Changes in the mechanism of Ca^{2+} mobilization during the differentiation of $\text{BC}_3\text{H1}$ muscle cells

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Ca^{2+} sequestration and release in $\text{BC}_3\text{H1}$ muscle cells is strongly dependent on the stage of differentiation. In proliferating cells, more than 90 % of the sequestered Ca^{2+} was $\text{Ins}(1,4,5)\text{P}_3$ -sensitive and 25 % was caffeine-sensitive. In differentiated cells, the Ca^{2+} accumulation was 5-fold higher and was InsP_3 -insensitive, but about 60 % of the sequestered Ca^{2+} was caffeine-sensitive. These changes were reversible upon addition of growth stimuli. Similarly, by measuring the intracellular Ca^{2+} concentration in single intact $\text{BC}_3\text{H1}$ cells, it was found that the number of histamine-responsive cells decreased and the number of caffeine-responsive cells increased during muscle cell differentiation. These data indicate that the development of the muscle phenotype in $\text{BC}_3\text{H1}$ myoblasts induces a major rearrangement of the mechanisms for Ca^{2+} mobilization.

INTRODUCTION

The $\text{BC}_3\text{H1}$ cell line, which was cloned from a nitrosoethyl-induced brain tumour in the C_3H mouse strain (Schubert *et al.*, 1974), has been shown to be an excellent model system for muscle cell differentiation. When maintained at subconfluence in the presence of high concentrations of foetal calf serum (FCS) these cells do not express muscle-specific genes, but they can be induced to differentiate, e.g. upon exposure to growth-factor-deficient medium. In these conditions $\text{BC}_3\text{H1}$ cells accumulate a variety of muscle-specific gene products, including: (1) enzymes such as muscle creatine kinase (Schubert *et al.*, 1974; Munson *et al.*, 1982; Olson *et al.*, 1983) and myokinase (Schubert *et al.*, 1974), (2) contractile proteins such as smooth-muscle and sarcomeric α -actin isoforms (Strauch & Reeser, 1989), troponin T and the sarcomeric muscle isoforms of myosin heavy and light chains and α -tropomyosin (Taubman *et al.*, 1989), (3) receptor molecules such as the nicotinic acetylcholine receptor (Patrick *et al.*, 1977; Olson *et al.*, 1984) and insulin receptors (Standaert *et al.*, 1984), and (4) the skeletal-muscle-type dihydropyridine-sensitive Ca^{2+} channels (Caffrey *et al.*, 1987).

Much less is known, however, about the effects of $\text{BC}_3\text{H1}$ cell differentiation on the regulation of the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Cell population measurements indicate that the activation of α_1 -adrenergic receptors (Brown *et al.*, 1984; Ambler & Taylor, 1986) as well as of H_1 -histaminergic receptors (Brown *et al.*, 1986) stimulates the rapid mobilization of intracellular Ca^{2+} . In the latter case there was in addition a large influx of Ca^{2+} into the cells. Imaging of single $\text{BC}_3\text{H1}$ cells revealed that the activation of both types of receptors resulted in oscillations of $[\text{Ca}^{2+}]_i$ (Ambler *et al.*, 1988). These cytosolic Ca^{2+} oscillations are caused by recycling of Ca^{2+} between the cytosol and intracellular Ca^{2+} pools (Berridge & Galione, 1988). Although the intracellular mediators of the oscillations in $\text{BC}_3\text{H1}$ cells are unknown, it was found that the oscillations were much more prevalent in subconfluent than in confluent cells. This may be related to an altered organization of the Ca^{2+} -sequestering

organelles upon cell differentiation (Ambler *et al.*, 1988). Recently it was shown by Northern blot analysis (Marks *et al.*, 1989) that $\text{BC}_3\text{H1}$ cells express mRNA for the ryanodine receptor, a protein complex which contains the Ca^{2+} -release channel of the sarcoplasmic reticulum. The mRNA for the ryanodine receptor was reversibly regulated by growth factors in a manner similar to the regulation of muscle-specific protein genes. This means that there was a marked induction of this mRNA within 24 h of switching subconfluent cells to serum-deprived medium.

The aim of the present work was to study the effect of muscle cell differentiation on $^{45}\text{Ca}^{2+}$ uptake into the intracellular stores of $\text{BC}_3\text{H1}$ cells permeabilized with *Staphylococcus aureus* α -toxin. In addition, the effect of differentiation on the sensitivity of the Ca^{2+} pools to $\text{Ins}(1,4,5)\text{P}_3$ and caffeine, an activator of Ca^{2+} -induced Ca^{2+} release (Endo, 1977), was investigated. These findings were then correlated with measurements of changes of $[\text{Ca}^{2+}]_i$ in single intact Indo-1-loaded cells upon addition of the Ca^{2+} -mobilizing agents histamine and caffeine. Differentiation was found to induce a reversible shift in the mechanism for Ca^{2+} release from InsP_3 -sensitive in proliferating cells to caffeine-sensitive in differentiated cells.

