Malignant hyperthermia in pigs: A search for abnormalities in Ca^{2+} binding proteins

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Malignant hyperthermia occurs in man and pigs as a hereditary disorder notably as a complication of halothane-induced anaesthesia. It involves an abnormality in the metabolism of Ca^{2+}. A search was made for abnormalities of calcium-binding proteins. Troponin C from normal pig muscle was found to differ in 2 of 159 amino acids from rabbit Tn C and 3 from man. No differences between normal and abnormal pig muscle were found. Two-dimensional electrophoresis of red cell calmodulin from normal and abnormal pigs also failed to demonstrate a difference.

Muscular hyperthermia *Pig troponin C* *Tryptic peptide mapping* *Calmodulin*

1. INTRODUCTION

Susceptibility to MH in humans and pigs can be diagnosed by in vitro testing of muscle biopsy samples immersed in a Ca^{2+}-containing Ringer solution [1,2] in which exposure to halothane-, succinylcholine- or KCl-induced contraction in MH-susceptible, but not normal, muscle. On the basis of these findings it has been proposed that the biochemical abnormality in MH involves a defect in the regulation of the $[Ca^{2+}]$ in the myoplasm.

We have been investigating the possibility that MH is due to an abnormal calcium binding muscle protein. Except for myoglobin which was found to be normal, attempts to isolate proteins from the muscles of a patient who had died of MH were unsuccessful because of severe post-mortem proteolysis. However, a condition similar to human MH occurs in pigs [3,4] which is linked to abnormal sensitivity to stress and to the formation of pale soft exudative meat on slaughter [5].

With the availability of specially bred halothane-sensitive pigs it became possible to obtain adequate quantities of fresh muscle for extraction of muscular proteins. Examination of extracts by 2D gel electrophoresis did not show any detectable differences in the major contractile proteins including the Ca^{2+}-binding light chains of myosin between normal and halothane-sensitive pigs. In addition, the 2D gel patterns of the water-soluble proteins, mostly glycolytic enzymes and creatine kinase, showed no difference. It was observed however that in a susceptible pig which had been exposed to halothane shortly before death there was a selective loss of glyceraldehyde-3-phosphate dehydrogenase and of aldolase which might contribute to heat production by diverting fructose 1,6-diphosphate into a futile cycle [6].

We report here a comparison of normal and abnormal pig for the major Ca^{2+}-binding protein Tn C and the results of 2D electrophoresis of calmodulin.

Abbreviations: MH, malignant hyperthermia; Tn, troponin; 2-D, two-dimensional; DEAE, diethylaminoethyl; DTT, dithiothreitol; EGTA, ethyleneglycolbis (aminoethylether) tetra-acetic acid; SDS, sodium dodecyl sulphate; IEF, isoelectric focusing; A, absorbance; UV, ultraviolet; M_r, relative molecular mass; T, tryptic peptide; R, electrophoretic mobility

2. MATERIALS AND METHODS

2.1. *Muscle samples*

Pigs homozygous for halothane sensitivity were

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obtained from the Agricultural Research Council Animal Breeding Research Organization (Edinburgh). During transport to Cambridge they were tranquilised with acetyl chloropromazine. They were kept for ~1 week before slaughter. Skeletal muscle was dissected from the hind legs and immediately cooled on ice and processed to ether-dried myofibrils [7] on the same day. Other samples were stored at −70°C. Fresh muscle from normal pigs was obtained from the Department of Surgery, University of Cambridge and from the Agricultural Research Council Institute of Animal Physiology (Babraham, Cambridgeshire).

2.2. *Isolation of Tn C*

Crude Tn complex was isolated from minced hind-leg muscle (~1 kg) by the method in [7] but omitting the second isoelectric precipitation of tropomyosin. The Tn complex was purified by ion-exchange chromatography [8] on a column of DEAE–Sepharose CL-6B equilibrated with 20 mM Tris–HCl (pH 7.5) containing 0.1 mM DTT and eluted with a linear gradient of KCl (0–0.45 M). The Tn complex was separated into its components by chromatography on a column of DEAE–Sepharose CL-6B equilibrated with 8 M urea–20 mM Tris–HCl (pH 8.0)–2 mM EGTA–0.5 mM DTT [9,10] and eluted with a gradient of NaCl (0–0.3 M) in 8 M urea buffer.

