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# Fura-2 detected myoplasmic calcium and its correlation with contracture force in skeletal muscle from normal and malignant hyperthermia susceptible pigs

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**Abstract.** Fura-2 was used to estimate myoplasmic $[Ca^{2+}]$ in intact intercostal muscle fibers from normal and malignant hyperthermia susceptible (MHS) pigs. Small muscle bundles (20–50 fibers) were loaded with the membrane-permeant form of the dye. Resting myoplasmic $[Ca^{2+}]$ were not significantly different in normal and MHS muscles. Halothane produced increases in myoplasmic $Ca^{2+}$ with associated contractures in MHS muscles, but not in normal muscles. These halothane effects were reversible. Caffeine produced increases in myoplasmic $Ca^{2+}$ and contractures in both MHS and normal muscles. The threshold concentrations were lower in the MHS muscles. The correlations between myoplasmic $[Ca^{2+}]$ and force in MHS and normal muscles were similar.

**Key words:** Intercostal muscle fibers – Resting $[Ca^{2+}]$ – Halothane – Caffeine – Fluorescence

## Introduction

Episodes of malignant hyperthermia are characterized by hypermetabolism and stiffness of the skeletal muscle. They can be observed in predisposed humans and animals exposed to triggering agents such as halothane or succinylcholine. Biopsied muscle from either humans or animals susceptible to malignant hyperthermia, but not normal humans or animals, go into contracture when exposed to low concentrations of caffeine (< 2.5 mM) or halothane (< 2.5%). Abnormal intracellular calcium regulation has been proposed as the primary defect in malignant hyperthermia susceptible (MHS) muscle (Greaser et al. 1969; Britt 1979; Gronert 1980). Several observations have supported this contention: (1) the low contracture threshold of MHS muscle to caffeine (Nelson et al. 1975), (2) functional defects in calcium regulation found in isolated vesicles of the sarcoplasmic reticulum (Gronert et al. 1979; Endo et al. 1983; Nelson 1984; Ohnishi et al. 1983, 1986); and (3) a possibly elevated intracellular $Ca^{2+}$ activity in resting intact MHS muscle fibers of human and swine (Lopez et al. 1985, 1986).

Fura-2 is a $Ca^{2+}$ specific fluorescent chelator that has already been used as a $Ca^{2+}$ indicator in a number of cell types (Almers and Neher 1985; Williams et al. 1985; Oakes et al. 1987). One of the main advantages of fura-2 is that it can be easily loaded into the certain cell types without injection (Tsien 1981). Once the membrane-permeant ester form of fura-2 is inside the cell it is hydrolyzed and supposedly trapped. On excitation at two different wavelengths, 340 and 380 nm, the dye fluoresces and the 340/380 fluorescence ratio is used to estimate the intracellular $[Ca^{2+}]$ (Grynkiewicz et al. 1985). Thus, the measurement is independent of the dye concentration and small movements of the preparation do minimally affect the results.

The present experiments were designed to estimate the myoplasmic $[Ca^{2+}]$ in MHS and normal muscles under control conditions and during the application of either halothane or caffeine. Simultaneously the contracture force of the muscle bundles was monitored.

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## Materials and methods

Biopsies of the external intercostal muscle (5th, 6th, or 7th intercostal space) were taken from 9 MHS and 6 normal German Landrace swine. The muscle specimens were removed while the animals were anesthetized with thiopental (Hormon-Chemie, München, FRG). To verify these animals as MHS or normal, halothane-caffeine contracture tests were performed on several intact muscle bundles according to the protocol of the European Malignant Hyperpyrexia Group (1984; see Table 1).

For the measurement of myoplasmic $[Ca^{2+}]$ or resting membrane potentials very thin sheets of 20–100 fibers intact from tendon to tendon were dissected and suspended in a plexiglas chamber (4.0 ml). Connective tissue appeared to be auto-fluorescent and thus was removed. For the measurement of isomeric 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 $35.0 \pm 0.5°C$ 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 $NaHCO_3$, 11.0 mM glucose. The pH was adjusted to 7.4 by gassing this solution with a mixture of 95% $O_2$ and 5% $CO_2$. Halothane was bubbled through the bath via a fluothane vaporizer (Fluotec 3, Cyprane Ltd., Keighly, GB) and actual halothane bath concentrations were determined as described by Van Dyke and Wood (1973). 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 s.

