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The use of Fura–2 to estimate myoplasmic [Ca^{2+}] in human skeletal muscle

P. A. IAIZZO*§, M. SEEWALD‡, S. G. OAKES† and F. LEHMANN-HORN*

Departments of Neurology and Animal Science‡, Technical University of Munich, Munich, West Germany*

Abstract — Fura–2 was used to estimate myoplasmic [Ca^{2+}] in intact fibers and fiber segments from normal and diseased human muscles. Small muscle bundles (20–50 fibers) were loaded with the membrane-permeant form of the dye (Fura–2 AM). High-performance liquid chromatography was utilized to study the ability of these cells to hydrolyze Fura–2 AM. Immediately after the 30 min loading period, Fura–2 (the Ca^{2+} indicator) was the predominant form of the dye in all preparations and the concentration within these fibers remained stable for over 4½ hours. In addition, the resting myoplasmic [Ca^{2+}] in fiber segments from normal subjects and those susceptible to malignant hyperthermia were the same. However, halothane administration (1.5%) induced correlated increases in myoplasmic [Ca^{2+}] and force only in fibers from the susceptible patients. In contrast, caffeine administration causes correlated increases in myoplasmic [Ca^{2+}] and force in both types of muscle, but lower concentrations were needed to do so in the fibers from the susceptible patients. The effects of halothane and caffeine were reversible. We conclude that Fura–2 can be used successfully to estimate resting levels and changes in myoplasmic [Ca^{2+}] in human skeletal muscle.

It has been reported that intracellular [Ca^{2+}] is elevated at rest in muscles affected by disease [1–4]. An elevated [Ca^{2+}] has been suggested to be associated with dystrophic changes in muscle. However, there is a paucity of reports in which myoplasmic [Ca^{2+}] in live human skeletal muscle has been estimated. This is in part due to the problems in obtaining intact muscle fibers from patients with various muscle diseases. However, it has been recently reported that Fura–2 can be used successfully to estimate [Ca^{2+}] in human skeletal muscle [5, 6]. In addition, we reported that these long muscle fiber segments repolarized several hours post-transsection and possessed electrical and mechanical properties of intact fibers [6]. There was no statistical difference in the mean 340/380 fluorescence ratios detected from intact or long cut muscle fibers (segments) from normal controls. Therefore, it may be possible to study additional properties of diseased muscle using material from routine muscle biopsies avoiding the need of fiber preparations, intact from tendon to tendon.

Fura–2, a Ca^{2+}-specific fluorescent chelator, has been used to estimate [Ca^{2+}] in a number of cell types including mammalian skeletal muscle [6–12]. A major advantage of Fura–2 is that it can be easily

§Present address : Department of Anesthesiology, Mayo Clinic, Rochester, MN, USA
†Present address : Department of Pharmacology, Mayo Clinic, Rochester, MN, USA

loaded into certain cell types without injection [13]. The ester groups of Fura–2 pentaacetoxymethyl ester (Fura–2 AM) are cleaved by cytoplasmic esterases to yield Fura–2 which is membrane impermeant. On excitation at various wavelengths, the hydrolyzed form of the dye fluoresces with differing intensities. Successful application of the ratio method to estimate intracellular [Ca^{2+}] requires that Fura–2 is the predominant fluorescent species present [11, 14, 15]. However, in certain cell types the conversion is minimal and all forms of the dye appear to leave the cells [11].

The present experiments were designed to evaluate the usefulness of Fura–2 to estimate the myoplasmic [Ca^{2+}] in fiber segments and intact fibers from both normal and diseased human skeletal muscles. We estimated how well the Fura–2 AM was hydrolyzed within the cell. Direct measurement of the hydrolysis of Fura–2 AM and cellular retention was made by high performance liquid chromatography [11]. In addition, Fura–2-loaded normal and diseased human skeletal muscle fibers were exposed to halothane and caffeine and changes in force and [Ca^{2+}] were monitored simultaneously.

