Intracellular Free $[Ca^{2+}]$ in Human Skeletal Muscle with Myopathic Carnitine Deficiency

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Abstract Carnitine is required for the transport of activated long chain fatty acids through the mitochondrial inner membrane. We measured the intracellular free calcium concentration ($[Ca^{2+}]_i$) by means of a calcium selective microelectrode in skeletal muscle biopsies obtained from nine patients in which myopathic carnitine deficiency (MCD) was diagnosed, and from six subjects with no evidence of neuromuscular disease. Intact intercostal muscle bundles were dissected and then split for electron microscopic studies and electrophysiological measurements. The $[Ca^{2+}]_i$ in muscle fibers from MCD patients was 0.46 ± 0.02 μmol $.l^{-1}$ (mean $\pm$ SEM) and 0.10 ± 0.01 μmol $.l^{-1}$ in control subjects. At the electron microscopic level, the predominant abnormality was the presence of lipid vacuoles between the myofibrils. These results show that in patients with myopathic carnitine deficiency there is a significant increase in the resting myoplasmic calcium concentration which might be related to a malfunction of some mechanisms responsible for the homeostasis of intracellular calcium.

Key words: Carnitine — Calcium — Skeletal muscle — Calcium microelectrode — Homeostasis

Introduction

Carnitine (3-hydroxy-4-trimethylaminobutyric acid) is a quaternary ammonium compound with multiple functions including transport of long chain fatty acids across the inner mitochondrial membrane delivering them for β oxidation. Carnitine is synthesized from methylated lysyl residues released by lysosomal protein degradation. However, the enzyme 4-butyrobetaine hydroxylase, catalyzing the last step, is present mainly in the liver, and not at all in skeletal

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muscle (Rebouche and Engel 1980; Tanphaichitr and Broquist 1973). Thus, the carnitine requirement of muscle cells must be entirely met by uptake from the blood, which seems to be a carrier mediated process (Rebouche 1977; Rebouche and Engel 1982; Bremer 1983).

Normally, the concentrations of carnitine in plasma and tissues remain relatively constant. However, there are clinical conditions in which the carnitine concentration can be modified. Carnitine deficiencies have been classified into 2 major groups: systemic carnitine deficiency (SCD) and myopathic carnitine deficiency (MCD). The systemic deficiency patient shows low muscle and low total plasma carnitine levels, and is characterized by multiple episodes of acute encephalopathy. This disorder has been associated to a biosynthetic carnitine defect in the liver. The myopathic form is limited to muscle. It is characterized by mild to severe muscle weakness and variable excess of lipids in skeletal muscle fibers, while carnitine levels in liver and plasma are in the normal range, indicating an abnormal carnitine transport into skeletal muscle and/or changes in carnitine efflux (Engel and Angelini 1973; Engel and Rebouche 1982).

In skeletal muscle, as well as in other excitable cells, the intracellular free calcium concentration ($[Ca^{2+}]_i$) is about four orders of magnitude smaller than the $[Ca^{2+}]$ in the extracellular medium (López et al. 1983). This concentration gradient is maintained by: i) the sodium calcium countertransport process, Na^+/Ca^{2+} exchange, which is powered by energy derived from the transmembrane sodium electrochemical gradient and is modulated by ATP (Gilbert and Meissner 1982); ii) a Ca^{2+}-pump at the plasma membrane in which the energy for Ca^{2+} extrusion is provided directly by ATP hydrolysis (Caroni and Carafoli 1981); and iii) an ATP dependent calcium transport system at the sarcoplasmic reticulum (Martonosi 1983). In the present study, we measured the $[Ca^{2+}]_i$ by means of a Ca^{2+} selective microelectrode in muscle biopsies from patients with myopathic carnitine deficiency (MCD), in order to directly assess whether the intracellular Ca^{2+} homeostasis was affected in those muscles in which a decrease in L-carnitine concentration existed.

Materials and Methods

Intact external intercostal muscle fibers were removed under local anesthesia (tetracaine) after securing consent from the patient involved and the approval from officials of the institution. Muscle biopsies were obtained from six subjects (four males, two females) aged 16 to 34 years, with no evidence of neuromuscular disease, who served as controls, and from nine patients (6 males, 3 females) with MCD, aged 8 to 18 years. Two of the MCD patients were biopsied for a second time, 6 months later, after receiving L-carnitine (orally, 1 g three times daily). MCD diagnosis was based on i) clinical findings, ii) electromyogram, iii) determinations of free [L-carnitine] in serum and skeletal muscle biopsies, using the enzymatic method described previously (Marquis and Fritz 1964).

