...ORMAL CALCIUM REGULATION IN CULTURED CELLS FROM NORMAL AND HALOTHANE ...CEPTIBLE PIGS

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...MARY

The effect of caffeine on intracellular calcium concentration was monitored in primary cultures of muscle cells from normal pigs and ...e from halothane susceptible (MHS) pigs. Calcium variations were estimated by recording changes in fluorescence ratio obtained on dual ...ation at 340 and 380 nm of myotubes loaded with the Ca^{2+} fluorescent probe Fura-2. Myogenic precursors were satellite cells obtained ... Masseter muscle of adult pigs previously tested for their sensibility to halothane. The cells were cultured until differenciation of ...nucleated myotubes. Measurements of fluorescence ratio were performed at room temperature in the absence or in the presence of 1 to 6 ... caffeine.

The resting fluorescence values were not significantly different in normal and MHS cells. Caffeine induced an increase in fluorescence ...in both types of cells. The magnitude of this increase was commonly higher in myotubes from MHS pigs for caffeine concentration of 1 ...mM. This result indicates an abnormal Ca^{2+} homeostasis in cultured cells from halothane susceptible pigs which may be a manifestation ...e lesion that causes Malignant Hyperthermia. Satellite cells isolated from muscle tissue could therefore provide a good model for ...stigation on sarcoplasmic calcium regulation.

...RODUCTION

The occurence of susceptibility to halothane in swine frequently leads to the development of meat of poor quality (PSE meat). The ...ted animals are also susceptible to develop Malignant Hyperthermia (MH) and have been largely used as a model for the study of the ...ar human syndrom.The molecular basis of MH is still poorly understood although the calcium release channel of the sarcoplasmic ...ulum (ryanodine receptor) has been shown to be modified in pig species (Fujii *et al.* 1991, Mac Lennan and Phillips 1992). On biopsied ...le from MH susceptible (MHS) individuals, a contracture can be initiated by low concentration of halothane or caffeine. This contracture ...related to an abnormal intracellular calcium regulation (Iaizzo *et al.* 1988). Altered calcium homeostasis has been reported in cells from ...r tissues (Iaizzo *et al.* 1991, Fink *et al.* 1992) but it would be of interest to investigate the expression of this alteration in developping ...e cells derived from MH individuals in order to relate it with the differenciation of the ryanodine receptor.

The aim of this study was to determine whether an abnormal regulation of intracellular calcium could be evidenced in cultured MH ...ptible pig muscle cells. We estimated and compared the calcium mobilization induced by caffeine in myotubes from MHS and normal ...s.

...ERIAL AND METHODS

Masseter muscles were obtained from 4 Pietrain pigs of 2 month old. The pigs have been tested by the halothane test (Olivier *et al.* ...), four pigs positive to the test (MHS pigs) and four negative (MHN pigs) were used. Just after stunning and exsanguination, the ...les were excised and immediately soaked in sterile phosphate buffered saline (PBS ; pH7.2) supplemented with 100 µg/ml gentamycine.

...ation of satellite cells : Muscles were washed with sterile PBS to remove potential surface contamination. Approximately 2 g of the ...le were dissected and minced in small pieces. For enzymatic dissociation, the samples were incubated for 2 hours at 37°C in a shaking ...r bath with DMEM (SIGMA Chemical Co.,USA), 10% FCS (foetal calf serum, GIBCO, UK), 0.15% protease (type XXV, pronase E,