Materials and Methods

Biopsies of the external intercostal, biceps, vastus lateralis, vastus medialis, latissimus dorsi or deltoid muscles were taken from normal humans and those with known muscle diseases. The muscle specimens were removed to determine if certain patients were susceptible to malignant hyperthermia; in vitro halothane-caffeine contracture tests were performed on several intact muscle bundles according to the protocol of the European Malignant Hyperpyrexia Group [16].

For the measurement of myoplasmic [Ca^{2+}] or resting membrane potentials, very thin sheets of 20–50 fibers intact from tendon to tendon were dissected and suspended in a plexiglass chamber (4.0 ml). For the measurement of isometric force, one tendon was fixed and the other fastened to a strain gauge (Akers, Horten, Norway). The experimental chamber was continuously perfused (1.0 ml/min). The temperature of the bathing solutions was maintained at 35.0 ± 0.5 °C with a temperature control unit.

The standard bathing solution contained: 118.3 mM NaCl, 3.5 mM KCl, 0.8 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$ and 11.0 mM glucose. The pH was adjusted to 7.4 by gassing this solution with a mixture of 95% O_2 and 5% CO_2. In several experiments, halothane was bubbled through the bath via a fluothane vaporizer (Fluotec 3, Cyprane Ltd, Keighley, UK) and actual halothane bath concentrations were determined as described by Van Dyke and Wood [18]. Caffeine (dehydrated; Roth, Karlsruhe, FRG), when added in single doses to the bathing solution, was at concentrations of 2.0, 4.0, 8.0 or 16.0 mM. Changes of the bathing solution were accomplished in less than 10 seconds.

Muscle fibers were loaded with Fura–2 AM (Molecular Probes, Eugene, OR, USA) by bathing them for 30 min at 35°C in a solution containing the membrane-permeant ester of the dye (2.0×10^{-7} M).

The experimental chamber was mounted on a Zeiss Universal microscope equipped for fluorescence and photometry. For excitation of fluorescence, the light from a 50 W mercury lamp (operated with a.c. and housed in a Zeiss 100 lamp housing; Osram HBO 50, Munich, FRG) passed through a locally constructed triggering unit, UV interference filters (Zeiss, center wavelengths of 340 or 380 nm; halfwidths 10 nm), a beam splitter (Zeiss, FT43T), into the microscope objective (Zeiss Achromat 40X water immersion; or an Olympus 101774 UVFL 401) and to the preparation. Fluorescence emitted from the fibers passed through an interference filter (500 nm center wavelength: 20 nm halfwidth) to a photomultiplier tube (Hamamatsu R268, Japan, factory selected for maximum sensitivity at 500 nm). A pinhole filter in the light path limited the region of the preparation from which fluorescence was collected to a circular area with a diameter of 40 μm [7, 9]. The voltage output of the photomultiplier was monitored with a Nicolet 535 signal averager. The averager was triggered by the peak light output of the unfiltered UV light via the triggering unit. Sixty-four samples of the fluorescence signal following excitation at each wavelength were averaged. The system is diagrammatically represented in Figure 1.