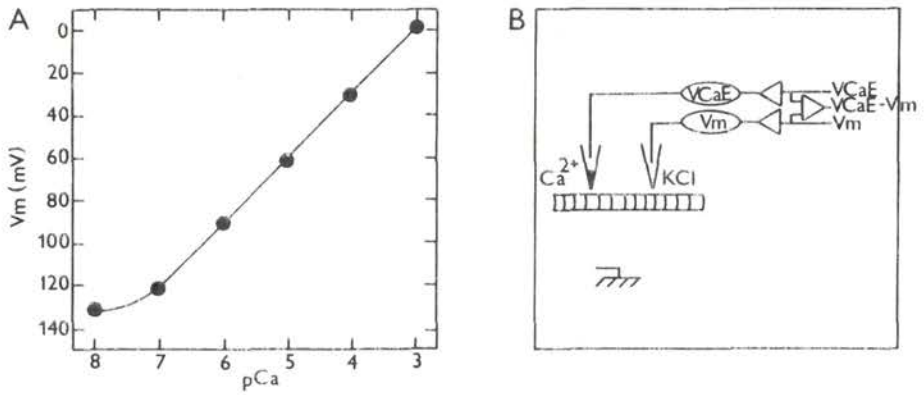

Fig. 1. *A*) Calibration curve of a submicron tip Ca^{2+} selective microelectrode using calibrating solution with pCa between 3 and 7, at constant background of 100 mmol $.l^{-1}$ KCl. *B*) Diagram of the recording arrangement for measuring resting membrane potential (*V*m) and intracellular free calcium concentration $[Ca^{2+}]_i$ in the same muscle fiber. *V*Ca*E* is the potential recorded through the calcium selective microelectrode and Vm the potential recorded by the KCl microelectrode. *V*Ca*E*—*V*m is the differential signal between the potential recorded through the Ca microelectrode and KCl microelectrode. This differential signal indicates the intracellular free calcium concentration ($[Ca^{2+}]_i$). The separation between the tips of *V*m and Ca^{2+} microelectrodes was about 300 μm.

Silanized tip microelectrodes were back filled with the liquid sensor based on the neutral synthetic ion carrier ETH 1001 (Simon et al. 1978) and 24 h later the microelectrode shank was similarly back filled with pCa7 solution (pCa = negative log $[Ca^{2+}]$). They were calibrated individually, before and after each period of successful intracellular measurement as described previously (López et al. 1983). Only those Ca^{2+} microelectrodes that showed a Nernstian response (30.5 mV per decade $[Ca^{2+}]$ at 37 °C) between pCa 3 and 7 were used experimentally (Fig. 1*A*).

Intact muscle bundles were split longitudinally for (a) electrophysiological measurements and (b) electron microscopy studies. (a) Individual muscle fibers were impaled with two glass microelectrodes (Fig. 1*B*) to record resting membrane potential and $[Ca^{2+}]_i$ (López et al. 1985). The voltage recorded by the resting membrane potential electrode was subtracted electronically from that recorded by the Ca^{2+} electrode to give a differential signal that was equivalent to the myoplasmic pCa. (b) The remaining muscle fibers held at constant length were fixed in ice cold 5% glutaraldehyde buffered with 0.1 mol $.l^{-1}$ sodium cacodylate (pH 7.3) and then processed according to standard methods for electron microscopy studies (Mokri and Engel 1975).

Data are presented as the mean ± standard error of the mean values. Differences in *V*m and $[Ca^{2+}]_i$ means from control and MCD patients were examined by the Student's *t* test for unpaired data. Differences were considered significant when $P < 0.05$.

Results

Fig. 2 shows a typical experiment of a simultaneous measurement of resting membrane potential and intracellular free Ca^{2+} concentration, from control (*A*)

