High Skeletal Muscle Adenylate Cyclase in Malignant Hyperthermia

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ABSTRACT Malignant hyperthermia occurs in humans with several congenital myopathies, usually in response to general anesthesia. Commonly, individuals who develop this syndrome lack symptoms of muscle disease, and their muscle lacks specific pathological changes. A biochemical marker for this myopathy has not previously been available; we found activity of adenylate cyclase and content of cyclic AMP to be abnormally high in skeletal muscle. Secondary modification of protein phosphorylation could explain observed abnormalities of phosphorylase activation and sarcoplasmic reticulum function.

INTRODUCTION

Often fatal syndromes characterized by sustained muscle contraction and hyperthermia occur in several mammalian species. The best known of these syndromes are human malignant hyperthermia (hyperprexia) (MH),[1] porcine malignant hyperthermia (fulminant hyperpyrexia stress syndrome) (FHSS), and, in wild animals, "overstraining disease" or "capture myopathy". Each of these syndromes can be provoked by physical stress (1–4), but the human syndrome is almost always attributed to anesthetic drugs (5).

The cause of these syndromes is unknown, but pigs susceptible to FHSS and humans susceptible to MH may be identified by increased sensitivity of biopsied muscle to halothane or caffeine, which can be used experimentally to trigger muscle contracture (6–8). The basis of this response has not been clarified by studies of isolated sarcoplasmic reticulum (SR) (9–11), which have arrived at contradictory conclusions.

In chemically skinned fibers from muscle of survivors of MH, we found hypersensitive responses to caffeine, due to abnormal function of SR; response of contractile proteins to Ca^{++} was normal (12). The abnormality was present in most fibers in quadriceps biopsies. In this preparation, the concentration of Ca^{++} outside the SR and the duration of Ca^{++} loading are fixed experimentally, and the amount of Ca^{++} available to be released by caffeine is a function of net uptake (13). If the Ca^{++} load in SR is increased, normal muscle can be made to respond to the low concentrations of caffeine to which MH muscle is responsive. Because SR in most fibers from MH muscle behaved as if it had a higher Ca^{++} load, we postulated that Ca^{++} uptake by SR was accelerated.

Adenylate cyclase (AC) mediates extracellular regulation of SR Ca^{++} transport. Activation of this enzyme, which is found in transverse tubules (14) and sarcolemma (15) of skeletal muscle, results in increased cytoplasmic 3'5' cyclic adenosine monophosphate (cyclic AMP), activation of cyclic AMP-dependent protein kinases, and phosphorylation of specific SR proteins. A 22,000 daltons protein, phospholamban, is the apparent substrate in slow twitch muscle; when it is phosphorylated by a cyclic AMP-dependent protein kinase, there follows activation of CaATPase and influx of Ca^{++} (16). A 100,000 daltons protein has been identified as a substrate in fast twitch muscle (17), but its role in regulating Ca^{++} flux is controversial.

Other considerations link a possible abnormality of AC to MH. Catecholamines are physiological agonists of AC and their serum content increases in

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[1] *Abbreviations used in this paper*: AC, adenylate cyclase; FHSS, fulminant hyperpyrexia stress syndrome; MH, malignant hyperthermia; PDE, phosphodiesterase; SR, sarcoplasmic reticulum.

humans during stress (18) or general anesthesia (19–21) and in pigs during attacks of FHSS (23–24). Halothane, an anesthetic frequently associated with attacks of MH and used experimentally to induce FHSS in susceptible swine, is another agonist of AC (25–26). Moreover, serum pyrophosphate, a product of AC, is increased in survivors of MH and susceptible relatives (27). We measured the content of cyclic AMP and activities of AC and cyclic nucleotide phosphodiesterase (PDE) in skeletal muscle of survivors of MH and relatives of survivors.

METHODS

AC was measured in skeletal muscle homogenates prepared by two methods. Initial studies were performed by homogenization of muscle in 0.15 M KCl-10 mM Tris buffer pH 7.5 with a Polytron (Brinkmann Instruments,Westbury, N. Y.), operated at maximal speed for 10 s (method 1); with this preparation basal activity and catecholamine activation were slowly unstable at 4°C. In the 10 min interval between homogenization and assay, up to 10% of basal activity and, independently, 20% of catecholamine activation were lost; therefore, all muscles were assayed within 10 min of homogenization. Ca^{++} concentrations were not controlled with this method. Other than in kinetic studies, AC activity was measured with 5 mM Mg and 1 mM ATP. For kinetic studies, Mg concentration was held at 5 mM and ATP concentration varied; because the K_m in normal skeletal muscle was reported to be 0.3 mM (15), concentrations of 0.1, 0.2, 0.4, and 0.5 mM ATP were selected. Kinetic constants were calculated by linear regression analysis of a Lineweaver-Burke transformation of the data obtained.