Muscle fibers were loaded with fura-2-AM (Molecular Probes, Eugene, OR, USA) by bathing them for 30 min at

**Table 1.** In vitro contracture test results

| Animal | Weight (kg) | Caffeine threshold (mM) | Halothane threshold (%) | Resting fura-2 340/380 mean ratios ($\bar{X} \pm$ SD) | Special remarks |
|---|---|---|---|---|---|
| Malignant hyperthermia susceptible | | | | | |
| $S_1$ | 60 | 1.0 | 3.0 (2.0[a]) | $0.029 \pm 0.013$ ($n = 10$) | – |
| $S_2$ | 64 | 1.0 | 1.0 | $0.037 \pm 0.005$ ($n = 8$) | – |
| $S_3$ | 68 | 1.0 | 1.0 | $0.058 \pm 0.014$ ($n = 10$) | deteriorated fibers |
| $S_4$ | 75 | 1.0 | 2.0 (1.0[a]) | $0.065 \pm 0.013$ ($n = 12$) | fought, R.T. |
| $S_5$ | 77 | 0.5 | 0.5 | $0.051 \pm 0.014$ ($n = 20$) | fought, R.T. |
| $S_6$ | 42 | 0.5 | 1.0 (0.5[a]) | $0.051 \pm 0.013$ ($n = 16$) | deteriorated fibers |
| $S_7$ | 24 | 1.0 | 1.5 (1.0[a]) | $0.053 \pm 0.011$ ($n = 30$) | [b] |
| $S_8$ | 30 | 1.0 | 1.0 | $0.043 \pm 0.006$ ($n = 30$) | [b], thin prep. |
| $S_9$ | 35 | 0.5 | 2.0 (0.5[a]) | $0.047 \pm 0.009$ ($n = 30$) | deteriorated fibers |
| | | | MHS group mean = | $0.049 \pm 0.013$ ($n = 164$) | |
| Normal | | | | | |
| $N_1$ | 24 | > 4.0 | > 4.0 | $0.042 \pm 0.010$ ($n = 30$) | [b], thin prep. |
| $N_2$ | 25 | 4.0 | > 4.0 | $0.043 \pm 0.010$ ($n = 16$) | thick prep. |
| | | | | $0.042 \pm 0.009$ ($n = 30$) | 2nd prep., thin |
| $N_3$ | 25 | > 4.0 | > 4.0 | $0.042 \pm 0.008$ ($n = 30$) | [b], thick prep. |
| $N_4$ | 31 | 3.0 | > 4.0 | $0.043 \pm 0.008$ ($n = 30$) | died in surgery |
| $N_5$ | 35 | > 4.0 | > 4.0 | $0.043 \pm 0.010$ ($n = 30$) | – |
| $N_6$ | 45 | > 4.0 | > 4.0 | $0.046 \pm 0.010$ ($n = 30$) | thick prep. |
| | | | Normal group mean = | $0.043 \pm 0.009$ ($n = 186$) | |

[a] Smaller than a 200 mg contracture. [b] Nearly all fibers with visible striations from tendon to tendon. R.T. = rectal temperature elevated

35° C in a solution containing the membrane-permeant ester of the dye ($2.0 \times 10^{-7}$ 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 AC and housed in a Zeiss 100 lamp housing; Osram HB0 50, München, FRG) passed through a locally constructed trigger unit, UV interference filters (Zeiss, center wavelengths of 340 or 380 nm; halfwidths 10 nm), a beam splitter (Zeiss FT425), into the microscope objective (Zeiss Achromat 40X water immersion; n.a. 0.75) and hence into 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 in the light path limited the region of the preparation from which fluorescence was collected to a circular area of 40 μm (Almers and Neher 1985). 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.

