Biochem. J. (1996) **316**, 759–764 (Printed in Great Britain)

Differentiation of BC_3H1 smooth muscle cells changes the bivalent cation selectivity of the capacitative Ca^{2+} entry pathway

Lisa M. BROAD*, David A. POWIS† and Colin W. TAYLOR*‡

*Department of Pharmacology, Tennis Court Road, Cambridge CB2 1QJ, U.K. and †Neuroscience Group, Faculty of Medicine and Health Sciences, The University of Newcastle, Newcastle, NSW 2308, Australia

Differentiation of BC_3H1 cells leads to expression of a variety of proteins characteristic of smooth muscle and to changes in the behaviour of intracellular Ca^{2+} stores. Treatment of both differentiated and undifferentiated cells with thapsigargin (2 μM) emptied their intracellular Ca^{2+} stores, and in the presence of extracellular Ca^{2+} caused an increase in cytosolic $[Ca^{2+}]$ that rapidly reversed after its removal. The amplitudes of these capacitative Ca^{2+} entry signals were 101 ± 8 nM ($n = 42$) in differentiated cells and 188 ± 16 nM ($n = 35$) in undifferentiated cells. Mn^{2+} entry in thapsigargin-treated cells, measured by recording the quenching of cytosolic fura 2 fluorescence, was $374 \pm 26\%$ ($n = 34$) and $154 \pm 7\%$ ($n = 41$) of control rates in differentiated and undifferentiated cells, respectively. Empty stores caused Ba^{2+} entry to increase to $282 \pm 20\%$ ($n = 8$) of its basal rate in differentiated cells and to $187 \pm 20\%$ ($n = 8$) in undifferentiated cells. Rates of Ca^{2+} extrusion, measured after rapid removal of extracellular Ca^{2+} from cells in which capacitative Ca^{2+} entry had been activated, were similar in differentiated ($t_{\frac{1}{2}} = 23 \pm 2$ s, $n = 7$) and undifferentiated (23 ± 1 s, $n = 6$) cells. The different relationships between capacitative Ca^{2+} and Mn^{2+} signals are not, therefore, a consequence of more active Ca^{2+} extrusion mechanisms in differentiated cells, nor are they a consequence of different fura 2 loadings in the two cell types. We conclude that during differentiation of BC_3H1 cells, the cation selectivity of the capacitative pathway changes, becoming relatively more permeable to Mn^{2+} and Ba^{2+}. The change may result either from expression of a different capacitative pathway or from modification of the permeation properties of a single pathway.

INTRODUCTION

In most cells, activation of receptors that stimulate hydrolysis of phosphatidylinositol 4,5-bisphosphate evokes both mobilization of intracellular Ca^{2+} stores and increased Ca^{2+} entry across the plasma membrane [1]. The role of inositol 1,4,5-trisphosphate ($InsP_3$) in mediating Ca^{2+} mobilization is established: $InsP_3$ binds to its intracellular receptor and Ca^{2+} then passes from the stores into the cytosol through the integral ion channel of the receptor [2]. The links between receptor-stimulated polyphosphoinositide hydrolysis and Ca^{2+} entry are less clear, although substantial evidence suggests that empty stores provide a signal that activates a Ca^{2+} entry pathway [3,4]; the signal itself has yet to be unequivocally identified [5–8]. Although $InsP_3$ is the physiological stimulus for emptying of intracellular Ca^{2+} stores and activation of the capacitative pathway, the pathway can also be activated in the absence of either receptor occupancy or inositol phosphates by emptying the intracellular stores with either Ca^{2+} ionophores [9] or inhibitors of the Ca^{2+}-ATPases of the endoplasmic reticulum [10,11]. Electrophysiological studies of mast cells [12] and *Xenopus* oocytes [13] have identified Ca^{2+}-selective channels (I_{CRAC}, Ca^{2+} release-activated Ca^{2+} current) that are probably responsible for capacitative Ca^{2+} entry, but most analyses have used fura 2-loaded cells to characterize the behaviour of this Ca^{2+} entry pathway. Many of these studies have taken advantage of the high affinity of Mn^{2+} for fura 2 and its ability to quench fura 2 fluorescence at all excitation wavelengths to examine unidirectional influx of Mn^{2+} through the capacitative pathway [5,14]. There are, however, conflicting reports of the Mn^{2+} permeability of the pathway in different cells. I_{CRAC} is highly selective for Ca^{2+}, but nevertheless has detectable permeability to Mn^{2+} [12,13,15]. Most studies with fura 2 have reported that Mn^{2+} permeates the capacitative pathway [5], but in some cells, notably hepatocytes ([16], but see [17]) and parotid acinar cells ([18], but see [19]), there is evidence, at least from some groups, that the capacitative pathway is not permeable to Mn^{2+}. There is presently no satisfactory explanation for these differences other than to suggest that different cells express different capacitative Ca^{2+} entry pathways. The utility of Mn^{2+} as a reliable monitor of Ca^{2+} entry is further challenged by the observation that a channel activated by inositol 1,3,4,5-tetrakisphosphate is equally permeable to Mn^{2+} and Ca^{2+} [20], and in A7r5 smooth-muscle cells the capacitative pathway is permeable to Mn^{2+}, but an additional vasopressin-regulated pathway is not [21].