Subsequent studies were performed with a modification of a method designed to stabilize enzyme activities (28): Mg added to the assay was reduced to 1.48 mM, which with 1.03 mM ATP gave final calculated concentrations of 0.94 mM MgATP, 0.5 mM Mg^{++}, and 0.1 mM ATP^{4-}; these calculations were based on the assumption that tissue Mg^{++} was diluted to a negligible concentration. With both methods of homogenization, AC was measured as conversion of [^{32}P]-ATP to [^{32}P]cyclic AMP during a 10 min incubation at 37°C. Reactions were initiated by addition of 50 μl of a reaction mixture containing 2 mM cyclic AMP, 20 mM creatine phosphate, 100 U/ml creatine kinase, and 30–50 cpm/pmol ATP to 50 μl of a 3.5% wt/vol homogenate. Reactions were linear with time for at least 20 min and with protein between 0.15 and 0.40 mg noncollagen protein. Cyclic AMP was separated from ATP by column chromatography (29); recovery was monitored with tritiated cyclic AMP.

Cyclic AMP was measured by radioimmunoassay (30) in TCA extracts of muscle. To obtain a linear assay, it was necessary to extract TCA with water-saturated diethyl ether and pass the aqueous phase through a 6 × 0.5-cm column of Dowex AG 50 W-X8 (Bio-Rad Laboratories, Richmond, Calif.). Inclusion of 5,000 cpm of tritiated cyclic AMP in the TCA permitted correction for recovery of 60–85%.

PDE was measured as the conversion of [^{3}H]cyclic AMP to [^{3}H]AMP, which was then converted to [^{3}H]adenosine by incubation with 5′-nucleotidase (31). Adenosine was separated from cyclic AMP by column chromatography on Dowex AG 1-X8 (Bio-Rad Laboratories); recovery of 90–95% was monitored with [^{14}C]adenosine. 10–15 mg of muscle were homogenized in 1 mM $KHCO_3$, pH 7.2, and centrifuged at 35,000 *g* for 10 min. The supernate was assayed without further treatment. The pellet was washed twice with 10 ml of the homogenizing buffer, in which it was finally suspended in a volume of 0.9 ml. Reactions were linear at 30°C for at least 60 min. PDE was determined against substrate concentrations of 1 and 200 μM; whether these two concentrations monitor isoenzymes or a single enzyme with nonlinear kinetics is unknown.

For enzyme and cyclic AMP assays, data were referred to noncollagen protein (32, 33); bovine serum albumin was used as standard. Results were analyzed by analysis of variance (34).

18 survivors of adverse anesthetic reactions and 15 relatives with muscle fibers abnormally sensitive to caffeine were studied. No survivors of MH were biopsied within 2 mo of an attack. Vastus lateralis was biopsied in all but one patient, who had a rectus abdominus biopsy during a cholecystectomy performed after 3 d of treatment with dantrolene sodium. Because available tissue was limited, not all measurements were performed on all biopsies, but all of them were examined histochemically and most by electron microscopy. Other than one physiologically abnormal relative who had evidence of active degeneration and regeneration, muscles of all patients included in this series appeared normal or had nonspecific abnormalities.

Control muscles were derived from several sources. Some muscles were from patients biopsied for complaints consistent with muscle disease, but who had normal physical examination, serum enzymes, and muscle histochemistry. One patient had ocular myasthenia gravis. Three muscles derived from patients biopsied because of a remote family history of MH, or because of unexplained elevation of serum creatine kinase; these muscles all were normal to physiological testing; values of AC fell within the range of other controls. Chemically indistinguishable, and also used as control muscle, were a piece of pectoral muscle obtained in the course of a radical mastectomy, and several quadriceps biopsies, obtained during orthopedic procedures on patients not suspected of neuromuscular disease. To control for inclusion of rectus abdominus muscle in the experimental series, a rectus abdominus biopsy was obtained from an individual without history of MH, who also was having an elective cholecystectomy. Most biopsies were performed at Columbia-Presbyterian Medical Center, where quadriceps biopsies were performed with intradermal procaine anesthesia, and muscle was immediately frozen in liquid N_2. Biopsies performed at other hospitals were shipped frozen on dry ice. All specimens were stored in liquid N_2 until assay. Controls varied in age at time of biopsy from 6–62-yr-old with a mean of 28.5, patients from 4.7–50-yr-old, with a mean of 20.8. Activities of AC and muscle content of cyclic AMP did not correlate with age in control or patient populations.