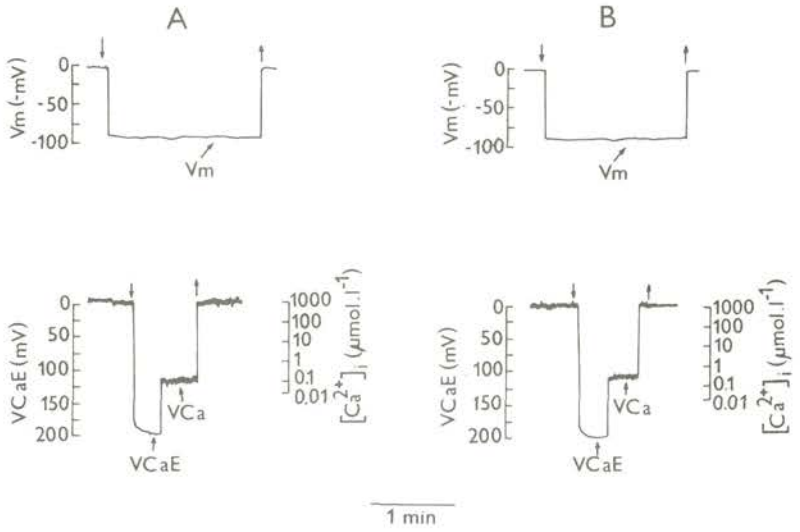

Fig. 2. Intracellular recording of resting membrane potential (Vm) and intracellular free calcium concentration (VCa) carried out on skeletal muscle fibers isolated from a control and a patient with myopathic carnitine deficiency. The trace labelled Vm (top trace) is the potential recorded by the 3 mol . l^{-1} KCl microelectrode, this was subtracted electronically from the initial potential recorded by the ion selective microelectrode, (VCaE) (lower trace), to give the myoplasmic $[Ca^{2+}]$. Downward arrows indicate the time when the muscle cell was impaled with the microelectrode and the upward arrow indicates its withdrawal. Calibration bars are shown on the left and right hand side in mV and μmol . l^{-1}. The actual values of the fiber impaled are shown at the bottom. *A*: $V_m = -85$ mV, $[Ca^{2+}]_i = 0.10$ μmol . l^{-1}. *B*: $V_m = -87$ mV, $[Ca^{2+}]_i = 0.38$ μmol . l^{-1}.

and MCD (*B*) muscles. It should be noted that the $[Ca^{2+}]_i$ was 3.8 times higher in the MCD muscle fibers than in control, with no detectable difference in the resting membrane potential. The mean value from twenty five satisfactory measurements of control muscle fibers was -84 ± 1 mV (M $\pm$SEM) and 0.10 ± 0.01 μmol . l^{-1} for the resting membrane potential and $[Ca^{2+}]_i$ respectively. Similar determinations carried out in thirty two muscle fibers from nine MCD patients showed a mean value for Vm of -86 ± 1 mV and 0.46 ± 0.02 μmol . l^{-1} for $[Ca^{2+}]_i$ respectively (Table 1).

In an attempt to determine whether the elevated intracellular free calcium concentration was due, in part, to a change in the L-carnitine concentration a second biopsy was performed to two of the MCD patients after 6 months of treatment with L-carnitine (oral L-carnitine, 1 g three times daily). Figure 3 shows the recording of resting membrane potential and intracellular calcium concentration from one of these patients before (*A*) and after (*B*) carnitine treatment during 6 months. The $[Ca^{2+}]_i$ was reduced in relation to previous

Table 1

	Vm ($-$mV)	$[Ca^{2+}]_i$ (μmol . l^{-1})	$[Ca^{2+}]_i$ (range)	Measurements (n)
Control	-84 ± 1	0.10 ± 0.01	0.09—0.13	25
MCD	-86 ± 1	0.46 ± 0.02	0.32—0.51	32
MCD + L-carnitine	-83 ± 2	0.32 ± 0.02	0.23—0.42	6

The difference between the resting membrane potential values recorded in control and MCD muscle fibers was not significant ($P > 0.20$). However, differences between the $[Ca^{2+}]_i$ values were significant with $P < 0.001$.