The BC_3H1 cell line, which was initially derived from a mouse neoplasm [22], shares properties with smooth muscle and has provided a model system in which to examine muscle differentiation. Removal of growth factors from proliferating BC_3H1 cells causes them to reversibly withdraw from the cell cycle and to rapidly begin expressing a variety of muscle-specific proteins [23]. This differentiation has been reported to be accompanied by changes in the sensitivity of the intracellular Ca^{2+} stores to $InsP_3$ and caffeine, suggesting that differentiation also leads to changes in the expression of intracellular Ca^{2+} channels [24]. Since the role of intracellular Ca^{2+} stores in regulating capacitative Ca^{2+} entry is widely accepted [5], though poorly understood, we have examined Ca^{2+} entry in BC_3H1 cells before and after differentiation. Our results indicate that while the intracellular Ca^{2+} stores of undifferentiated cells responded only to $InsP_3$, those of differentiated cells responded to both $InsP_3$ and caffeine/ryanodine. Furthermore, although both differentiated and undifferentiated cells express a capacitative Ca^{2+} entry pathway,

Abbreviations used: AM, acetoxymethyl ester; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; HBS, Hepes-buffered saline; I_{CRAC}, Ca^{2+} release-activated Ca^{2+} current; $InsP_3$, inositol 1,4,5-trisphosphate; λ_{ex} and λ_{em}, excitation and emission wavelengths respectively; $R_{340/380}$, fluorescence ratio at λ_{ex} = 340 nm/λ_{ex} = 380 nm.

‡ To whom correspondence should be addressed.

the bivalent cation selectivity of the pathway changes during differentiation.

MATERIALS AND METHODS

Cell culture and differentiation

BC_3H1 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, U.K.) and used between passages 9 and 37 after receipt. Cells were grown (37 °C, 5 % CO_2) in Dulbecco's modified Eagle's medium supplemented with 20 % (v/v) foetal calf serum, penicillin (85 units/ml), streptomycin (85 μg/ml), L-glutamine (3.5 mM) and 0.9 % non-essential amino acids. They were passaged every 3–4 days when still subconfluent. For Ca^{2+} measurements, cells were subcultured into the same medium on to either rectangular (no. 2, 9 mm × 22 mm) or round (no. 1.5, 22 mm diameter) glass coverslips and used before the cells were confluent (2–4 days). Cells were differentiated by incubation of 2–4-day-old plated cells for a further 9–12 days in medium containing a low concentration of serum (0.5 %). Previous publications have reported that this treatment leads to expression of muscle-specific proteins [23] and to changes in morphology [22]. For both proliferating and differentiating cells, the growth medium was changed every 3 days.