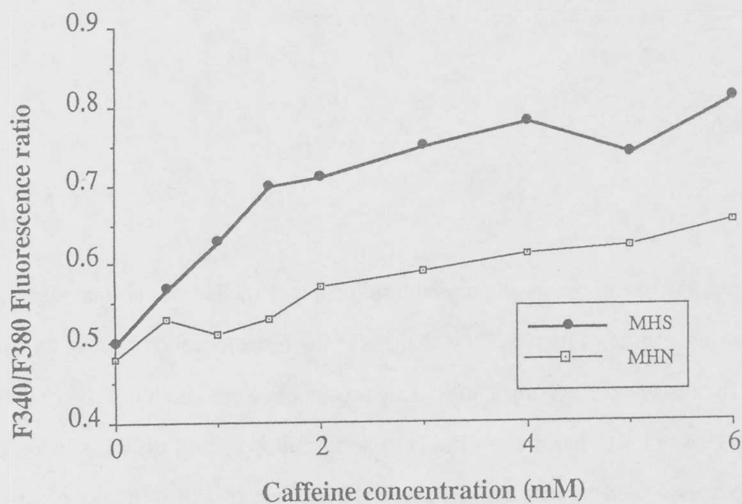

Figure 1: Typical effect of increasing caffeine concentration on the fluorescence ratio in myotubes from MHS and MHN pigs. Each point is the peak value obtained within the time needed for reaching a steady state level (2 to 5 min after the application of the drug).

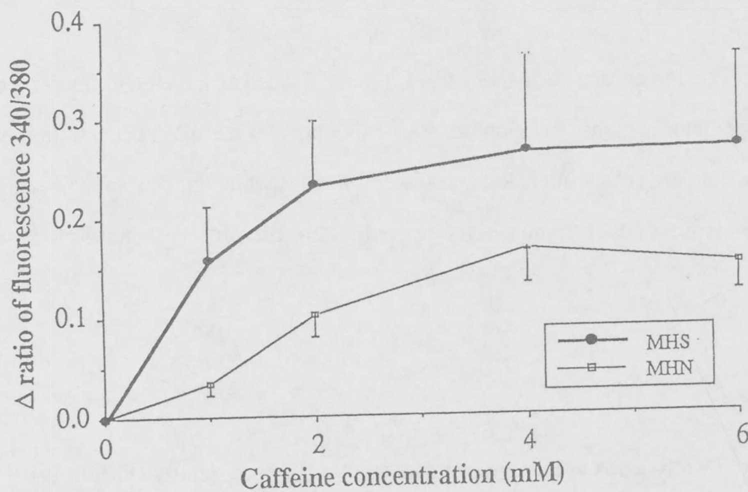

Figure 2: Effect of caffeine on mean augmentation in fluorescence ratio in cells from the 4 MHN and the 4 MHS pigs of this study. Augmentations are greater in MHS and the curve with these animals separates well from the MHN population. (Means ± SE).

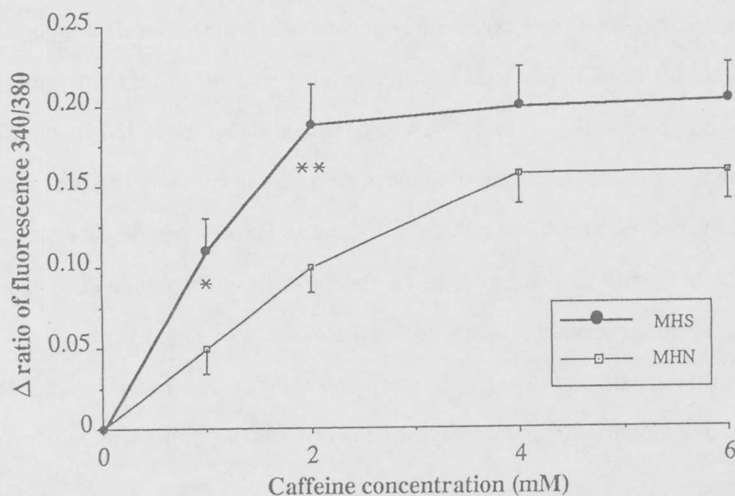

Figure 3: Means of Δ fluorescence ratio in MHN myotubes (n=20; Means ± SE) and MHS myotubes (n=29; Means ± SE) as a fonction of caffeine in the incubating medium. For 1 and 2 mM caffeine, the augmentation of the ratio is significantly higher in MHS cells (* = p< 0.05 , ** = p< 0.01).