2.3. *Isolation and characterization of tryptic peptides*

Tn C was aminoethylated [11] and digested with trypsin and the tryptic peptides were fractionated by gel filtration on a column of Sephadex G-50 equilibrated with 50 mM NH_4HCO_3. Further fractionation was carried out by ion-exchange chromatography on DEAE–Sepharose CL-6B or by preparative finger-printing on Whatman 3MM paper [12]. One large tryptic peptide (residues 45–78) was digested further with pepsin and the digest was fractionated by gel filtration on Sephadex G-25.

Peptides isolated on paper were detected by lightly staining with 0.02% (w/v) ninhydrin in acetone. Peptides were hydrolysed for 18–24 h at 108° in 6 M HCl containing 0.01% (w/v) DTT and 0.05% (w/v) phenol to reduce losses of methionine and tyrosine. Hydrolysates were analysed on a Rank-Hilger Chromaspek or a Durrum amino acid analyser.

2.4. *Isolation of erythrocyte calmodulin*

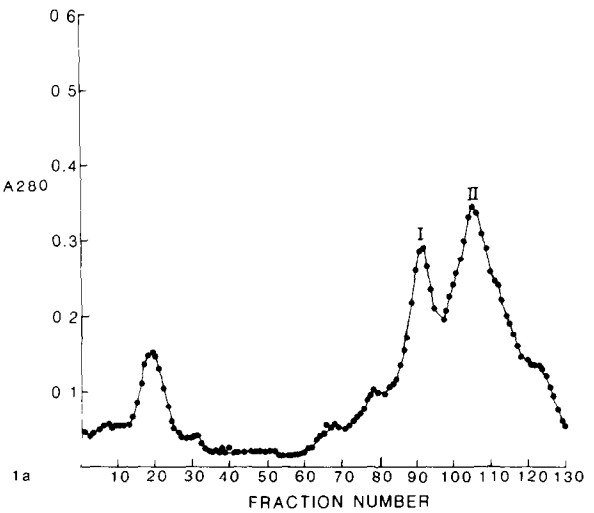
Calmodulin was isolated from pig and human erythrocytes by a modification of the method in [13,14]. Erythrocytes were washed with 130 mM NaCl–20 mM Tris–HCl (pH 7.4), and lysed in 14 vol. 2 mM EGTA (pH 7.4). After removal of erythrocyte ghosts by centrifugation, the haemolysate was made 0.1 M in NaCl adjusted to pH 6.5 with 1 M CH_3COOH and stirred with DEAE-cellulose (5 g dry wt/l) for 1 h. The DEAE-cellulose was filtered-off, washed with 10 mM imidazole (pH 6.5) containing 0.5 M NaCl and packed into a column. The column was eluted with 10 mM imidazole (pH 6.5) buffers containing successively 0.15 M, 0.3 M and 0.6 M NaCl. Calmodulin was isolated from the 0.6 M NaCl eluate either by gel filtration and ion-exchange chromatography [13] or by affinity chromatography on fluphenazine–Sepharose 4B [15].

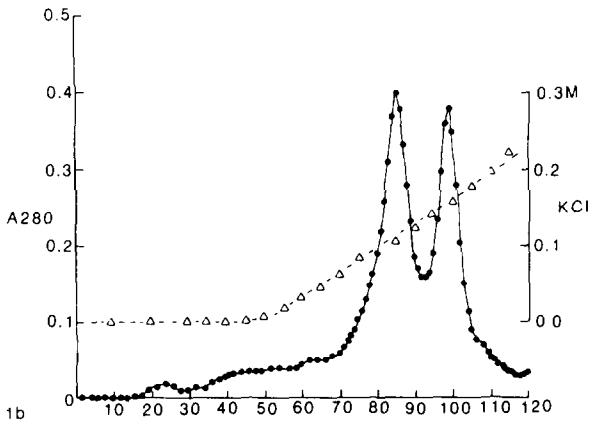
Calmodulin samples were analysed by IEF in tube gels containing 5% acrylamide and a pH 2.5–9 ampholine. The gels were stained with 0.25% (w/v) Light Green SF in 10% (v/v) acetic acid for 30 min [16]. Samples were also analysed on SDS gels by the method of Laemmli [17].