EXPERIMENTAL

$\text{BC}_3\text{H1}$ cells were obtained from A.T.C.C. (Rockville, MD, U.S.A.) and used within 12 passages of receipt. Cells were cultured at 37 °C in a humidified air/ CO_2 (91:9) atmosphere. The growth medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % (v/v) FCS, 3.5 mM-L-glutamine, 0.9 % (v/v) non-essential amino acids, 85 μg of streptomycin/ml and 85 units of penicillin/ml (Flow Laboratories, Irvine, Scotland, U.K.). Cells were subcultured at regular time intervals to prevent the cultures from reaching confluency. Cells were seeded either in 175 cm^2 flasks for the flux experiments or in coverglass chambers (Nunc Inc., Naperville, IL, U.S.A.) for the fluorescence experiments. Differentiation was induced by

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; HBSS, Hanks balanced salt solution; SR, sarcoplasmic reticulum; T-tubule, transverse tubule.

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switching the still subconfluent cells to DMEM containing 0.5% (v/v) FCS (Spizz *et al.*, 1986). The cultures were supplied with fresh growth medium three times a week.

After trypsin treatment the cells were resuspended in 140 mM-KCl/10 mM-NaCl/2.5 mM-MgCl₂/4 mM-dithiothreitol/5% (w/v) Dextran-4/10 mM-Hepes/KOH (pH 7.0) at a density of (250–500) × 10³ cells/ml. The cells were permeabilized by incubation for 5 min at 37 °C in the presence of 12 µg of *Staphylococcus aureus* α-toxin/ml. Dextran-4 was added to the solutions in order to counteract osmotic lysis. The plasma membrane permeabilization by α-toxin allows only relatively small molecules (pores of around 2 nm diameter) to pass, so that the toxin itself is excluded from the cell interior and the intracellular protein content is largely retained (Bhadki & Tranum-Jensen, 1987). Subsequently cells were washed and resuspended in the same buffer, but without α-toxin. The permeabilized cells were kept on an overhead rotator at 5 °C for up to 3 h. Ca²⁺-uptake experiments in permeabilized cells were performed at 37 °C using a rapid filtration technique, modified from that of Chueh *et al.* (1987). GF/C glass microfibre filters (Whatman International Ltd., Maidstone, Kent, U.K.) were used. Permeabilized cells were incubated at a density of (100–300) × 10³ cells/ml in uptake medium containing 140 mM-KCl, 10 mM-NaCl, 2.5 mM-MgCl₂, 2 mM-dithiothreitol, 1 mM-ATP, 0.25 mM-K-EGTA, 0.14 mM-CaCl₂, 5 mM-NaN₃, 5% (w/v) Dextran-4, 20 mM-potassium oxalate and 10 mM-Hepes/KOH (pH 7.0). It was calculated, using the association constants published previously (Parys *et al.*, 1986), that the free Ca²⁺ concentration amounted to 0.3 µM. Radioactive ⁴⁵Ca²⁺ was added to about 20 µCi/ml. The radioactivity remaining on the filter was determined by scintillation spectrometry, using Ready Safe (Beckman Instruments Inc., Fullerton, CA, U.S.A.) as scintillation liquid.

For fluorescence measurements, the cells were loaded with the Ca²⁺-sensitive dye Indo-1 by incubation for 20 min at 35 °C in Hanks balanced salt solution (HBSS) containing 2 µM-Indo-1/AM. After washing twice with HBSS at room temperature, the chambers were mounted in an ACAS 570 interactive confocal laser cytometer (Meridian, Okemos, MI, U.S.A.). Excitation was done with the 351–363 nm lines of the argon laser, and the emitted fluorescence was detected at 405 and 485 nm. Image scans (100 × oil objective lens, Olympus, NA 1.3) of 252 µm × 252 µm in optical slices with a thickness of 1 µm were made every 30 s. Background fluorescence was subtracted and the average ratio of the emissions, as an estimate of [Ca²⁺]_i, was calculated for each cell observed in the image.

Creatine kinase activity was tested in BC₃H1 cell homogenates using an optimized test (Sigma Diagnostics 47-10). Myokinase activity was tested according to Missiaen *et al.* (1988). Protein concentration was measured by the method of Lowry *et al.* (1951), with BSA as standard, after precipitation of the proteins with 10% (w/v) trichloroacetic acid.

Biochemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Indo-1/AM was from Molecular Probes (Junction City, OR, U.S.A.) and ⁴⁵Ca was from Amersham International. α-Toxin was generously provided by Professor Dr. N. Blanckaert (Laboratory for Biological Chemistry, K.U. Leuven) and Dr. F. Vanstapel (Biomedical N.M.R. Unit, K.U. Leuven). The α-toxin was purified from culture broth of *S. aureus* by sequential glass adsorption chromatography (Cassidy & Harshmann, 1976) and cation-exchange chromatography (Lind *et al.*, 1987).

RESULTS AND DISCUSSION

We have measured the ATP-driven accumulation of ⁴⁵Ca²⁺ in non-mitochondrial stores of permeabilized BC₃H1 cells (Fig. 1). Mitochondrial Ca²⁺ uptake is expected to be small at a free Ca²⁺

Fig. 1. Ca²⁺ uptake in permeabilized differentiated BC₃H1 cells

Cells were seeded at 10000 cells/cm² in DMEM with 20% (v/v) FCS. After 3 days, cells were switched to DMEM with 0.5% (v/v) FCS for 8 days. Cells were then permeabilized using *S. aureus* α-toxin. Ca²⁺ uptake was measured using a rapid filtration procedure at 37 °C in the following media: ●, control medium [140 mM-KCl, 10 mM-NaCl, 2.5 mM-MgCl₂, 2 mM-dithiothreitol, 1 mM-ATP, 0.25 mM-K-EGTA, 0.14 mM-CaCl₂, 5 mM-NaN₃, 5% (w/v) Dextran-4, 20 mM-potassium oxalate and 10 mM-Hepes/KOH (pH 7.0)]; ○, control medium + 10 µM-A23187; ■, control medium without oxalate. The calculated free Ca²⁺ concentration was 0.3 µM. Values are means ± S.D. (n = 3).

concentration of 0.3 µM (Gill & Chueh, 1985). We have nevertheless included 5 mM-azide in the uptake medium in order to suppress any mitochondrial contribution. The Ca²⁺ uptake in control conditions without oxalate reached a maximum after 5 min. At that time Ca²⁺ accumulation was typically 10–20 times higher than the equilibrium value obtained in the presence of a 10 µM concentration of the Ca²⁺ ionophore A23187. Cells permeabilized with 12 µg of α-toxin/ml sequestered more Ca²⁺ than cells permeabilized with 50 µg of saponin/ml. Ca²⁺ accumulation, however, decreased at higher concentrations of α-toxin.

Ca²⁺ accumulation was greatly stimulated in the presence of Ca²⁺-precipitating anions. Oxalate at 20 mM or pyrophosphate at 10 mM (results not shown) were equally effective in this respect. Such stimulation by oxalate has also been observed in suspensions of permeabilized neuronal and smooth muscle cells (Chueh *et al.*, 1987; Mullaney *et al.*, 1987). In the present study we have selected Ca²⁺ accumulation during 5 min in the presence of 20 mM-oxalate as a standard condition for the evaluation of the Ca²⁺-sequestering activity in BC₃H1 cells.

The effect of cell differentiation on Ca²⁺ handling by the non-mitochondrial Ca²⁺ pools was further investigated (Fig. 2). Ca²⁺ accumulation increased about 5-fold upon differentiation and this increase occurred in parallel with the expression of other muscle cell properties, such as muscle creatine kinase and myokinase (Fig. 2a). Fig. 2(b) illustrates the amount of Ca²⁺ released by 20 µM-InsP₃, by 7 mM-caffeine or by the combination of both, relative to the release induced by 10 µM-A23187, as a function of the differentiation time. These agents were added to the loading solution, instead of first allowing the pools to sequester Ca²⁺, because the time course of the Ca²⁺ release by InsP₃ (Henne & Söling, 1986), A23187 (Enouf *et al.*, 1987), and especially caffeine (Palade, 1987), is slowed down in the presence of oxalate. The

Fig. 2. Functional changes in differentiating BC₃H1 cells

Cells were seeded at 10000 cells/cm² in DMEM with 20% (v/v) FCS. After 3 days, cells were switched to DMEM with 0.5% (v/v) FCS for the times indicated in the Figure. (a) Comparison of Ca²⁺ uptake activity and of muscle enzyme activity, as described in the legend in Fig. 1; ●, Ca²⁺ uptake measured in control conditions; Δ, creatine kinase activity; ▽, myokinase activity. (b) Ca²⁺ release by InsP₃ and caffeine. Ca²⁺ uptake was measured in control conditions, supplemented with 20 μM-InsP₃ (▼), 7 mM-caffeine (▲) or 20 μM-InsP₃ + 7 mM-caffeine (■). Ca²⁺ release is expressed as a percentage of maximal release, as estimated from uptake experiments in the presence of 10 μM-A23187. All values are the means of between two and six observations; S.D.s were less than 10%.