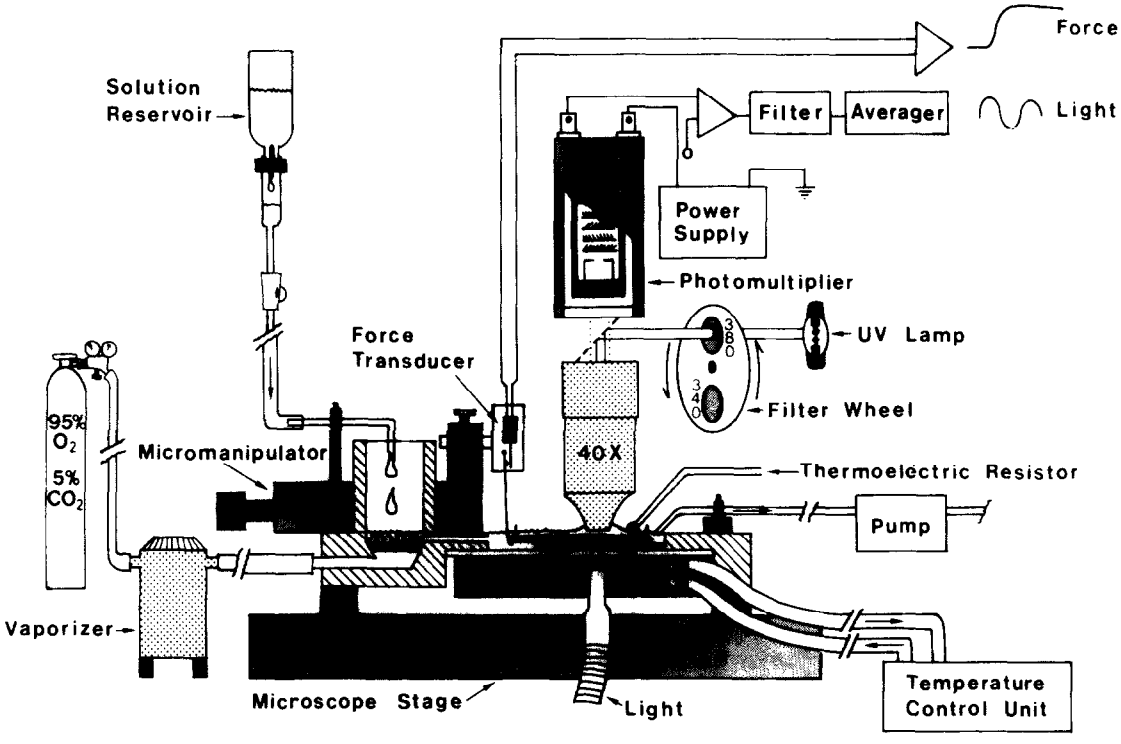

Fig. 1 Schematic diagram of the experimental set-up used to record fluorescence from the human skeletal muscle fibers

Fluorescence measurements were made at several locations along the length of individual fibers and 5–20 fibers were studied in each preparation. At the start of each experiment the whole bundle was stretched until the average striation spacing was approximately 2.50 μm. The distribution of the dye within an individual fiber was not always uniform and measurements were not taken from fibers in which a difference was visually noticeable. A measurement was made every 1½ to 2 minutes; the position and appearance of the fiber was observed before and after the fluorescence was recorded. When a fiber appeared damaged (clots) or if striations were not visible, the measurement was not used. When a spontaneous contracture developed under resting conditions (while the preparation was in normal bathing solution), the preparation was discarded.

The relationship between the Fura–2 fluorescence ratio and $[Ca^{2+}]$ was approximated as previously described [9]. Resting membrane potentials were measured as described by Lehmann-Horn et al. [18]. Statistical differences were determined using Scheffe's multiple contrast (non-parametric) test.

The fluorescence ratios which we measure are somewhat less than those reported by others (e.g. Williams and co-workers [12]), but this is related to the properties of the system we used [14]. Nevertheless, during the calibration experiments, the intensity of the Fura–2 emission changed upon excitation with either wavelength (340 or 380 nm) when the $[Ca^{2+}]$ was altered. Thus, we do not believe our measurements were limited by the resolution of the recording system.

High performance liquid chromatography (HPLC) assay

Fura–2 and its metabolites, in 20 μl of extract, were assayed using the methods previously described by Oakes and co-workers [11]. The extracts were prepared by placing a known quantity of washed muscle into 2 ml of acetonitrile. Bundles of both

