR's muscle had increased so that the signal intensity ratio with respect to ATP was twice as large as that of normal controls. Assuming that the spin-lattice relaxation time of phosphomonoesters is similar to that of P_i in both patient and controls, the concentration of phosphomonoesters in the patient was 5.0, SD 1.3 mM at the end of exercise and in controls was 2.7, SD 0.6. In the aerobic exercise study

Table 1 Metabolites and pH in resting muscle

Subject	Verapamil	n	pH	Ratios, Signal Intensity			Concentration		
				PCr/ P_i	PCr/ATP	P_i /ATP	PCr (mM)	P_i (mM)	ADP (μM)
Normal men	—	18	7.03*	9.4	3.07	0.34	38.3	4.1†	6
Patient KR	+	10	± 0.02	± 2.0	± 0.14	± 0.07	± 1.7	± 0.8	± 3
			7.04†	11.1	3.13	0.36	39.1	4.2	5
			± 0.02	± 4.2	± 0.21	± 0.06	± 2.7	± 0.7	± 4
Normal women	—	5	7.07*†	8.3	3.18	0.47	39.7	5.7†	4
			± 0.01	± 3.2	± 0.08	± 0.14	± 1.0	± 1.7	± 2
			7.01	9.4	3.14	0.34	39.1	4.1	4
Patient AW	—	3	± 0.03	± 1.8	± 0.16	± 0.06	± 2.0	± 0.7	± 3
			7.04	8.0	3.24	0.40	40.4	4.8	3
			± 0.04	± 3.0	± 0.15	± 0.02	± 1.9	± 0.2	± 3

*Sig. different at $p < 0.0001$

†Sig. different at $p = 0.03$

‡Sig. different at $p < 0.0001$

Fig 2 The effect of aerobic dynamic exercise on (a) pH, (b) phosphocreatine concentration, and (c) ADP. The exercise protocol and metabolite quantification are described in Methods. Symbols: controls, ■ (n = 18, mean ± SD); KR on verapamil, ● (n = 10, mean ± SD); KR off verapamil, □, ○.

on AW, phosphomonoester rose to 4.8 mM (controls, 3.0, SD 0.6 mM). In muscle the increase in this signal during exercise is probably due to an increase in the concentration of sugar phosphate intermediates of the glycolytic pathway.

In normal subjects there was a small mean decrease in pH of 0.10, SD 0.09 units after contraction ceased. This is due to H⁺ release as net PCr synthesis takes place.¹⁸ KR's muscle usually reached a much lower pH than normal (mean, 6.20). The subsequent decrease of 0.04, SD 0.08 units cannot be compared with the

control value because at low pH the PCr net resynthesis rate and associated H⁺ release is slow.²⁴ However, in three exercise studies the final pH was in the normal range (6.57, SD 0.16), and the decrease in pH during the first half minute of recovery was 0.25, SD 0.09 units. These three studies were performed within 6 hours of a previous MRS exercise study. These results were treated separately from the others because in those investigations, the MRS study was the first exercise study of the day. For comparison with normal subjects, four men of similar age to the patient were examined within 6 h of a previous investigation; the final pH was 6.80, SD 0.22, and the subsequent pH decrease was only 0.05, SD 0.02. Thus, when the pH of the patient's muscle fell within the normal range during exercise, the subsequent pH decrease was about three times normal. AW showed a normal decrease (0.12 pH units) after the aerobic exercise study.

Recovery from aerobic dynamic exercise

The mean half recovery time for phosphocreatine was three times longer in KR than in controls (table 2). The data from Arnold *et al*²⁴ show that low pH is associated with slow PCr recovery. However, recovery times are also longer than normal in patients with blocks in oxidative metabolism^{9 12 24} and deficient oxygen delivery,²⁵ so it is important to distinguish between the low pH and deficient oxidative metabolism as a cause of the slow recovery. ADP recovery is a sensitive index of oxidative capacity in muscle,^{11 25} and its mean recovery half time was the same in KR and in controls (about 15 s). This suggests that the slow metabolic recovery in KR was due to the low intracellular pH and not to a decrease in oxidative capacity or oxygen delivery. AW, who did much less work than controls and KR, did not reach such a low intracellular pH, and her recovery times were in the normal range (table 2).

In both KR and controls there was a 1–2 min lag period before the intracellular pH began to increase;

Table 2 Recovery from exercise

Subject	n	Exercise	Verapamil	T _{1/2} (s)	
				PCr	Pi
Normal	20	aerobic	—	44	26
Controls*				± 16	± 7
Patient KR	9	aerobic	+	123	43
				± 40	± 16
Patient KR	1	aerobic	—	81	24
Patient AW	1	aerobic	—	51	18
Normal	6	ischaemic	—	78	44
Controls†				± 15	± 7
Patient KR	1	ischaemic	+	93	51
	1	ischaemic	+	120	80
Patient AW	1	ischaemic	—	84	48

*13 men, 7 women
† 4 men, 2 women

the rate of recovery thereafter was about 0.1 pH unit/min in patient and controls (data not shown). Signal-to-noise was not adequate to measure pH recovery in AW.