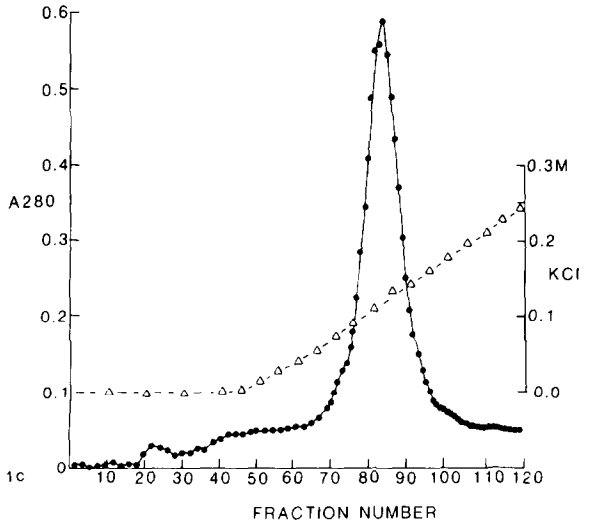
3. RESULTS

The isolation of the Tn complex is shown in fig.1a, 1b and 1c. The separation of the Tn complex into Tn's C, I and T is shown in fig.2 and 3.

Measured by A the amount of Tn complex/100 g dried myofibrils from normal and abnormal homozygote muscle were 378 mg and 362 mg, respectively (100 g fibrils ≈ 720 g wet muscle). Tn C from normal and halothane-sensitive pigs was eluted from DEAE–Sepharose CL-6B at 0.2 M NaCl in 8 M urea buffer. Both samples migrated as a single band in SDS gels with an app. M_r 18000. The UV spectra of Tn C from normal and abnormal pigs were very similar and closely resembled that reported for rabbit Tn C [18] with fine structure peaks at 253, 259, 269 and 276 nm and a shoulder at 28.2 nm. Calculation of the Phe:Tyr ratio from the A_{260}: A_{280} ratio based on data for the free amino acids [19] gave values of 5.1:1 and 4.5:1 for normal and MH pig Tn C, respectively, consistent with the observed ratio (10:2) in rabbit Tn C. The ratio in MH pig Tn C 4.5:1 or 9:2 could indicate the replacement of one







residue of Phe, but no evidence for a substitution was found in the amino acid composition data, and the difference falls probably within experimental error.

Peptides corresponding to the entire sequence of rabbit Tn C were obtained for both the normal and MH-susceptible pig Tn C (table 1). They all had the expected amino acid compositions except that in peptide T-12 (105–120) one of the three residues of Ala in rabbit Tn C was replaced by Met, and in peptide T-13 (121–136) the one Ile at position 131 in rabbit was replaced by Leu in pig (fig.4). The Ala⟶Met substitution in peptide T-12 was located at position 106 by CNBr cleavage. These differences were present in Tn C isolated from two normal pigs and from the halothane-sensitive pig. There was no evidence for any difference between the Tn C from normal and halothane-sensitive pigs. As amino acid composition data do not distinguish between acidic amino acids and their amides, predicted electrophoretic mobilities of tryptic peptides of pig Tn C were calculated as in [20]. In all cases the observed and predicted mobilities [18] were in reasonable agreement.

Calmodulins isolated from normal and MH-susceptible pigs behaved identically on SDS gel electrophoresis. They ran as single bands on IEF gels with no evidence for doublets in mixtures.

4. DISCUSSION

Troponin C is present in the muscle as a complex with Tn I and Tn T probably as a tetramer Tn–T_1I_2C [21] bound to tropomyosin in the thin filaments. Binding of Ca^{2+} to Tn C induces a conformation change which relieves the inhibition by Tn I of the actomyosin ATPase allowing contrac-

Fig.1a. Typical separation of the crude troponin complex on DEAE-Sepharose. The vertical axis indicates A_{280} (A280). Tn appears as a double peak except when Ca^{2+} is added – see fig.1b,c.

Fig.1b. Rechromatography of peak I (fig.1a) in the absence of 0.1 mM $CaCl_2$. The KCl concentrations (—Δ—) (measured by conductivity) are indicated to show the KCl gradient.

Fig.1c. As fig.1b but in the presence of 0.1 mM $CaCl_2$.