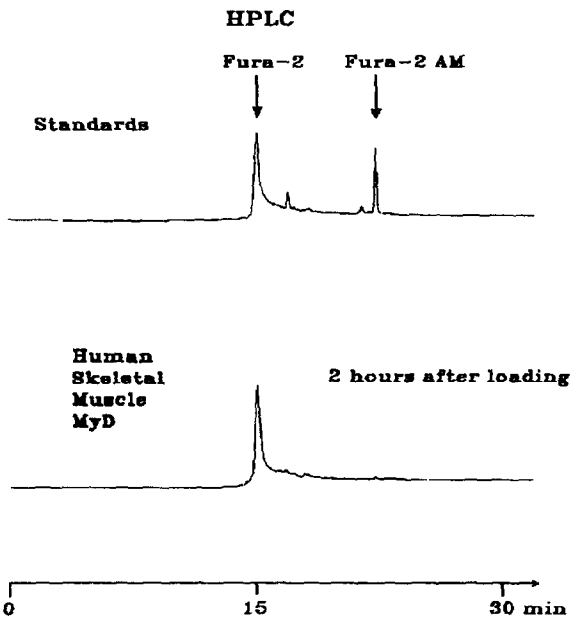

Fig. 2 Chromatograms showing the assay calibration and the uptake and metabolism of Fura-2 AM. A 10 pmol of both Fura-2 AM and Fura-2 were injected onto the column. Metabolites were present indicating the presence of breakdown products. B A 20 µl sample of extract from muscle fiber segments from a patient with myotonic dystrophy was injected. The muscle bundle was extracted 2 h after it was loaded with Fura-2 AM

intact and cut (segments) fibers were prepared for study. To determine if there were any changes in the concentrations of Fura-2 AM and its metabolites with time, several bundles of intact fibers were prepared from the same intercostal muscle biopsy and simultaneously loaded with the permeable form of the dye. After the 30 min loading period, the bundles were washed and either placed in acetonitrile or maintained in an oxygenated Krebs solution. At various times following loading, additional bundles (2–3 per time) were placed in acetonitrile. Subsequently, the concentrations per gram of muscle of Fura-2 AM and its metabolites were estimated. These concentrations were calculated by calibrating the apparatus with a known concentration of each standard and correcting for the effects of the solution front. Prior to these HPLC experiments, the resting membrane potentials of several fibers were recorded as a measure of the relative viability of a given preparation.

Results

HPLC assay of myoplasmic Fura-2 and metabolites

The HPLC assay provided good separation of Fura-2 AM and its metabolites. The retention times under the HPLC conditions employed here were 15.6 min for Fura-2 and 22.6 min for Fura-2 AM. The retention times for the other metabolites were assumed to fall between these two extremes. Figure 2 shows two typical chromatograms. The upper record was obtained when known standards were injected into the apparatus. The samples were not pure and contained several different Fura-2 AM metabolites. The lower record in Figure 2 was that obtained following the injection of a sample prepared from fiber segments from a patient diagnosed to have myotonic dystrophy. These fibers were placed in the acetonitrile 2 h after loading. The estimated concentrations of various forms of the dye in this sample were the following: 101.4 µM of Fura-2, 0.007 µM of Fura-2 AM and 6.5 µM for the combined concentration of the other metabolites. The resting myoplasmic $[Ca^{2+}]$ of these fibers was estimated to be normal (0.10 µM).

The concentration of Fura-2 AM and its metabolites appeared to change little in time. Several bundles ($n = 14$) of intact fibers prepared from the muscle of a normal subject were simultaneously loaded with Fura-2 AM. Immediately after loading the majority of Fura-2 AM was hydrolyzed to Fura-2 and only a trace amount of the ester form remained (see Fig. 3). One hour post-loading, the presence of Fura-2 AM within the cell was not detectable and the concentrations of the minor metabolites also decreased. The average concentration of Fura-2 in all bundles sampled was 286.4 ± 50.0 µM ($n = 14$) which was nearly 50-fold greater than the combined concentration of all other forms of the dye which were located within the cells.

The ability of a bundle to take up and hydrolyze Fura-2 AM appeared to be related to the type and condition of a preparation. For example, the estimated concentration of Fura-2 present within long fiber segments from a patient with polymyositis was an order of magnitude less than that observed for similar fibers prepared from a