The major manifestations of MH include a rapid, sustained, and otherwise unexplained rise of temperature, muscle rigidity", metabolic acidosis, and skeletal muscle damage, resulting in myoglobinuria or markedly increased serum creatine phosphokinase (CPK) activity. Hyperthermia may occur without rigidity and it is controversial which of the manifestations or which combination provides evidence of certain diagnosis. Because collection of information about attacks of MH was not systematic in the hospitals that referred patients for study, information about CPK, acidosis, or arrhythmia was often unavailable. We therefore defined MH as a hyperthermic response to general anesthesia. 14 of the 18 patients also had involuntary, sustained muscle contractions, and the remaining 4 had lactic acidosis, arrhythmias, or CPK elevation coincident with hyperthermia. All but one of the 18 subjects had physiological evidence

of increased caffeine sensitivity of skinned single fibers; the exceptional patient had a rectal temperature of 107°F, metabolic acidosis, and rise in serum CPK, but no rigidity during general anesthesia. The probability of inappropriately assigning to this series a patient with fever rather than hyperthermia should therefore, have been remote. By definition, all relatives classified as "susceptible to MH" had muscle fibers with abnormal caffeine sensitivity.

RESULTS

Basal AC activity was abnormally increased in muscle of MH reactors and those relatives with abnormal single fiber responses to caffeine, compared to control muscles (Table I). The abnormality was apparent with either method of homogenization, but was not seen if calcium-chelated homogenates (method 2) were assayed in the presence of free Mg^{++} concentrations above 1 mM. Increased basal activity was due to an apparent increase in maximum velocity (V_{max}) (22.2±12.6, patients; 6.42±2.33, controls ($P < 0.01$); the K_m of AC for ATP was 0.28 mM for patients and controls. Addition of 0.1 mM isoproterenol (method 1) or 10 μM guanylylimido-diphosphate and 0.1 mM isoproterenol (method 2) to the reaction mixture resulted in higher AC activity in the patients and susceptible relatives than controls. (Guanyl nucleotides alone stimulate skeletal muscle AC activity <5% (28). Fluoride stimulated activity was also higher in MH muscle.

Values obtained on survivors and physiologically abnormal relatives were not statistically different, and there was no differences in basal activity or catecholamine or fluoride responses of rigid or nonrigid survivors.

Drugs used as premedication for biopsies, prior consumption of caffeinated beverages, and serum epinephrine concentration may influence muscle cont. nt of cyclic AMP; these variables were not controlled, and no patients were biopsied more than once to determine biological variation. Nonetheless, the content of cyclic AMP was increased in muscle of MH survivors and physiologically abnormal relatives (Table II). The mean of values for reactors and relatives had large standard deviations, due to several outlying values. When data from reactors and relatives were pooled, the mean was 18.9±15.7 pmol/mg noncollagen protein, and that of controls was 5.45±3.76 ($p < 0.001$). Measured activities of cyclic AMP PDE in the muscle of six survivors of MH were not different from activities in the muscle of six age-matched controls (Table II).

DISCUSSION

Because hyperthermia suggests mitochondrial dysfunction and sustained contracture, a disorder of SR, most past research on MH or its veterinary models has focussed on these organelles. Recently, an electrophysiological abnormality was identified in surface membranes of FHSS-susceptible pigs (35). Skeletal muscle AC is highly localized to sarcolemma and transverse tubules (14, 15). The observation that AC activity is increased in MH muscle indicates that surface membranes of skeletal muscle also are abnormal in humans at risk for MH.