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 1½–2 min; 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. If a bundle was too thick it was more difficult to inspect the whole length of an individual fiber. When a spontaneous contracture developed under resting conditions (i.e. 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 with two different $Ca^{2+}$ buffering systems. One set of solutions contained only EGTA (see Allen et al. 1977), and the other set was made with several different $Ca^{2+}$ buffers with differing pH values (see Tsien and Rink 1981). A very small amount, 0.4 μg, of fura-2 penta-potassium salt (Molecular Probes, Eugene, OR, USA) was added to 250 μl of each calibration solution which was placed inside an opaque cup (250 μl) mounted inside the experimental chamber. The fluorescence amplitude from this amount of dye was in the same range of amplification as that in the actual experiments. All calibration measurements were made at $35.0 \pm 1.0$° C. When tested at several $[Ca^{2+}]$ up to $10^{-4}$ M the fluorescence ratios obtained with the two buffer systems were not statistically different. A cubic spline interpolated calibration curve of our indicator system is shown in Fig. 1.

Resting membrane potentials were measured as described by Lehmann-Horn et al. 1981. Statistical differences were determined using Scheffé's multiple contrast (nonparametric) test.

## Results

### *Resting $[Ca^{2+}]$*

The mean 340/380 fluorescence ratio measured from MHS muscle was $0.049 \pm 0.013$ ($\bar{X} \pm$ SD; $n = 164$) and this value was not significantly different ($p > 0.25$) from that of normal muscle which was $0.043 \pm 0.009$ ($n = 186$). Accord-

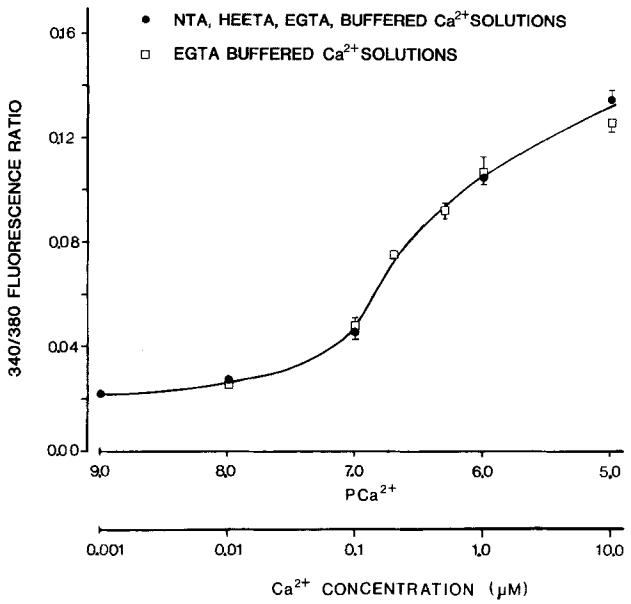



**Fig. 1.** Relationship between fura-2 (penta-potassium salt) 340/380 fluorescence ratio and $[Ca^{2+}]$. Two different $Ca^{2+}$ buffering systems were used to produce two sets of calibrating solutions (□ only EGTA; ● several chelators given in the key). The *fitted curve* is a result of a cubic spline interpolation. Symbols represent $\bar{X} \pm SD$

ing to the calibration measurements these values correspond to intracellular $[Ca^{2+}]$ of 0.10 μM and 0.08 μM respectively. Variation in the measured fluorescence ratio, when observed along the length of an individual fiber, was always less than ½ of the total standard deviation of all measurements from that preparation. 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 were still between −75 and −85 mV.

Figure 2 shows histograms of the mean fluorescence ratios measured in 9 MHS and in 6 normal muscles: (1) under resting conditions; (2) during exposure to halothane; (3) during exposure to caffeine; and (4) after the washout of caffeine. The fluorescence ratios under resting conditions were very similar for all normal muscles, but quite variable for the MHS muscles. Yet, the standard deviation was within a given preparation similar for each group. From one normal animal (N2), two preparations were studied and identical intracellular $[Ca^{2+}]$ were estimated. In 3 MHS preparations the mean fluorescence ratios were actually less than those determined from normal muscle. In two MHS animals the core temperature was elevated at the time of biopsy (see Table 1), which may suggest that these animals were in the initial stage of an MH episode at the time of surgery (however no muscle stiffness was observed).