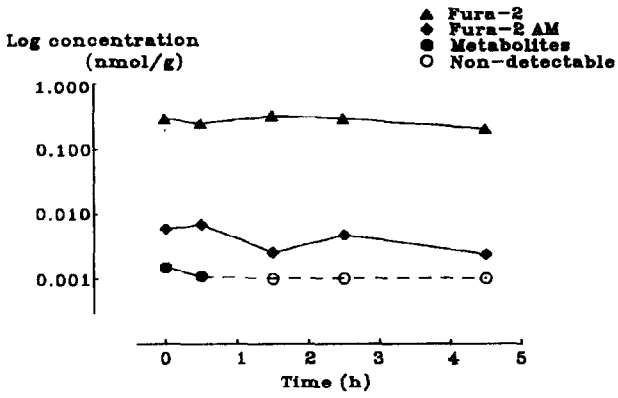

Fig. 3 Time course of Fura-2 AM hydrolysis to its metabolites. The incubations were of normal human skeletal muscle fibers which were loaded for 30 min at 35°C. The relative concentrations of Fura-2 AM and metabolites were estimated from 14 carefully dissected bundles of intact fibers which were prepared from the same biopsy. Note that the relative concentration of Fura-2 per gram of muscle at each time period was nearly 50-fold greater than its metabolites

normal subject. The resting membrane potentials of these fibers were considered normal, but inflammatory changes were noted histologically.

Resting [Ca^{2+}]

As previously reported variation in the measured fluorescence ratios, when observed along with length of an individual fiber, was always less than half of the total standard deviation of all measurements for a given preparation [8]. Fura-2 did not appear to have an effect on the resting membrane potentials of the muscle fibers: at the end of the fluorescence measurements the resting potentials of both intact and fiber segments were still between –70 and –85 mV. However, it should be noted that the dye concentration in the region of the last few millimeters of the transsected fibers appeared to be somewhat higher.

We previously reported that the estimated myoplasmic [Ca^{2+}] did not differ between: 1) intact skeletal muscle fibers prepared from normal swine and those susceptible to malignant hyperthermia [9]; and 2) intact and long fiber segments (> 3.0 cm long) isolated from human skeletal muscle fibers [5, 6]. The estimated [Ca^{2+}] from fiber segments obtained from normal humans and those susceptible to malignant hyperthermia did not differ significantly ($P > 0.25$). The mean fluorescence ratios were the following: 0.053 ± 0.010 (X ± S.D.; n = 158) for fibers prepared from six different patients which had positive contracture tests for malignant hyperthermia (and no other known muscle disease), and 0.052 ± 0.006 (n = 212) for fibers prepared from ten normal subjects. According to our calibration measurements these mean fluorescence ratios corresponded to myoplasmic [Ca^{2+}] of 0.10 μM.

The estimated resting myoplasmic [Ca^{2+}] was correlated to the length of the fiber segments. For example, the resting [Ca^{2+}] was estimated to be 0.10 μM in the fiber segments from the patient with myotonic dystrophy which were subsequently used for the HPLC assay (Fig. 2). These fiber segments were approximately 4.0 cm long and had resting membrane potentials more negative than –75 mV at the time of the fluorescence measurements. In contrast, in fiber segments of lengths less than 2.0 cm, the mean resting potentials were –60 mV or more positive, and the average 340/380 fluorescence ratio was elevated. The fibers visually appeared deteriorated (e.g. clots) and the resting [Ca^{2+}] was estimated to be elevated to levels between 0.2–0.3 μM.

Effects of halothane and caffeine

Halothane at concentrations below 1.5% induced contracture and an increase in 340/380 fluorescence ratio in long fiber segments from patients susceptible to malignant hyperthermia ($P < 0.001$). These effects were reversible on washout of halothane (Fig. 4). The changes in the calculated [Ca^{2+}] and force were well correlated during halothane exposure. Concentrations of halothane up to 4% did not cause contractures or the mean resting intracellular [Ca^{2+}] to change in normal muscles. When muscle fiber segments from normal subjects, those susceptible to malignant hyperthermia or those with a known neuromuscular disease, were exposed to caffeine concentrations of greater than 4.0 mM they went into contracture and the fluorescence ratios increased (see Fig. 5).