most remarkable change is the decrease in InsP₃-sensitive Ca²⁺ release from more than 90% of the sequestered Ca²⁺ in proliferating cells to an undetectable level in fully differentiated cells. This partial release was not caused by enhanced InsP₃ metabolism, because the Ca²⁺ release by 20 μM-InsP₃ after 4 days of differentiation (68.9 ± 0.4%, *n* = 4) was the same as with 40 μM-InsP₃ (65.3 ± 3.4%, *n* = 4). Caffeine-sensitive Ca²⁺ release, in contrast, increased from 25% of the sequestered Ca²⁺ in proliferating cells to more than 60% in fully differentiated cells. Since the amount of sequestered Ca²⁺ also increased 5-fold with differentiation, the total amount of Ca²⁺ which is released by caffeine increased by about 12-fold. A fraction of the sequestered Ca²⁺ remained insensitive to both InsP₃ and caffeine, but could be released by A23187. This fraction also increased as a function of the differentiation time. The observed effects cannot be ascribed to changes in the oxalate-sensitivity during differentiation, since very similar data were obtained by measuring Ca²⁺ uptake in the presence of 10 mM-pyrophosphate instead of oxalate, and also in the absence of Ca²⁺-precipitating anions.

In order to investigate whether the effects of caffeine indeed reflected the activation of the Ca²⁺-induced Ca²⁺-release channel, we have studied the effect of Ruthenium Red, a blocker of that

channel (Ohnishi, 1979), on the caffeine-induced Ca²⁺ release in cells allowed to differentiate for 8 days. Whereas 7 mM-caffeine released 50.1 ± 6.4% (*n* = 4) of the accumulated Ca²⁺, this did not occur in the presence of 5 μM-Ruthenium Red. Half-maximal inhibition of Ca²⁺ release occurred at a 1 μM concentration of the polycation. This blockade of the caffeine effect by Ruthenium Red indicates that the caffeine-induced Ca²⁺ release reflects the activation of the Ca²⁺-induced Ca²⁺-release channel. The Ca²⁺ uptake in the presence of 7 mM-caffeine plus 5 μM-Ruthenium Red was 13.4 ± 3.5% (*n* = 4) higher than in control conditions. A similar (10.8 ± 3.5%, *n* = 4) increase in Ca²⁺ uptake occurred in the presence of 5 μM-Ruthenium Red alone. Such stimulation of the Ca²⁺ uptake rate by Ruthenium Red has been reported for skeletal-muscle sarcoplasmic reticulum (Ohnishi, 1979), and indicates that the Ca²⁺-induced Ca²⁺-release channel is already activated to some extent at the concentrations of Ca²⁺ used to monitor Ca²⁺ uptake.

With the exception of the undifferentiated cells, the InsP₃-sensitive and caffeine-sensitive Ca²⁺ pools did not appreciably overlap, because addition of both InsP₃ and caffeine induced a nearly additive Ca²⁺ release. Spatially different InsP₃- and caffeine-sensitive Ca²⁺ pools have already been reported in bovine chromaffin cells (Burgoyne *et al.*, 1989), sensory neurons (Thayer *et al.*, 1988) and smooth muscle (Saida & van Breemen, 1983; Kanaide *et al.*, 1987), and present a key feature in some models of cytosolic Ca²⁺ spiking (Berridge & Galione, 1988).

The observed changes could be reversed by replating differentiated BC₃H1 cells in the presence of 20% (v/v) FCS (Fig. 3). There was a pronounced decrease in the Ca²⁺-uptake activity within 2 days, and the InsP₃-sensitivity reappeared within the same time period. The caffeine-sensitivity decreased, but this adaptation took 5–7 days. Obviously this kind of experiment reflects not only the contribution of the original cell population but also of the new cells appearing in the culture. This is in contrast with the previous experiment, where the changes were observed in the absence of cell proliferation.