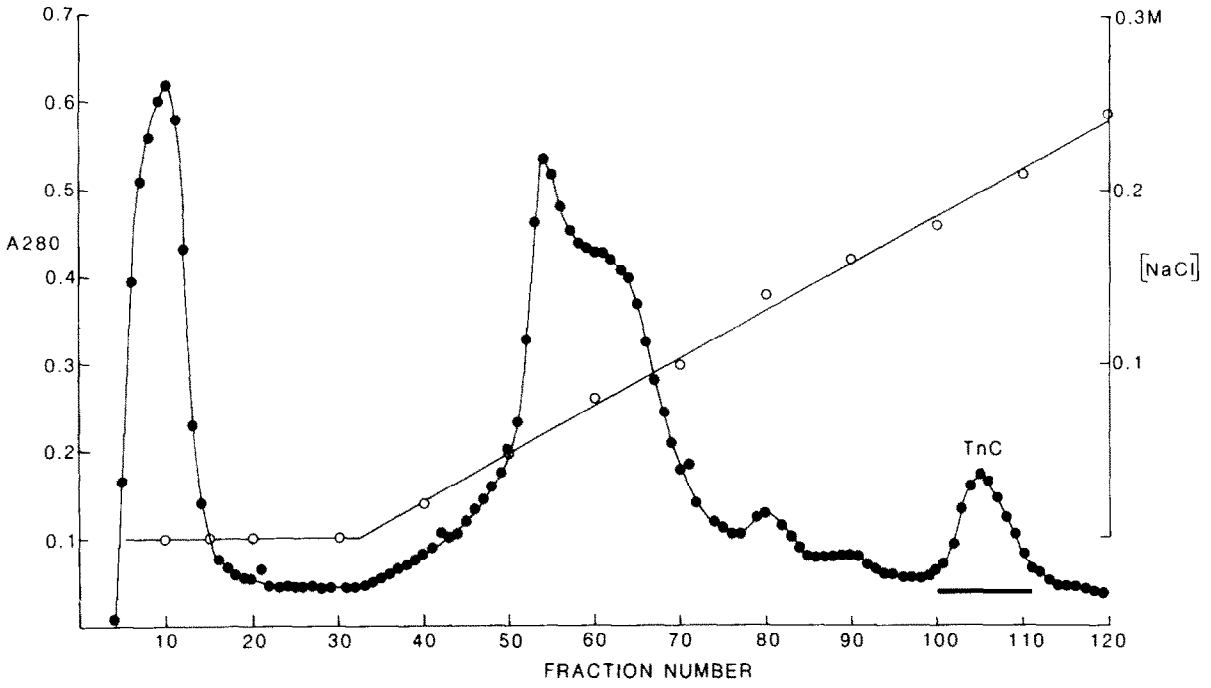


Fig.2. Chain separation of Tn complex on DEAE–Sepharose and 8 M urea to obtain Tn C. For analysis of the initial peak see fig.3. The second peak was shown by SDS electrophoresis and fingerprinting to consist of some Tn T and degradation products of Tn T. The Tn C peak is on the right.

tion to occur. Tn C may also play a second role as a regulatory δ' subunit of phosphorylase kinase conferring Ca^{2+} sensitivity on the enzyme and synchronizing glycogenolysis with muscular contraction [22].

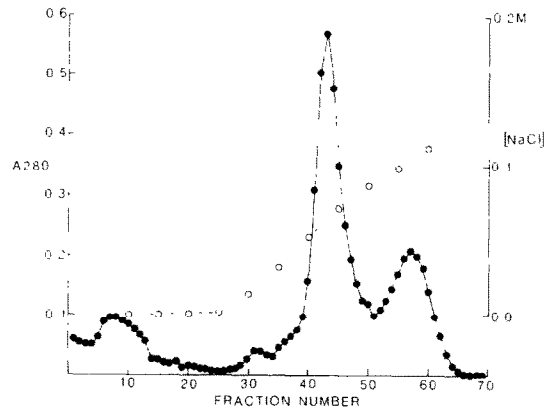


Fig.3. Rechromatography on CM cellulose of the initial peak indicated in fig.2 yielding Tn I and a degradation product of Tn T.

At present the primary structure of skeletal muscle Tn C is known for rabbit [23], man [24], chicken [25] and frog [26] and for bovine cardiac muscle Tn C [27]. A partial sequence for residues 75–119 of rat fast muscle Tn C has been deduced from nucleic acid sequence data [28]. Mammalian Tn C's are very similar: Man and rabbit Tn C differ only in one amino acid (table 2), pig and rabbit in two, and rat and rabbit Tn C are identical between positions 75 and 119. Tn C's of chicken and frog, however, show a greater number of differences from mammalian Tn C. Both have three additional residues at the N-terminus and 17 amino acid substitutions relative to rabbit Tn C. Because Tn C is part of a complex regulatory system in which it interacts with at least two other proteins it might be expected that its amino acid sequence would be under severe evolutionary constraint as amino acid substitutions in the regions of intersubunit contact might impair the function of the troponin complex. The present analysis indicates that Tn C from normal and halothane-sensitive pigs are identical so that it may be concluded that