*Effects of halothane*

Whenever MHS muscles were exposed to halothane at concentrations below 1.5% they went into contracture and the 340/380 fluorescence ratio increased ($p < 0.001$). These effects were reversible on washout of halothane (Figs. 2 and 3). The changes in the calculated $[Ca^{2+}]$ and force correlated very well during the presence or halothane ($n = 6$). To emphasize the reproducibility of this correlation, the time course of halothane administration in 2 different MHS preparations is shown in Fig. 3; note different rates at which the halothane effects reversed. The estimated mean intracellular $[Ca^{2+}]$ during halothane application was 0.60 μM $Ca^{2+}$ (fluorescence ratio of 0.092 ± 0.022; $n = 66$) compared to 0.10 μM $Ca^{2+}$ under resting conditions. After halothane was discontinued and the experimental chamber was flushed the mean intracellular $[Ca^{2+}]$ returned to 0.13 μM (fluorescence ratio of 0.059 ± 0.19; $n = 68$) and this was not significantly different from control ($p > 0.10$). However, the contracture often took 10–15 min to decrease to 10% of the maximum amplitude (see Fig. 3). The mean intracellular $[Ca^{2+}]$ when contracture amplitude was 10% of maximum or less, was the same as the control value, 0.01 μM (fluorescence ratio of 0.015 ± 0.015; $n = 26$).

When normal muscle was exposed to halothane up to 4%, no contractures were observed and the mean resting intracellular $Ca^{2+}$ was not altered. The mean estimates were 0.08 μM $Ca^{2+}$ (fluorescence ratio of 0.043 ± 0.009; $n = 186$) before halothane and 0.07 μM $Ca^{2+}$ (fluorescence ratio of 0.042 ± 0.012; $n = 22$) during application of halothane (see Fig. 2).

*Effects of caffeine*

When MHS or normal muscle bundles were exposed to caffeine at concentrations of > 4.0 mM they went into contracture and fura-2 signals changed (see Fig. 2). At 2.0 mM caffeine, only MHS muscle went into contracture. This force development was always well correlated with increased intracellular calcium. In normal fibers the mean fluorescence ratio was the same at rest and in 2.0 mM caffeine (rest, 0.043 ± 0.009; 2 mM caffeine, 0.044 ± 0.008). The mean 340/380 fura-2 ratios during exposure to the various concentrations of caffeine are shown in Fig. 4 and these values were used to estimate the following $[Ca^{2+}]$: 0.29 μM $Ca^{2+}$ ($n = 47$) for MHS fibers and 0.08 μM $Ca^{2+}$ ($n = 31$) for normal fibers in 2 mM caffeine; 0.44 μM $Ca^{2+}$ ($n = 63$) for MHS and 0.16 μM $Ca^{2+}$ ($n = 47$) for normal fibers in 4 mM caffeine; 0.35 μM $Ca^{2+}$ ($n = 43$) for MHS and 0.37 μM $Ca^{2+}$ ($n = 47$) for normal fibers in 8 mM caffeine; and 0.62 μM $Ca^{2+}$ ($n = 35$) for MHS and 0.37 μM $Ca^{2+}$ ($n = 41$) for normal fibers in 16 mM caffeine. Figure 4 shows not only the shift of the mean 340/380 fluorescence ratios in MHS compared to normal fibers at each caffeine concentration, but also a similar shift in contracture development; the values for the MHS fibers were shifted to the left (lower caffeine concentrations). The mean ratios were statistically different between the normal and MHS muscle at 2.0 and 4.0 mM caffeine ($p < 0.001$). A fluorescence ratio equal to or greater than 0.062 (0.16 μM $Ca^{2+}$) was correlated with the occurrence of a contracture.