The amplitude of the 340/380 fluorescence ratio was well correlated with the amplitude of the

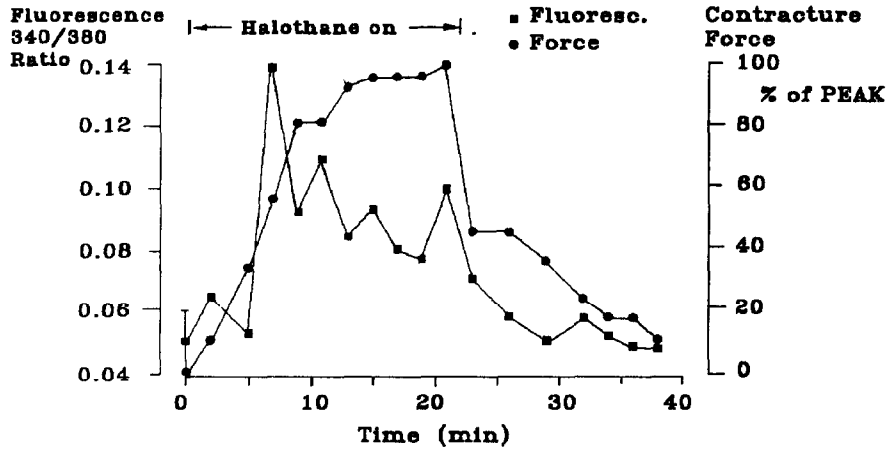

Fig. 4 The effects of halothane on Fura-2 fluorescence ratio and force (%). Time course recorded from fibre segments of vastus medialis from a patient susceptible to malignant hyperthermia. Note that the halothane (1.5%) effects were reversible

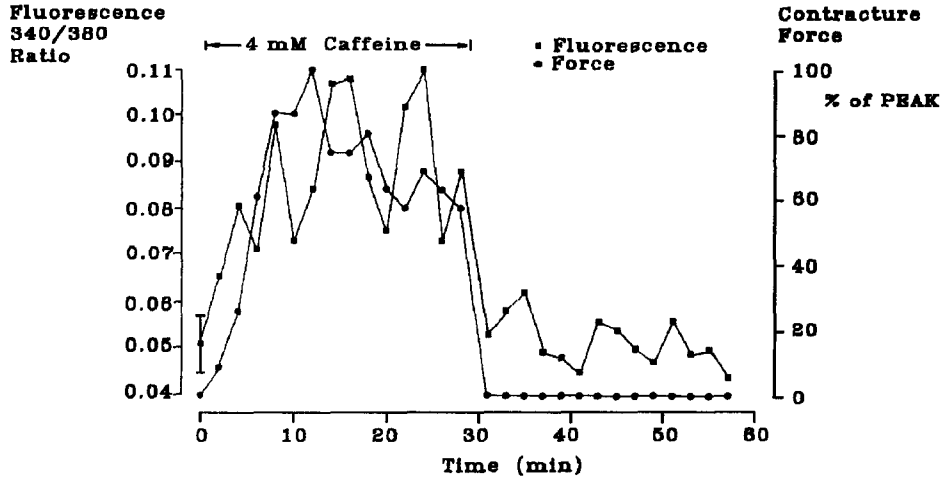

Fig. 5 The effects of caffeine on Fura-2 fluorescence ratio and force (%). Time course recorded from fiber segments prepared from the vastus lateralis muscle of a normal subject. Note that the caffeine (4.0 mM) effects were reversible

contracture. In preparations from both normal humans or those susceptible to malignant hyperthermia, increasing concentrations of caffeine produced a graded increase in both fluorescence and force. It should be noted that the recorded contractures were the summed response of all fibers within a preparation, whereas the intracellular [Ca^{2+}] was measured only in a small percentage of fibers.