Table 1

Amino acid compositions in molar ratios of tryptic peptides of troponin C from normal (N) and halothane-sensitive (M) pigs compared with rabbit (R)

	T-1	T-2	T-3	T-4	Peptic peptides of T 5-6a (45-78)					T-6b
	1-8	9-20	21-37	38-44	45-55	45-57	61-72	73-75	76-78	79-81
	N M R	N M R	N M R	N M R	N M R	N M R	N M R	N M R	N M R	N M R
Asp	1.0 1.3 1		3.9 4.0 4			1.0 1.1 1	3.1 2.9 3			
Thr	1.0 1.0 1			1.0 0.9 1	1.8 2.0 2	2.0 1.9 2	0.9 1.0 1			
Ser	0.2	1.5 1.7 2	1.0 1.0 1		0.5	0.3	1.0 1.1 1			
Glu	3.0 3.0 3	2.5 3.1 3		0.9 0.9 1	2.9 2.6 3	3.4 3.0 3	2.0 1.8 2	2.0 1.8 2		
Pro					0.7 1.0 1	0.9 1.0 1				
Gly	0.2		3.2 3.0 3	1.0 1.0 1	1.1 1.2 1	1.2 1.2 1	2.1 2.2 2			
Ala	1.9 2.0	0.9 1.0 1	2.9 3.1 3			0.9 0.7 1				
Val			0.8 0.9 1	1.0 1.1 1			0.9 1.1 1		1.0 1.0 1	1.0 1.0 1
Met		1.0 1.0 1	1.0 0.9 1	1.1 0.8 1	0.7 1.0 1	0.7 0.7 1			1.0 0.7 1	0.6 0.8 1
Ile		1.0 1.0 1	1.1 1.1 1				0.9 1.1 1			
Leu		1.0 1.0 1		1.0 1.0 1	2.1 2.0 2	2.0 1.8 2			0.8 0.8 1	
Tyr		1.0 1.0 1								
Phe		1.0 1.0 1	2.0 1.9 2				0.6 0.6 1	1.0 1.0 1		
His										
Lys		1.1 1.3 1	0.9 1.0 1		1.0 1.1 1	1.0 1.1 1				
Aec										
Arg	1.0 1.0 1			1.0 1.1 1						1.0 1.0 1
R obs	0.59	0.32-0.37	0.45	0	0.17	0.33	0.92	0.85	0	-0.38
R calc.	0.54	0.41	0.55	0	0.23	0.41	1.09	0.92	0	0.48
Charge	-2	-2	-3	0	-1	-2	-5	-2	0	+1

	T-7	T 7-8	T-8	T-9	T 9-10	T-11	T-12	T-13	T 14-15	T-16	T-17
	82-84	82-88	85-88	89-90	89-100	101-104	105-120	121-136	137-145	146-153	154-159
	N M R	N M R	N M R	N M R	N M R	N M R	N M R	N M R	N M R	N M R	N M R
Asp		1.0 1.1 1	1.0 1.0 1			1.0 1.0 1	3.2 3.1 3	1.3 1.0 1	5.2 5.0 5	1.9 2.0 2	
Thr								0.9 1.0 1			
Ser					1.1 0.9 1			2.1 2.0 2			
Glu	0.6 1.0 1	2.0 2.0 2	1.0 1.0 1		4.0 4.1 4		3.1 3.0 3	4.0 4.0 4		1.1 0.9 1	2.0 2.1 2
Pro											
Gly				0.6 0.7 1	0.9 0.9 1		1.1 1.1 1	1.2 1.2 1	2.1 1.9 2		1.2 1.2 1
Ala		1.0 1.0 1	1.2 0.9 1		1.1 1.1 1		1.9 1.9 3	0.8 1.1 1			
Val								0.7 0.9 1			1.0 0.9 1
Met	1.0 1.0 1	1.0 0.9 1					0.9 1.0 0	0.8 0.9 1			2.0 1.6 2
Ile						1.0 1.0 1	1.9 2.1 2	0.0 0.0 1		0.9 1.0 1	
Leu					0.9 1.0 1		1.0 1.0 1	2.1 2.0 1		1.0 1.0 1	
Tyr							0.9 1.0 1				
Phe					0.9 1.0 1	1.0 1.0 1	1.0 1.0 1			2.0 2.0 2	
His								0.8 0.9 1			
Lys	1.0 1.2 1	1.9 1.8 2	1.0 1.0 1	1.0 1.0 1	1.1 1.1 1			1.0 1.0 1	1.0 1.0 1	1.0 1.0 1	
Aec					(+) 0.8 1						
Arg					1.1 1.1 1	1.0 1.0 1	1.0 0.8 1		1.0 1.0 1		
R obs	-0.38	0	0.45	-0.65	0.13	0	0.60	0.43	0.26	0.47	0.37
R calc	-0.38	0	0.45	-0.80	0.20	0	0.69	0.54	0.26	0.51	0.33
Charge	+1	0	-1	+1	-1	0	-4	-3	-1	-2	-1

MH is not caused by a defect in Ca^{2+}-binding to the thin filaments.