The findings on permeabilized cells were compared with measurements of changes in intracellular Ca²⁺ in intact individual cells loaded with the fluorescent Ca²⁺ indicator Indo-1. The response of cells to a saturating dose of histamine (0.1 mM) or

Fig. 3. Reversibility of BC₃H1 cell differentiation

Cells were seeded as 12000 cells/cm² in DMEM with 20% (v/v) FCS and switched after 3 days to DMEM with 0.5% (v/v) FCS for 10 days. At that time, the differentiated cells were collected by trypsin treatment and replated at 5000 cells/cm² in the presence of 20% (v/v) FCS. Ca²⁺ uptake (●) and Ca²⁺ release in the presence of InsP₃ (▼) and caffeine (▲) were measured as a function of the time after replating. Ca²⁺ release is expressed as a percentage of the maximal release obtained in the presence of 10 μM-A23187. All values are means of four observations; the S.D.s were less than 10%.

Fig. 4. Ca^{2+} mobilization in single intact $\text{BC}_3\text{H1}$ cells

The cells were seeded at 2000 cells/ cm^2 in DMEM with 20% (v/v) FCS in coverglass chambers. Experiments were done on proliferating cells after 1 day in culture (a) or on differentiating cells after switching to 0.5% FCS for 7–9 days (b). Cells were loaded with Indo-1 and the analysis was done as described in the Experimental section. The broken lines indicate the effect of 0.1 mM-histamine and the solid lines indicate the effect of 7 mM-caffeine. The addition of histamine or caffeine is indicated by arrows. The responses of representative individual cells are shown.

caffeine (7 mM) was compared during the early proliferation phase and during the differentiating phase (Fig. 4). After stimulation with histamine, $[\text{Ca}^{2+}]_i$, as estimated from the Indo-1 F_{405}/F_{485} ratio, rose and then slowly declined. In proliferating cells (1 day after seeding) a similar response was observed in 76% of the cells ($n = 102$) investigated. Fewer than 10% ($n = 98$) of the cells showed a response in $[\text{Ca}^{2+}]_i$ during stimulation with caffeine. In the differentiated stage the histamine-sensitive response was observed in only 17% ($n = 63$) of the cells, whereas the effect of caffeine was present in more than 80% of the cells ($n = 160$). In most cases caffeine evoked an initial Ca^{2+} transient followed by a maintained plateau value. As also reported by O'Neill *et al.* (1990), caffeine quenched equally the 405 nm and the 485 nm signals, resulting in a constant ratio. We have observed significant cell-to-cell differences in the courses of the Ca^{2+} transients. Nevertheless, the statistical evaluation of the change in responsiveness during differentiation is in agreement with our observations in permeabilized cells that there is a shift in the type of Ca^{2+} pool, from InsP_3 -sensitive in proliferating cells to caffeine-sensitive in differentiated cells.

We have no direct evidence that the caffeine-induced Ca^{2+} release observed in this study reflects the activation of the ryanodine receptor. Experiments with ryanodine in permeabilized cells were not conclusive, since the long incubation at 37 °C which is required for the binding of ryanodine (Pessah *et al.*, 1986) also destroyed the uptake activity. Our results on the reversible development of the caffeine-induced and Ruthenium-Red-sensitive Ca^{2+} release in differentiating $\text{BC}_3\text{H1}$ cells are nevertheless in agreement with the reversible expression of the junctional channel complex mRNA in these cells (Marks *et al.*, 1989). This junctional channel complex contains both the foot structure, which serves as the junctional connection with the T-tubule, and the Ca^{2+} -release channel of the SR itself (Imagawa *et al.*, 1987). Our data therefore suggest that the Ca^{2+} -release channel becomes functionally expressed in the $\text{BC}_3\text{H1}$ cells. This expression occurs in parallel with an increase in the Ca^{2+} uptake activity, whereas the InsP_3 -sensitivity decreases and disappears. The mechanism involved in the generation of cytosolic Ca^{2+} oscillations in $\text{BC}_3\text{H1}$ cells, as in many non-muscle cells, is still unknown (Berridge & Galione, 1988; Jacob, 1990). The 'two-pool' model assumes that an interaction between anatomically distinct InsP_3 -sensitive and Ca^{2+} -induced Ca^{2+} -release-sensitive Ca^{2+} pools is the underlying phenomenon. The finding that, in $\text{BC}_3\text{H1}$ cells, the ratio between both pools can be easily controlled

makes this cell line especially suitable for further study of the underlying phenomena in the establishment of cytosolic Ca^{2+} oscillations.

The observed changes in Ca^{2+} handling are concomitant with the development of the contractile phenotype. This suggests that muscle-specific regulatory proteins such as myogenin, which is expressed in $\text{BC}_3\text{H1}$ cells to a high level (Brennan *et al.*, 1990; Brunetti & Goldfine, 1990), could be directly or indirectly involved.

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