Discussion

Fura-2 was found to be well suited for qualitative investigations of resting myoplasmic free [Ca^{2+}] and changes in concentrations induced by various drugs. Although Fura-2 has been shown to have limited value as an indicator for absolute [Ca^{2+}] in certain cell types [7, 11], the results of the present study support the idea that Fura-2 may also be used as a quantitative calcium indicator in skeletal muscle. In both intact and long fiber segments from human

skeletal muscle Fura–2 AM was easily loaded into the cells and rapidly hydrolyzed to Fura–2 which remained trapped in high concentrations. The Fura–2 was fairly uniformly distributed along the total length of both the intact and cut fibers, with slight accumulations of the dye at the very ends of the transsected fibers. As reported earlier, Fura–2 did not appear to have any toxic effects on these muscle preparations [9]. Nevertheless, as was recently suggested [9], in skeletal muscle the most dependable use of Fura–2 as a calcium indicator may be in a qualitative fashion (e.g. to study the correlation between isometric force and changes in the fluorescence ratio).

It was previously reported that the presence of Fura–2 AM and metabolites other than Fura–2 in the cell will make the measurement of changes in intracellular [Ca^{2+}] by the Fura–2 ratio method difficult [11]. However, this difficulty should be of minor importance in human skeletal muscles, because the ratio between Fura–2 and the other forms of the dye within a given cell is relatively large. In contrast, this difficulty may be a concern in muscles that are affected by disease. We observed that the extent of loading and hydrolysis was impaired in certain preparations which were known to be diseased.

The findings of the experiments which compared the resting [Ca^{2+}] in long muscle fiber segments from normal humans or those susceptible to malignant hyperthermia confirmed our previous finding in which intact muscle fibers from normal or susceptible pigs were studied [9]. Once again this result is not in agreement with those of Lopez and co-workers who reported elevated [Ca^{2+}] at rest in muscle fibers from either humans or swine which were susceptible to malignant hyperthermia [19, 20]. Recently, Gronert and co-workers [21] have also questioned the results of Lopez et al. [20]. They suggested that it would be somewhat paradoxical to find increases in intracellular [Ca^{2+}] at rest without any particular increase in basal metabolism, such as that proposed by Williams [22]. Furthermore, if myoplasmic [Ca^{2+}] is elevated at rest in muscle from patients susceptible to malignant hyperthermia, then one might expect that dystrophic changes of the muscles whould be evident. This prediction is based on the suggestions of Bodensteiner and Engel [1] that intracellular calcium overloading may be an important mechanism of muscle degeneration, and the findings of Duncan [2] who demonstrated that when intracellular [Ca^{2+}] was artificially elevated, by either treatment with an ionophore or caffeine, myofilament degradation quickly followed. This idea was also supported by our previous findings that the myoplasmic [Ca^{2+}] at rest was elevated in both intact and fiber segments obtained from a patient who was diagnosed to have symptoms characteristic of Schwart-Jampel Syndrome and who had muscle which showed histological evidence of dystrophic changes [6]. However, the contrary is true for malignant hyperthermia, abnormalities of muscle histology are considered rare and unspecific [23].

It was noteworthy to determine that long muscle segments are suitable for the study of myoplasmic [Ca^{2+}]. Reversible increases in myoplasmic [Ca^{2+}] and contractile force were regularly observed in such preparations. This is of particular interest for the study of skeletal muscles from patients who would be placed at risk if an intercostal muscle biopsy were to be removed for the in vitro study of intact muscle fibers (i.e. patients with myotonic dystrophy and those susceptible to malignant hyperthermia).

We conclude that Fura–2 can be successfully used to estimate the resting myoplasmic [Ca^{2+}] in normal and in diseased human skeletal muscle. Myoplasmic [Ca^{2+}] was elevated in myopathies with associated dystrophic changes, but not in muscle susceptible to malignant hyperthermia. Fura–2 AM is rapidly converted to the Ca^{2+} binding form of the dye which remained within the cell at a very high concentration for several hours. Thus, it is possible to use this indicator to quantitatively determine myoplasmic [Ca^{2+}] in human skeletal muscle. Finally, because contractures induced in either normal (caffeine) or MHS (caffeine and halothane) intact muscle fibers were clearly correlated with increases in myoplasmic Ca^{2+}, these methods could be applied to the study of other drugs on myoplasmic free [Ca^{2+}].

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Please send reprint requests to : Dr P.A. Iaizzo, Department of Anesthesiology, Mayo Clinic, Rochester, MN 55905, USA

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