Ischaemic dynamic exercise

The response to exercise in the absence of oxidative metabolism was assessed by occluding blood flow to the muscle. Two exercise studies were performed in KR and one in AW. In one study from each patient blood samples were drawn for lactate and pyruvate analyses (1) before exercise, (2) when muscle contraction had ceased but ischaemia was maintained, and (3) for 30 min after blood flow had resumed.

The results in table 3 show the pH and metabolite concentrations reached in ischaemic exercise; results were similar in most respects in the patients and the control group, but the patients did less work than any of the control subjects. In the second investigation on KR, phosphomonoesters reached concentrations that were higher than normal, and recovery times were abnormally long. Recovery of intracellular pH was within the normal range for all three patient studies.

Lactate concentrations in blood drawn from the antecubital vein after the patients had been at bed rest for about half an hour were 0.32 mM (KR) and 0.31 mM (AW). This is well within the normal range for the six control subjects (0.22–0.94 mM, $\chi = 0.58$). Following exercise, lactate increased to a maximum of 1.53 mM (KR) and 1.86 mM (AW) 3 min after restoration of blood flow. The maximum concentration in six controls was also reached at about 3 min and at that point varied from 1.50–3.90 mM (mean, 2.72). The concentration of lactate in the patients' and controls' blood then declined in parallel, and after 28 min was 0.52 mM (KR), 0.77 mM (AW) and 0.69, SD 0.21 mM (controls). KR's lactate:pyruvate ratio remained in the normal range throughout the study. This ratio was also normal in AW except for the first min of recovery when it was 2–3 times normal.

Effect of verapamil on muscle metabolism

KR discontinued his verapamil for 4 days. His muscle symptoms were noticeably worse 18 hr after the last

Taylor, Brosnan, Arnold, Bore, Styles, Walton, Radda dose and continued to worsen over the 4 day period. Four MRS studies of resting muscle were carried out 18–88 hours after the last dose of verapamil (table 1), and aerobic dynamic exercise was performed 84 h after the last drug dose (fig 2 and table 2).

When the patient was not taking verapamil his muscle Pi concentration at rest was significantly higher than in controls ($p = 0.03$). The pH of the resting muscle was also significantly elevated ($p < 0.0001$ compared to normal controls and $p = 0.0005$ compared with his own muscle pH when he was taking verapamil). The pattern of excessive metabolite and pH changes during exercise was present when KR was off verapamil (fig 2). This was also true for recovery from exercise (table 2). Overall, the data suggest that the abnormal MRS results observed in the patient during exercise and recovery were not due to effects of verapamil on muscle metabolism. This is corroborated by the results from AW. She was not receiving the drug but exhibited abnormal changes during exercise similar to those of her brother (fig 2). Additional data come from two patients with an exertional muscle pain syndrome who were verapamil responsive.²⁶ The drug did not affect the MRS-observable results of aerobic or ischaemic exercise.

ATP utilisation during exercise

This was determined 36, 60 and 86 h after KR discontinued his verapamil and was found to be 12.9, 12.7 and 9.9 mmol ATP/l of intracellular H₂O for each bout of exercise (see *Methods*). When the patient was feeling well again, 24 h after he restarted verapamil, ATP utilisation was lower at 9.1 and 9.4.

ATPase activity

Ca²⁺-ATPase and Mg²⁺-ATPase specific activities of the sucrose density fractions prepared from purified sarcoplasmic reticulum are shown in fig 3. The elution patterns of the activities were similar in the fractions from patient and control, but the patient's Ca²⁺-ATPase activity was very much reduced. No human control muscle was available at the time of the patient's biopsy, so the control results in fig 3 are from rat skeletal muscle. Subsequently, muscle from two

Table 3 The effect of fatiguing ischaemic exercise on pH and metabolite concentrations

Subject	n	Work	pH	Metabolite Concentrations				
				PCr (mM)	Pi (mM)	ATP (mM)	ADP (μM)	PME (mM)
Normal Controls*	6	2.8†	6.14	13.2	30.7	6.9	15	10.2
(SD)		0.6	0.14	5.6	7.1	0.6	9	1.8
Patient KR	1	1.7	6.00	8.2	41.3	6.9	17	11.2
	1	1.7	5.95	17.8	22.8	6.6	6	24.9
Patient AW	1	1.4	6.51	8.3	33.4	7.1	66	9.5