The intracellular $[Ca^{2+}]$ from all measurements taken after caffeine was flushed from the chamber were 0.12 μM (fluorescence ratio of 0.057 ± 0.019; $n = 39$) for MHS muscle and 0.10 μM (fluorescence ratio of 0.049 ± 0.011; $n = 51$) for the normal muscle. These values were not significantly different from the resting ratios ($p > 0.25$). The contracture often took 10–15 min to decrease to 10% of the maximum amplitude. The mean 340/380 fluorescence ratios, when contracture amplitudes were 10% of maximum or less, were close to the values recorded under control conditions.

The amplitude of the 340/380 fluorescence ratio was well correlated with the amplitude of the contracture. In some preparations, both from normal and MHS animals, in-

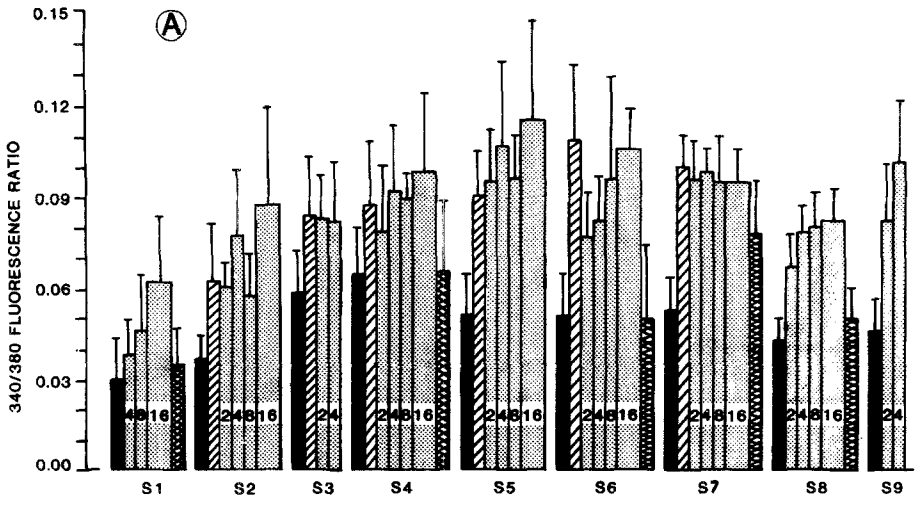
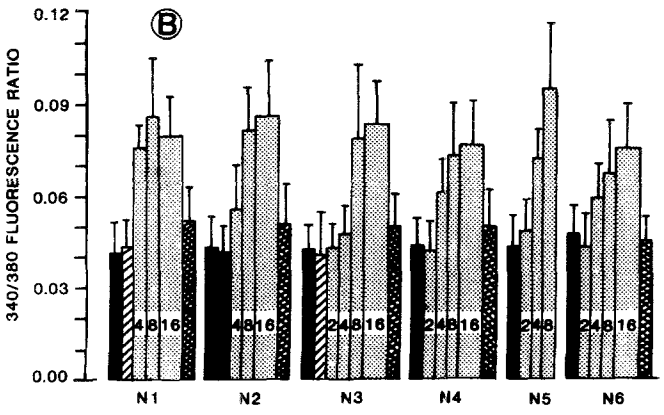



**Fig. 2A, B**
Mean fura-2 340/380 fluorescence ratios at rest ■, during halothane ▨ or caffeine administration ▩, and following washout ▧. Halothane caused an increase in fluorescence ratio only in MHS muscle. Caffeine concentrations of 2.0, 4.0, 8.0 and 16.0 mM were used (as indicated on the *bars*). Caffeine produced a somewhat graded increase in fluorescence in both normal and MHS muscle. Only caffeine concentrations greater than 2.0 mM produced an increase in the normal fibers (**A**, MHS animals; **B**, normal animals; $\bar{X} \pm$ SD; the labels $S_1 - S_9$ and $N_1 - N_6$ are the same as in Table 1)