Fura 2 loading and fluorescence measurements

Cells were incubated in Hepes-buffered saline (HBS), which had the following composition (mM): NaCl (135), KCl (5.9), $MgCl_2$ (1.2), $CaCl_2$ (1.5), Hepes (11.6), $NaHCO_3$ (5) and glucose (11.5) at pH 7.3; Ca^{2+}-free HBS was prepared by omitting $CaCl_2$ from HBS. Cells were loaded with fura 2 by incubating them for 60–90 min at 22 °C in HBS supplemented with fura 2 acetoxymethyl ester (AM) (2 μM), Pluronic F-127 (0.2 mg/ml) and BSA (1 mg/ml). After the loading period, the coverslips were incubated in HBS containing BSA for at least 1 h to allow complete de-esterification of the fura 2 AM and then mounted vertically in a perfused cuvette as previously described [25]. The temperature of the perfusate was maintained at 22 °C, and at the perfusion rates used (10–15 ml/min), medium within the cuvette exchanged with a half-time of about 11 s, and exchange was essentially complete within 60 s. Fluorescence [excitation wavelength (λ_{ex}) = 340, 359 and 380 nm; emission wavelength (λ_{em}) = 510 nm] was recorded in either a Perkin-Elmer LS 50B or an Hitachi F4500 spectrofluorimeter. Autofluorescence, which was always less than 10 % of the initial fura 2 fluorescence signal, was determined at the end of each experiment by addition of ionomycin (1 μM) and $MnCl_2$ (1 mM) in Ca^{2+}-free HBS. Fluorescence ratios ($R_{340/380}$; λ_{ex} = 340 nm/λ_{ex} = 380 nm) were calculated after subtraction of the autofluorescence signal and then calibrated to free [Ca^{2+}] by reference to a look-up table created from Ca^{2+} standards in media containing (mM): KCl (100), Mops (10), $MgCl_2$ (1), K_2EGTA (10), pH 7.2, and appropriate concentrations of $CaCl_2$.

For measurements of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in single cells, coverslips were mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope and excitation light of appropriate wavelength was provided by a high-pressure xenon arc lamp (100 W) mounted behind a computer-controlled filter wheel housing narrow-band interference filters. Emitted light collected by the objective passed through a dichroic mirror (400 nm) and high-pass barrier filter (480 nm) before detection by an intensified CCD camera (Photonic Science) [21]. Video images were digitized and stored in the memory of an Improvision imaging system and, after correction for autofluorescence, the images were analysed using IonVision 3 software (Improvision, Coventry, U.K.) [26]. Medium was perfused over the coverslip at 5–6 ml/min; the half-time for exchange of medium was about 4 s, and exchange was essentially complete within 12 s.

Mn^{2+} entry to cells was measured by briefly incubating cells in Ca^{2+}-free HBS supplemented with $MnCl_2$ (50 μM) and recording the quenching of fura 2 fluorescence at the isosbestic wavelength for Ca^{2+} (λ_{ex} = 359 nm). The rate of fluorescence quench, reflecting the rate of Mn^{2+} entry, was determined by fitting a straight line by least-squares linear regression to the fluorescence trace recorded after the [Mn^{2+}] bathing the cells had reached its equilibrium concentration. In more than 30 independent experiments, the basal rate of Mn^{2+} entry varied by up to 5-fold between experiments; all stimulated rates of Mn^{2+} entry are therefore expressed relative to the basal rate of entry, measured in the same cells before emptying their Ca^{2+} stores. Basal rates of Mn^{2+} entry were similar in differentiated (93 ± 11 units/s, $n = 78$) and undifferentiated (81 ± 9 units/s, $n = 74$) cells. Similar methods were used to measure Ba^{2+} entry, except that the perfusion medium included $BaCl_2$ (free [Ba^{2+}] ~ 4 mM) and the fluorescence ratio ($R_{340/380}$) was recorded. Because Ba^{2+} is very poorly transported by Ca^{2+}-ATPases [27], the rate of Ba^{2+} entry was quantified by fitting a straight line to the increases in $R_{340/380}$ measured after the [Ba^{2+}] bathing the cells had reached its equilibrium concentration. Stimulated rates of Ba^{2+} entry are expressed relative to the basal rate of Ba^{2+} entry recorded before thapsigargin treatment of the same cells.