*4 men, 2 women
†range, 2.0–3.6

Fig 3 ATPase activity of sucrose gradient fractions of a purified muscle preparation (see Methods). Symbols: Mg²⁺-ATPase activity from control (□) and patient (■); Ca²⁺-ATPase activity from control (—) and patient (◇). Note that the Mg²⁺-ATPase activity of muscle from patient and control is similar, but Ca²⁺-ATPase activity in the patient is very low compared to normal. Specific activity is expressed as U/mg protein.

normal human subjects was analysed, and the results are presented in table 4. It can be seen that the total protein recovered in the purified sarcoplasmic reticulum was similar for rat muscle and all three human muscle samples, but the Ca²⁺-ATPase activity from the patient was only about 10% of the rat and human control activity. Mixing experiments showed that the low activity in the patient's sarcoplasmic reticulum was not due to the presence of an inhibitor.

Discussion

Ca²⁺-ATPase deficiency is a rare disorder of skeletal muscle. The investigations carried out on the few reported cases have dealt primarily with identifying the defect¹⁴ and with molecular aspects of the enzyme deficiency.¹⁵ Ca²⁺-ATPase of mammalian muscle is encoded by two genes. The product of one is found in cardiac and slow twitch skeletal muscle while that of the second is located in the fast twitch fibres. The fast

twitch form exhibits developmental regulation.²⁷ In the patients investigated by Karpati *et al*¹⁵ there was a marked reduction in immunoreactive Ca²⁺-ATPase which was confined to the fast twitch fibres. The concentration of a 100 kD phosphoprotein, probably the Ca²⁺-ATPase, was very much reduced in the muscle microsomal fraction. In our patients the genetic basis of the defect is not yet known, but it appears unlikely that there is an absence of Ca²⁺-ATPase protein itself. This ATPase is known to compose most of the protein in the sarcoplasmic reticulum, and the yield of protein in this fraction was the same per gram of tissue in KR as in controls. Thus in KR (and AW) the Ca²⁺-ATPase protein is probably present but inactive. The defect may reside in the Ca²⁺-ATPase itself or may be of a more integrated nature and involve the interaction of the ATPase with other macromolecules.

Initially neither KR nor AW suffered from impaired muscle relaxation, so Ca²⁺-ATPase deficiency was not suspected. The MRS findings, however, revealed an unusual metabolic response to exercise which suggested abnormal Ca²⁺ handling. The most striking feature was rapid and excessive intracellular acidification of the exercising skeletal muscle accompanied by a substantial decrease in the PCr concentration. The patient was a marine engineer, a job which requires extensive use of forearm muscles. His forearms were well developed and he had no muscle weakness. Similarly fit and muscular control subjects show only small changes in phosphocreatine concentration and pH during the aerobic, dynamic exercise protocol, so the patient's metabolic response to exercise was unexpected.

Decrease in muscle pH during exercise is due to lactate production, so the results indicate that either glycolysis was more active than normal, less pyruvate than usual was being oxidised via the Krebs cycle, or acid washout from the muscle was slow. The rate of recovery of pH after exercise was normal in patient KR (it could not be measured in AW), so it is not likely that slow acid washout was the cause of the over-acidification of the muscle. As already discussed the normal recovery of ADP after aerobic exercise is consistent with normal oxidative metabolism.

The results obtained in ischaemic exercise also make it unlikely that the abnormal results in aerobic exercise were due to slow oxidative metabolism. Reduced blood flow is therefore also ruled out as a cause of the metabolic abnormalities. The increase in phosphomonoester concentration which accompanied the large decrease in muscle pH in aerobic exercise suggests that glycolysis was more active in the patient than in control subjects. The decrease in pH that occurred in some aerobic studies after the end of exercise is also consistent with glycolysis remaining

Table 4 Ca²⁺-ATPase activity and protein content of sarcoplasmic reticulum

Subject	Ca ²⁺ -ATPase activity (U/mg protein)	Protein yield (mg/g wet wt muscle)
Normal control 1	0.407	0.93
Normal control 2	0.593	1.43
Rat skeletal muscle	0.483	1.05
Patient KR	0.056	1.12

active for a longer time than in normal muscle. Results from KW's ischaemic exercise studies, in which blood flow and oxidative metabolism were eliminated, are also consistent with increased glycolytic flux. In ischaemic exercise the patient's muscle reached the same pH and PCr concentration as control muscle while phosphomonoester concentrations were as high as, or higher than, control values. However, these changes were achieved with significantly less work than in control subjects. In aerobic exercise the patient did a similar amount of work to controls but the changes in metabolite concentrations and pH were greater. Thus, it appears that the Ca^{2+} -ATPase-deficient muscle is less efficient than normal muscle.