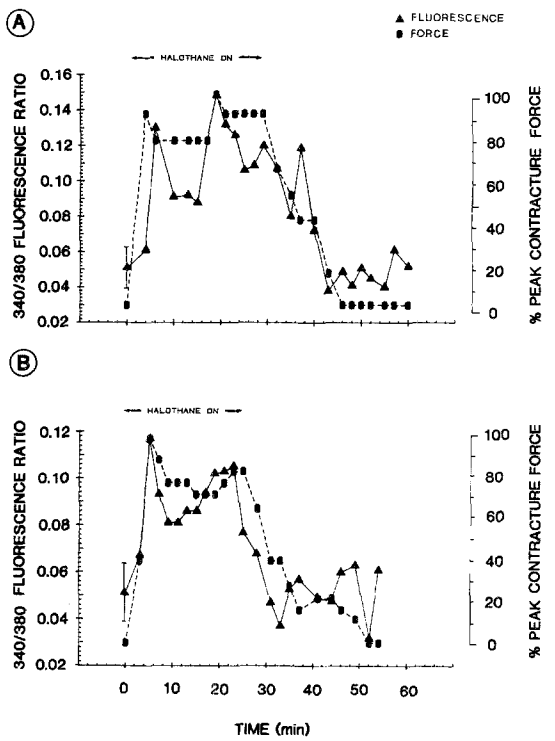




**Fig. 3A, B.** The effects of halothane on fura-2 fluorescence ratio and force (%). **A** Time course recorded from animal $S_6$; **B** time course from animal $S_7$. Note that the halothane (1.5%) effects were reversible

creasing concentrations of caffeine produced a graded increase in both fluorescence ratio and force (Fig. 5). However, in some preparations the largest mean fluorescence ratios and peak contracture forces occurred at caffeine concentrations less than 16.0 mM. Yet, in most preparations a good correlation between fluorescence and force remained (Fig. 5). Only in MHS preparation $S_1$ upon caffeine exposure was the increase in the intracellular $[Ca^{2+}]$ much less than the elicited contracture. It should be noted, that a recorded contracture was the summed response of all fibers within a preparation, whereas the intracellular $[Ca^{2+}]$ was estimated only in a small percentage of fibers. It is possible that in preparation $S_1$ the mean myoplasmic $[Ca^{2+}]$ during caffeine exposure included estimates from fibers which had not gone into a state of strong contracture.

## Discussion

It has long been supposed that contractures of MH muscle are due to an increase in myoplasmic calcium (Greaser et al. 1969; Nelson et al. 1975; Gronert 1980; Ohnishi et al. 1983, 1986). Our study verifies this assumption by estimating myoplasmic calcium correlating it to contracture force. Reversible contractures induced in normal muscle by caffeine or in MHS muscle by caffeine or halothane were clearly correlated with an increase in myoplasmic free $Ca^{2+}$. When the contracture amplitude increased or decreased so did the fluorescence ratio. MHS muscles are known to have a higher sensitivity to caffeine. Correspondingly, 2.0 mM caffeine caused an increase in the myoplasmic $[Ca^{2+}]$ and the development of contractures only in MHS muscle. These findings are consistent with reports that the sarcoplasmic reticulum isolated from MHS muscle has a higher sensitivity to these substances (Endo 1983; Ohnishi et al. 1983, 1986).

The determination of absolute myoplasmic free-$Ca^{2+}$ by the use of fura-2, like other calcium indicators, may have limitations (some known and others yet to be identified; see Almers and Neher 1985; Oakes et al. 1987). In these intact skeletal muscle fibers, fura-2 was easily loaded, remained trapped (no noticeable loss of dye after 3 h), and was fairly uniformly distributed along the total length of the fiber. Fura-2 did not appear to have a toxic effect on these muscle bundles: (1) resting membrane potentials were between $-75$ and $-85$ mV after the fluorescence measurements; (2) the optical appearance of the individual fibers