Although the skeletal muscle is the site of the most significant metabolic defect in MH, there is evidence for abnormalities in other tissues. Erythrocytes from halothane-sensitive pigs are

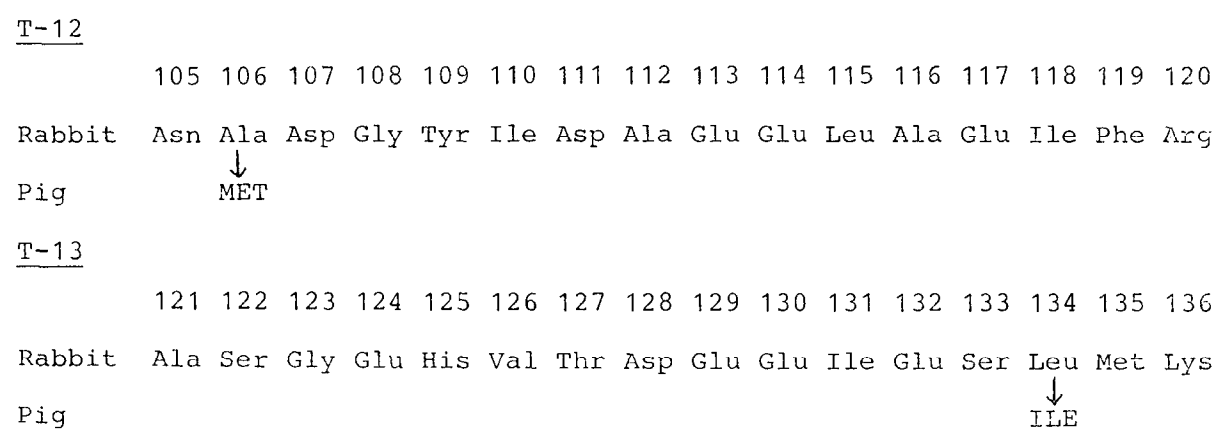


Fig.4. Amino acid sequences of peptides T-12 (105–120) and T-13 (121–136) which contain amino acid substitutions in pig Tn C.

more readily haemolysed by osmotic shock in 0.6% NaCl than those from normal pigs [29]. Platelets from human MH patients are reported to show a highly significant depletion of ATP on exposure to halothane [30] which could form the basis for a blood test for screening for MH. In 13 MH patients tested [31] there was a statistically significant increased rise in plasma insulin in response to a glucose load, compared to normal control. In [32] evidence was found for the involvement of the liver in porcine MH. Administration of halothane was followed within 10 min by a large efflux of K^+ from the liver and there was also a large release of glucose mainly derived from glycogenolysis in the liver.

One possible cause of MH is a generalised abnormality in the permeability of plasma membrane to Ca^{2+}, affecting most significantly the sarcolemma but also involving other organs. These features could suggest a possible involvement of calmodulin in the aetiology of MH. Erythrocytes contain a well characterized calmodulin-stimulated $Ca^{2+} + Mg^{2+}$-ATPase which maintains the internal $[Ca^{2+}]$ in the μM range [33]. Impairment of this enzyme might contribute to the increased osmotic fragility of erythrocytes from halothane-sensitive pigs. Calmodulin-sensitive $Ca^{2+} + Mg^{2+}$-ATPase activities have also been found in rat pancreatic islet cells [34] and in synaptic plasma membranes [35], and calmodulin is present in platelets [36].

Table 2

Differences between mammalian skeletal troponins C

	106	112	131
Rabbit	Ala	Ala	Ile
Rat	Ala	Ala	?
Man	Ala	PRO	Ile
Pig	MET	Ala	LEU

Calmodulin was isolated from erythrocytes which represented a more convenient source than muscle. Samples isolated from normal and halothane-sensitive pigs were indistinguishable by IEF indicating that they have the same electric charge. However, this does not preclude the possibility of an electrically neutral substitution, which would require a more detailed analysis of the protein for its detection.

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