Our measurements of both Mn^{2+} and Ba^{2+} entry provide measures of their unidirectional influx because, after removal of either ion from the extracellular medium, the changes in fura 2 fluorescence that resulted from their permeation of the capacitative pathway did not reverse (see Figures 2A and 2B), indicating that neither ion is rapidly removed from the cytosol.

Materials

Cell culture media were from Gibco BRL (Paisley, Scotland, U.K.). Fura 2 AM, Ca^{2+} standards and Pluronic F-127 were from Molecular Probes (Leiden, The Netherlands). Ionomycin, ATP and Hepes were from Calbiochem. Thapsigargin and ryanodine were from Alamone Laboratories (Jerusalem, Israel). All other reagents, including caffeine and histamine dihydrochloride, were from Sigma. Media containing Ba^{2+} were prepared by addition of 5 mM $BaCl_2$ to HBS supplemented with EGTA (1 mM) to minimize the effects of contaminating Ca^{2+}; the final free [Ba^{2+}] was therefore ~ 4 mM.

RESULTS

Differentiation of BC_3H1 cells changes the responses to Ca^{2+}-mobilizing stimuli

After 9–12 days in medium containing a low concentration of serum (0.5 %), BC_3H1 cells had substantially changed from the flattened fibroblast-like cells characteristic of undifferentiated cells to the more smooth muscle-like elongated morphology that characterizes the differentiated state [22]. Populations of undifferentiated BC_3H1 cells responded to histamine (100 μM) and ATP (50 μM) with an increase in $[Ca^{2+}]_i$, but caffeine (10 mM) and ryanodine (5 μM) evoked no detectable response (Figure 1A). The increases in $[Ca^{2+}]_i$ evoked by histamine were invariably smaller (~ 17 %) than those evoked by ATP, reflecting the results from single-cell analyses in which all cells responded to ATP, but $\leqslant$ 50 % responded to histamine (results not shown). Populations of differentiated cells invariably responded to ATP (50 μM) and to caffeine (10 mM)/ryanodine (5 μM) with an