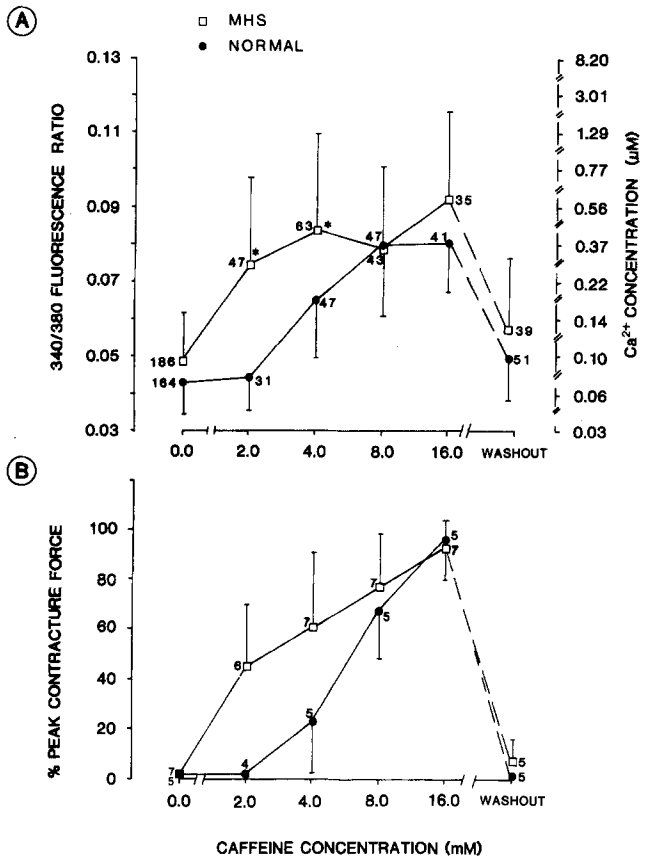



**Fig. 4.** Effects of increasing caffeine concentrations on the fura-2 fluorescence ratio (**A**) and contracture force (**B**). No change in fluorescence or force was observed when normal fibers were exposed to 2.0 mM caffeine. In addition, when normal fibers were exposed to 4.0 mM caffeine the response was less than that observed for the MHS fibers exposed to 2.0 mM caffeine (* = statistically different at $p < 0.001$)

remained the same; (3) spontaneous contractures were very rare; and (4) at the end of the experimental protocol similar resting fluorescence ratios were obtained. Nevertheless, the use of fura-2 in a quantitative sense may be the most dependable, e.g., in the correlation between isometric force and the fluorescence ratio. Monitoring force allowed us to observe the time course of the halothane and caffeine induced contractures, and it was useful as an indicator of the condition of the muscle bundle in normal solution (in addition to the optical inspection of each fiber). On the other hand, if one assumes the calibration curve to be valid, then the correlation between these parameters may also provide an estimate as to how much $Ca^{2+}$ is needed for the activation of contraction. The results from this study, would indicate that a myoplasmic free $[Ca^{2+}]$ corresponding to the mechanical threshold is between 0.08 and 0.16 μM, which is similar to those reported by others (for a review on this topic see Lüttgau and Stephenson 1986). However, it should be re-emphasized that all $[Ca^{2+}]$ cited within this report are at best, only estimates of the true $[Ca^{2+}]$ present in the fibers.

In terms of resting $[Ca^{2+}]$ in MHS muscle our findings do not uniformly support those of others. The resting myoplasmic $[Ca^{2+}]$ of MHS fibers was not found to be statistically different from that of normal fibers (qualitatively or quantitatively). This is in disagreement with the reports of Lopez and co-workers, who used $Ca^{2+}$-selective microelectrodes to determine resting levels and observed elevations in the MHS fibers. They report resting $[Ca^{2+}]$ in MHS fibers that were comparable to those we observed during contractures. However, with the $Ca^{2+}$-selective microelectrodes, the mean resting myoplasmic $[Ca^{2+}]$ in normal fibers was similar to that determined with fura-2. There are several possible explanations for the discrepancy in MHS fibers, such as: (1) the conditions of the preparations were different in the two studies; (2) the $Ca^{2+}$-selective electrodes may traumatize the cell membranes to the extent that an MH stress response is initiated; (3) Fura-2 is less sensitive than the microelectrodes used by Lopez et al. (1985); (4) the number of fibers investigated by Lopez et al. (1985) was too small; and (5) neither method is appropiate for making such measurements. Furthermore, Lopez and co-workers did not verify that the $Ca^{2+}$-selective microelectrode was in the same cell that the resting membrane potential was simultaneously recorded from (see Lopez et al. 1986). A difference of 5–10 mV in resting membrane potentials, which is common from one cell to the next, could have resulted in a large difference in the derived $[Ca^{2+}]$.