PIG #	n	Caff 0mM	Caff 1mM	Caff 2mM	Caff 4mM	Caff 6mM
MHN 1	5	0.46 ± 0.01	0.47 ± 0.01	0.55 ± 0.03	0.71 ± 0.04	0.69 ± 0.04
MHN 2	5	0.49 ± 0.03	0.51 ± 0.02	0.55 ± 0.03	0.59 ± 0.03	0.60 ± 0.03
MHN 3	8	0.58 ± 0.02	0.64 ± 0.05	0.68 ± 0.03	0.72 ± 0.03	0.72 ± 0.02
MHN 4	2	0.57 ± 0.03	0.60 ± 0.02	0.73 ± 0.06	0.74 ± 0.04	0.69 ± 0.02
mean MHN	20	0.52 ± 0.06	0.55 ± 0.08	0.63 ± 0.09	0.69 ± 0.07	0.67 ± 0.05
MHS 1	9	0.46 ± 0.03	0.60 ± 0.05	0.67 ± 0.05	0.67 ± 0.05	0.68 ± 0.05
MHS 2	13	0.50 ± 0.02	0.54 ± 0.02	0.66 ± 0.04	0.67 ± 0.03	0.67 ± 0.03
MHS 3	5	0.61 ± 0.01	0.76 ± 0.05	0.74 ± 0.03	0.74 ± 0.04	0.76 ± 0.03
MHS 4	2	0.62 ± 0.01	0.92 ± 0.02	1.04 ± 0.06	1.17 ± 0.07	1.16 ± 0.08
mean MHS	29	0.55 ± 0.03	0.70 ± 0.17	0.78 ± 0.18	0.81 ± 0.24	0.82 ± 0.23

Table 1: Fluorescence ratio recorded from myotubes loaded with Fura-2 and exposed to graded caffeine concentration. Values are means ± SD of n myotubes.

MA, USA) and 0.05% collagenase (type V, SIGMA, USA). Following incubation, the cell suspension was filtered through four layers eesecloth, then centrifuged at 1500 g during 10 min. The harvested satellite cells were resuspended in DMEM with 10% FCS and 10^{-6}M methasone (SIGMA, USA), and washed 3 times in this medium.

culture : Cells were seeded in gelatinised Petri dishes with DMEM, 20% FCS, 10^{-6}M dexamethasone and 50 µg/ml gentamycine at 10^5cells/ml for multiplication.When cells reached sub-confluence, they were trypsinated and a part of them was seeded in silicon wells gelatinised glass slides (flexiperm, Heraeus) at 10^5 cells/ml. At confluence, the proliferation medium was replaced by fusion promoting ium : DMEM with 2% horse serum (GIBCO, UK), 10 µg/ml insulin (SIGMA, USA) and 5 µg/ml transferin (siderophilin, SIGMA, A). Myoblasts ceased dividing and fused to form multinucleated myotubes in 3 to 7 days after exposure to fusion medium.

loading with Fura-2/AM : Cells were incubated with 10 µM Fura-2/acetoxy methyl ester (Fura-2-AM; SIGMA, USA) and 0.02% onic F 127 in Hepes buffered basal medium Eagle (BME) without phenol red (SIGMA, USA). The incubation time ranged from 35 to 45 at room temperature, in darkness. Cells were then rinsed in BME during at least 30 min before fluorescence measurements.

rofluorimetry : The fluorescence was recorded using an epifluorescence microscope equiped for dual excitation fluorescence surement (Olympus IMT2-OSP3). Two excitation wavelengths were used : 340 nm and 380 nm, and emission at 510 nm was measured y 0.1 sec. An average on 1 sec of the fluorescence ratio (F_{340}/F_{380}) was recorded every 5 sec. The relation between the fura-2 rescence ratio and the calcium concentration was estimated using various calcium buffering systems.

pinhole in the light pass of the microscope limited the region of the preparation to a circular area of about 15 µm from which fluorescence collected. The effect of caffeine (0 to 6 mM) was tested by perfusing the recording solution in the incubating chamber. Increasing eine concentrations were applied every 2 to 5 min.