How does diminished Ca^{2+} -ATPase in the sarcoplasmic reticulum lead to this pattern of abnormal muscle metabolism? The role of the sarcoplasmic reticulum in excitation-contraction coupling is well established. The excitation of the transverse tubules initiates the release of Ca^{2+} from the lumen of the cisternae into the myofibrillar compartment. This elevation in Ca^{2+} concentration acts to bring about muscle contraction and fuel mobilisation. The Ca^{2+} concentration of the cytosol is restored to basal levels through uptake of the ion into the lumen by the Ca^{2+} -ATPase. Hence the proper functioning of this membrane-bound transport system is critical for the normal relaxation of muscle fibres. The result of a Ca^{2+} -ATPase deficiency will be that, within the cytoplasm, the Ca^{2+} concentration will remain elevated for a longer time than normal after release from the cisternae. It might also reach a higher concentration than normal. Thus, patients with a deficiency of this enzyme may present with an inability to relax the muscle after a contraction.^{14 15}

Ca^{2+} plays a regulatory role in muscle energy production. Muscle glycogen is the major fuel source for glycolysis, and myophosphorylase is required for glycogen breakdown. The activity of the phosphorylase depends on several factors including the proportion of the *a* and *b* forms. Ca^{2+} activates phosphorylase kinase which in turn catalyses the conversion of phosphorylase *b* to the more active phosphorylase *a*. Thus, in Ca^{2+} -ATPase-deficient muscle the altered Ca^{2+} concentration may lead to a more active phosphorylase or to phosphorylase which is active for a longer proportion of the contraction/relaxation cycle than in normal tissue. The result would be an unusually high rate of lactate production.

It is interesting to speculate about the cause of increased use of energy in the patient's muscle during exercise. Pumping of Ca^{2+} requires a large proportion of the energy used in contracting muscle,¹³ so uncoupling of the Ca^{2+} -ATPase could lead to the observed inefficiency. This cannot be the cause in our patient, however, because the assay for enzyme activity

Taylor, Brosnan, Arnold, Bore, Styles, Walton, Radda

actually measures ATP utilisation, and this was very low. An alternative explanation is that a high cytosolic Ca^{2+} concentration leads to Ca^{2+} cycling by the mitochondria through the Ca^{2+} uniport system and Na^{+} -dependent efflux, resulting in dissipation of energy. Both of these pathways are present in skeletal muscle mitochondria, but the role of the mitochondrion in buffering Ca^{2+} is thought to be minor under normal physiological conditions (see ref 28) because the K_m of the uniport mechanism for Ca^{2+} is around 10 μM whereas the cytosolic free Ca^{2+} is normally in the sub μM range.

Whatever additional mechanisms, if any, contribute to the removal of Ca^{2+} from the cytoplasm, basal concentrations must be maintained near normal. The disruptive effects of a high cytosolic Ca^{2+} are well known, but in our patients serum creatine kinase activities were normal and there was no histological evidence of tissue necrosis.

The results from KR suggest that verapamil did affect muscle metabolism but was not responsible for the rapid decline in pH and PCr concentration during exercise. Qualitatively the drug did not alter the metabolic response to exercise, but too few examinations were performed for conclusions to be drawn about quantitative differences. The data from resting muscle were significantly different when he was on and off the drug. Four examinations of resting muscle were carried out in the absence of verapamil, and the spectra reveal that the pH was slightly but significantly elevated and the Pi concentration was increased when compared with normal. In other words, the verapamil apparently normalised the resting muscle. Our results also suggest that in KR verapamil reduced the amount of ATP hydrolysed for a given number of voluntary contractions.

What is the basis of the therapeutic effect of verapamil on skeletal muscle? Lane *et al*²⁸ reported that four out of eight patients with an exertional muscle pain syndrome of unknown origin responded positively to verapamil, but the mechanism of action was not apparent. Verapamil has been shown to have different effects on different tissues. It is generally used in humans as a cardiac anti-arrhythmic drug and it has also been used in the treatment of angina and hypertension; it blocks the slow Ca^{2+} channels in myocardial tissue, thereby reducing myocardial contractility.³⁰ In rats it also appears to decrease the erythropoietic response to hypoxemia.³¹ Studies *in vitro* have shown that verapamil has several actions on skeletal muscle. It inhibits Ca^{2+} -induced release of muscle enzymes.³² It inhibits the increase in O_2 consumption produced by high external $[\text{K}^{+}]$ but not by DNP or caffeine.³³ It also depresses the contractures of skeletal muscle due to acetylcholine but not to caffeine or potassium, and this inhibition is of a non-competitive nature.³³ Damage to

electrically stimulated, hypoxic mouse muscle can be diminished by verapamil, probably because of its inhibitory effect on contractility.³⁴

Thus, in our patient, the effect of verapamil could be in lowering intracellular Ca^{2+} concentration or in an anaesthetic effect at the neuromuscular junction. Either or both mechanisms could lower the rate of energy utilisation in muscle, thereby affecting the rate of glycolysis and lactate production.

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