In our study it was often difficult to control the status of the MHS animals prior to the biopsy (especially in the larger animals); extreme activity of MHS animals prior to the surgery (elevated core temperature) appeared to be correlated with a slightly higher resting $[Ca^{2+}]$. Furthermore, the biopsy and dissection procedures may be more traumatic to the MHS fibers, as they have been reported to be more injury sensitive (Nelson and Flewellen 1979; Gallant et al. 1986). In some MHS preparations, myoplasmic calcium was elevated, but this may be a response to the invasive procedure, due to the impaired $Ca^{2+}$ regulation within MHS muscle.

We conclude that the resting myoplasmic $[Ca^{2+}]$ in normal and MHS swine are not different until the muscle has undergone some degree of stress or trauma. In addition, contractures induced in either normal (caffeine) or MHS (caffeine and halothane) intact muscle fibers are clearly correlated with increases in myoplasmic $Ca^{2+}$.

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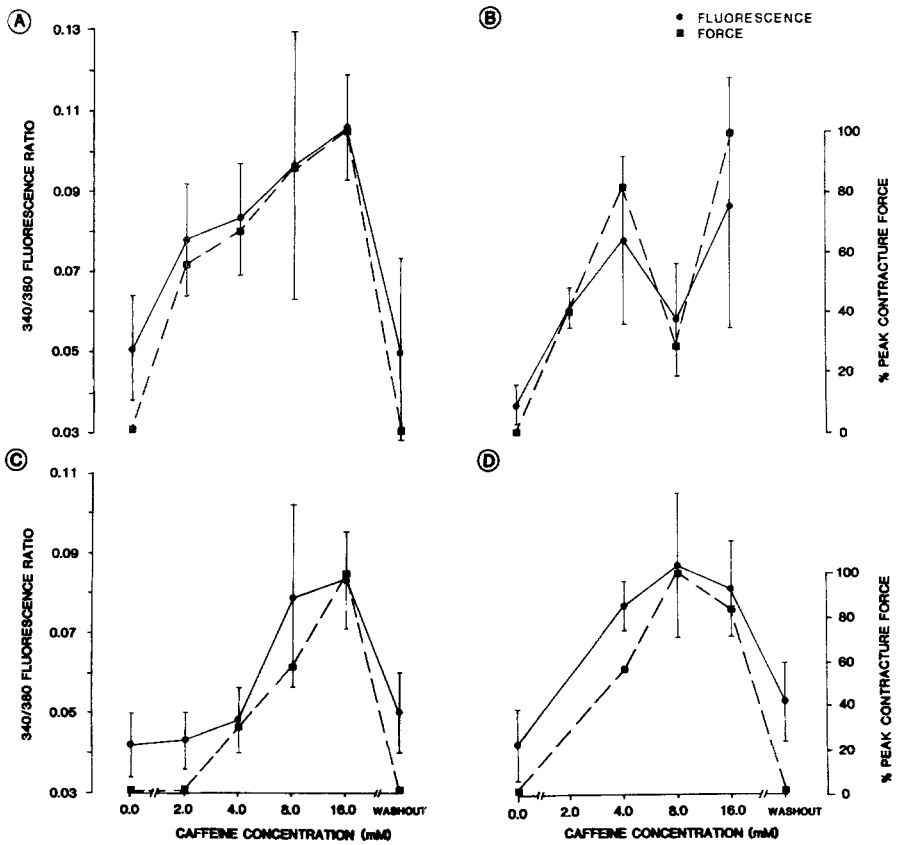


**Fig. 5A–D**
Dependence of mean fura-2 fluorescence ratio and contracture force on increasing caffeine concentrations. Shown is this dependence for two MHS muscles [(**A**) and (**B**)] and two normal muscles [(**C**) and (**D**)]. Note the good correlation between the mean fluorescence ratios and force


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