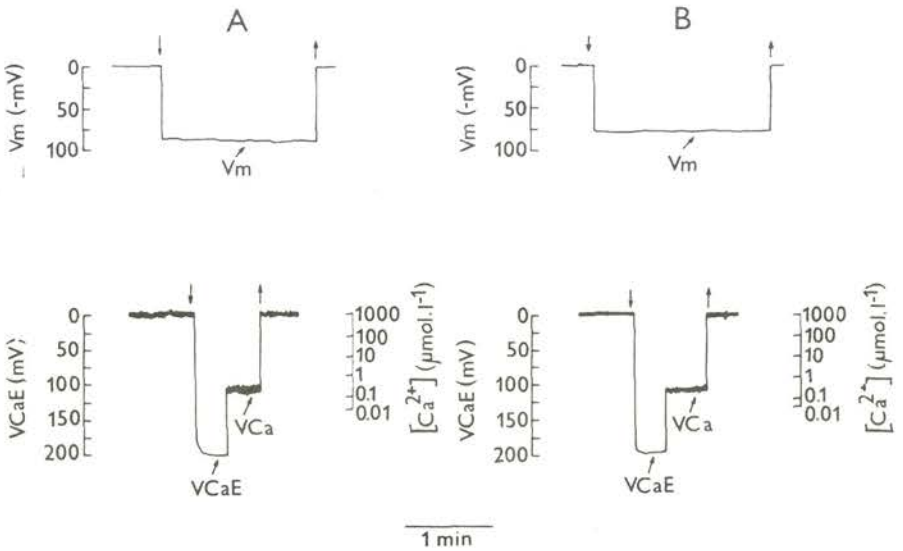

Fig. 3. Effects of L-carnitine on $[Ca^{2+}]_i$. *A*) Simultaneous measurements of resting membrane potential (*top trace*) and myoplasmic free $[Ca^{2+}]$ (*lower trace*) before L-carnitine treatment. *B*) Determination of Vm and $[Ca^{2+}]_i$ after L-carnitine administration (second biopsy from the same patient) that induced a detectable decrease on the $[Ca^{2+}]_i$ (from 0.36 μmol . l^{-1} to 0.28 μmol . l^{-1}) without any significant change in the resting membrane potential. On the left and right side of each record are the calibration bars in mV and μmol . l^{-1}. *A*: $V_m = -86$ mV, $[Ca^{2+}]_i = 0.36$ μmol . l^{-1}. *B*: $V_m = -80$ mV, $[Ca^{2+}]_i = 0.28$ μmol . l^{-1}.

determinations from 0.36 to 0.28 μmol . l^{-1}. This change in $[Ca^{2+}]_i$ appeared to be associated with a moderate increment in myoplasmic L-carnitine concentration (from 1.48 to 1.95 μmoles per gram wet weight).

Fig. 4. Electron micrograph illustrating lipid droplets (*) in a skeletal muscle biopsy obtained from a MCD patient. x 12,000.

Figure 4 shows an electron micrograph of a longitudinal section of a muscle biopsy obtained from a MCD patient. The predominant abnormality observed in the MCD fibers was the presence of several lipid vacuoles between the myofibrils as it was described previously by Engel and Rebouche (1982).

Discussion

As has been stated, previously MCD patients exhibit an abnormally low level of myoplasmic L-carnitine, however in the serum the concentration is normal. Such deficiency in [carnitine] has been related primarily to an abnormal transport system for L-carnitine at the plasma membrane (Engel and Angelini 1973).

In the present study, the intracellular free calcium concentration was signifi-

cantly higher in the MCD muscle fibers as compared to controls, with no detectable change in resting membrane potential between both groups. This finding represents the first direct demonstration that in MCD muscle fibers the intracellular calcium homeostasis is abnormal. Alterations in intracellular calcium concentration seem to be a common feature of several primary muscle pathologies, and not a unique finding. Patients susceptible to malignant hypertermia syndrome (López et al. 1985) and those suffering from Duchenne muscular dystrophy (Sánchez et al. 1988) also show elevation in the resting intracellular free $[Ca^{2+}]$.

In skeletal muscle, carnitine transport against a concentration gradient has been suggested to occur down the Na^+ concentration gradient perhaps involving an Na^+ dependent cotransport system (Rebouche 1977). The apparent effect of Na^+ on carnitine transport appears to be not directly dependent on the Na^+—K^+ ATPase activity and/or on membrane depolarization (Vary and Neely 1982). Although so far, there is no direct evidence indicating that the Na^+ electrochemical gradient is modified in MCD muscle fibers, changes in this gradient might lead to an increase of the intracellular Ca^{2+} concentration, via the Na^+/Ca^{2+} exchange mechanism.

In addition, a low myoplasmic L-carnitine concentration may induce some alteration in the oxidative anaerobic pathway, since the long chain fatty acids cannot cross the inner mitochondrial membrane to be oxidized. This accumulation of long chain fatty acids in the myoplasm induces the formation of lipid vacuoles between myofibrils. It is possible that changes in the oxidative pathway modify the amount of ATP synthesis by the mitochondria. All of the high affinity Ca^{2+} transport systems require ATP in order to maintain the physiological intracellular Ca^{2+} concentration (Caroni and Carafoli 1981; Martonosi 1983). Changes in ATP concentration would modify some of these energy dependent Ca^{2+} regulatory mechanisms. In this regard, we found that the calcium uptake by membrane vesicles isolated from sarcoplasmic reticulum is significantly lower in MCD muscle than in control muscle (López et al. 1986). Administration of L-carnitine over a period of six months in two patients induced a reduction of $[Ca^{2+}]_i$ in the order of 16% which supports the previous working hypothesis. Although no conclusion can be drawn in terms of the possible alteration in the intracellular $[Ca^{2+}]$ regulatory mechanisms and/or the metabolic impact, differences in muscle performance observed between MCD and controls might be influenced by the imbalance of the intracellular Ca^{2+} homeostasis.

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