In several extensively studied families, MH occurred in numerous individuals with a pattern suggestive of autosomal dominant inheritance (36, 37). MH probably occurs with increased incidence in families with central core disease (38–40), an autosomal dominant myopathy. It has been associated in children, primarily

TABLE I
Adenylate Cyclase Activity of Skeletal Muscle Homogenates

	Basal	Catecholamine-activated	Fluoride-activated
Units	picomoles/milligram of noncollagen protein/minute		
Method 1			
Controls (n = 11)	5.41±1.75 (2.23–7.19)	11.3±5.41 (4.17–20.4)	
MH reactors and relatives (n = 8)	*13.6±6.54 (5.28–25.3)	*31.6±16.5 (19.1–54.2)	
Method 2			
Controls (n = 18)	1.42±0.64 (0.61–2.83)	4.01±2.66 (0.91–10.4)	9.78±5.17 (4.35–17.2)
MH reactors (n = 14)	*7.22±4.56 (2.19–16.8)	‡21.7±10.4 (9.7–43.1)	‡26.3±9.73 (10.2–38.1)
MH relatives (n = 6)	*4.74±1.89 (2.20–6.86)	‡15.2±4.90 (11.0–24.2)	‡20.2±3.30 (14.5–23.4)

Data are presented as means±SD (range). While with both methods, control and subject values overlapped, means were significantly higher for activity in muscle of anesthetic reactors or relatives with abnormal single fiber physiology. *$P < 0.005$; ‡$P < 0.001$.

TABLE II
Skeletal Muscle Cyclic AMP PDE Activity and Cyclic AMP Content

	Cyclic AMP PDE			
	Supernate		Particulate	
Substrate concentration:	1 μM	200 μM	1 μM	200 μM
Units:	picomoles/milligram of noncollagen protein/minute			
Controls (n = 6)	34.3±13.3	332±90.1	14.5±4.41	87.1±12.5
MH reactors (n = 6)	25.1±7.68	307±151	19.6±7.04	128±72.0

	Cyclic AMP content picomoles/milligram of noncollagen protein
Controls (n = 29)	5.45±3.76 (0.96–15.0)
MH reactors (n = 17)	16.5±15.5 *(3.41–68.9)
MH relatives (n = 9)	24.2±14.4 *(6.09–50.6)

Skeletal muscle cyclic AMP PDE activity (mean±SD) and cyclic AMP content ((mean±SD) and (range)) from survivors of MH reaction, relatives, or control subjects. PDE activity, determined against two substrate concentrations in supernate and particulate fractions, was not different from control; cyclic AMP content was increased in MH reactors and relatives. * $P < 0.001$.

boys, with a unique dysmorphic syndrome of unknown inheritance pattern (41). In no patient we studied was there a family history of anesthetic reactions or deaths. The inheritance of susceptibility to MH in these sporadic cases is unknown; therefore, it is impossible to know if increased skeletal muscle AC activity describes a single hereditary muscle disease.

Paradoxically, increased activity of AC occurred in muscle of individuals when they were asymptomatic. The normal function of AC provides an explanation for this paradox and may imply a unique mechanism of genetic disease. Although an activity of AC can be readily measured in vitro, cyclic AMP is synthesized at a relatively low rate in vivo in the absence of physiological stimulants, and it is rapidly degraded by PDE. Cyclic AMP content and the extent of activation of cyclic AMP-dependent protein kinases are correspondingly low in unstimulated tissues. The usually asymptomatic myopathy associated with MH is therefore associated with abnormality of an enzyme that normally requires stimulation to be catalytically active. Catecholamine secretion and anesthetics could provide that stimulation (18–26).

Increased enzyme activity would be an unusual primary expression of a mutant gene, especially for what may be a dominant mutation. The normal K_m for MgATP implies that the affinity of the catalytic subunit for substrate is not the cause of altered enzyme activity. Abnormal boundary lipids, affecting the coupling to catalytic subunits of the guanine nucleotide binding subunit of AC (N-protein [42]), or a change in function of N-protein could, theoretically, increase AC activity.

Whatever its molecular basis, this change in AC activity is likely to have physiological significance. The increased cyclic AMP content of muscle, by catalyzing phosphorylation of phosphorylase kinase, could be the cause of the activation of phosphorylase in MH muscle (43), which may predispose to the development of lactic acidosis during attacks of MH. Increased phosphorylation of SR proteins by cyclic AMP-dependent protein kinases could contribute to the abnormality of SR function detected in chemically skinned fibers (12), which may underlie the contracture tests used to identify individuals susceptible to MH or pigs with the FHSS phenotype.

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