ULTS

Satellite cells reached confluence in 2 to 4 days. The first myotubes appeared within 3 days after switching in differenciation medium. otubes developed on a large proportion of non fusing cells. The rate of fusion was variable from one animal to another and from one e well to another. We usually obtained between 10 and 30% of fused nuclei in our cultures. All data shown in this study were recorded multinucleated myotubes.

In myotubes from both normal and MHS pigs, increasing concentrations of caffeine generally induced a graded increase in rescence ratio. However, this increase was commonly higher in MHS myotubes (Fig.1). Table 1 shows the mean fluorescence ratios sured in cells from the MHS and MHN groups of pig without caffeine and after the addition of the drug. Under resting conditions feine 0 mM), the ratios were not significantly different ($p > 0.13$). During exposure to low concentration of caffeine, the ratios increased tended to be higher in MHS groups but the values were not statistically different between the two groups ($p>0.09$ and $p>0.07$ at 1 and 2 caffeine respectively). At higher concentration of caffeine, the ratios were not significantly different between the two groups ($p>0.25$). e more pronounced augmentation of fluorescence ratios in MHS pigs is illustrated in Fig.2. The plotted values represent the shift of rescence ratio measured after exposure to caffeine compared to the resting fluorescence. The shift was higher in MHS group at every feine concentration.

When we compared altogether the data obtained for myotubes from MHS pigs with those from MHN pigs, the responses to low centrations were significantly greater in MHS cells ($p<0.05$ at 1 mM and $p<0.01$ at 2 mM) (Fig.3). At higher concentrations (above 2 M), the fluorescence ratios were not significantly different between the two groups.

DISCUSSION

The satellite cells are a source of myogenic cells obtainable from adult animals and this is of great interest in the case of study on MH. We used Masseter muscle because the yield in satellite cells obtained was better as compared to *Longissimus dorsi* muscle for instance. Moreover, the carcass depreciation is minimized when removing only this muscle. The problem is the high proportion of non-fusing cells contaminating the cultures as already described in the same species (Seewald *et al.* 1991). Here, we could select a precise myotube before measuring calcium variations, contaminating cells did therefore not disturb the analysis.

The resting cytosolic fluorescence in our cultured myotubes was not different between MHS and MHN cells. This is in agreement with the study of Iaizzo *et al.* (1988) on myoplasmic calcium in pig muscle fibers using the same technique for calcium estimation.In our study, it was difficult to control the physiological status of the myotubes and this may account for the very variable responses from one cell to another. The cells exhibiting abnormal high or low fluorescence ratios in the absence of caffeine were discarded.

When the cells were exposed to caffeine at low concentration (< 3 mM), the fura-2 fluorescence changed with a greater amplitude in myotubes from MHS pigs. This may indicate an abnormal regulation of intracellular calcium but does not seem to be assimilable to contracture that occur in muscle fibres. In this case, calcium variation reached 440 nM in response to caffeine (Iaizzo *et al.*1988) whereas our maximum increase would correspond approximately to 30 nM augmentation in calcium concentration according to a titration made on our equipment. At higher caffeine concentration (> 3mM), the fluorescence signal changed to a low extent in MHS cells as well as MHN ones. Caffeine is supposed to interact with internal calcium storage site in skeletal muscle cells (Klein *et al.* 1990). Thus, the caffeine-sensitive storage site in our myotubes seems to be practically depleted after the third application of caffeine whatever the myotube origin. The occurence of a higher increase of calcium in MHS cells in response to caffeine may be associated with genetic abnormalities expressed *in vitro*. Other abnormalities have been described in cultured cells from MH individuals (Wieland *et al.* 1989), but not related to calcium regulation. The expression in culture of altered calcium regulation shows that the mechanism of MH could be searched on this model at different stages of differenciation of the triadic junctions, i.e. before and after ryanodine receptor is set.

Acknowledgments

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