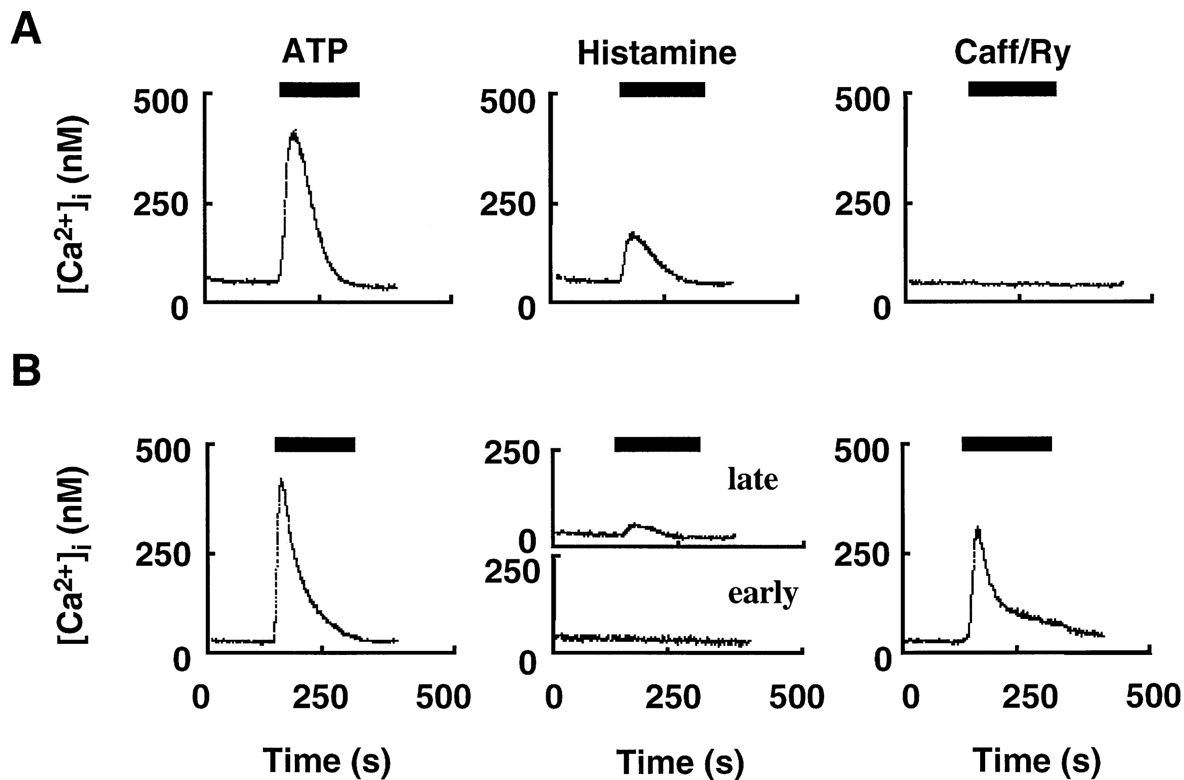

Figure 1 Responses of undifferentiated and differentiated BC_3H1 cells to Ca^{2+}-mobilizing stimuli

Populations of undifferentiated (**A**) or differentiated (**B**) BC_3H1 cells were stimulated with ATP (50 μM), histamine (100 μM) or caffeine (10 mM) and ryanodine (5 μM) (Caff/Ry), all in Ca^{2+}-free HBS, for the periods shown. Differentiated cells differed in their responses to histamine according to passage number: cells from early passages did not respond to histamine, whereas cells from later passages did respond, albeit with very small increases in $[Ca^{2+}]_i$. All traces, with the exception of the single trace from a late passage (30), were from passage numbers 22–27. All traces are typical of records from at least 10 similar recordings from independent experiments.

increase in $[Ca^{2+}]_i$ (Figure 1B). Differentiated cells from early passage numbers (receipt +9–27) lost their ability to respond to histamine (100 μM), although cells that were differentiated from later passages (receipt +29–37) responded, albeit with a small increase in $[Ca^{2+}]_i$, to histamine (Figure 1B).

The changes in the behaviour of intracellular Ca^{2+} stores were further examined by permeabilizing cells with saponin in cytosol-like medium [28]; both differentiated and undifferentiated cells then sequestered $^{45}Ca^{2+}$ into their intracellular stores after addition of ATP. A maximal concentration of InsP_3 (10 μM) released 80 ± 2 % ($n = 3$) of the ionomycin-releasable Ca^{2+} stores of undifferentiated cells and 65 ± 1 % ($n = 3$) of the stores of differentiated cells. Only the differentiated cells responded to caffeine (10 mM) and ryanodine (5 μM), which caused release of 45 ± 1 % ($n = 3$) of the stores. Addition of caffeine (10 mM), ryanodine (5 μM) and InsP_3 (10 μM) to differentiated cells caused release of 74 ± 1 % ($n = 3$) of the intracellular Ca^{2+} stores. These results are consistent with observations of intact differentiated cells in which the Ca^{2+} mobilization evoked by caffeine was ~ 70 % of that evoked by ATP (results not shown). In differentiated BC_3H1 cells, therefore, the InsP_3- and caffeine/ryanodine-sensitive stores substantially overlap, and only ~ 9 % of the stores are sensitive to only caffeine/ryanodine.

Capacitative Ca^{2+} and Mn^{2+} entry in undifferentiated and differentiated BC_3H1 cells

Incubation of either undifferentiated or differentiated BC_3H1 cells with HBS containing thapsigargin (2 μM) for 150 s, followed by incubation in Ca^{2+}-free HBS for a further 400 s, completely emptied the agonist-sensitive intracellular Ca^{2+} stores, as shown by the subsequent lack of increase in $[Ca^{2+}]_i$ after addition of ATP (100 μM) (results not shown). In both cases, a small residual pool (~ 10 %) of Ca^{2+}, which remained stable for 30 min after thapsigargin treatment, could be released by ionomycin (1 μM). These results confirm that the brief (150 s) treatment of BC_3H1 cells with thapsigargin (2 μM) used in all subsequent experiments is sufficient to ensure that the ATP-sensitive stores remain completely empty throughout the experimental protocol.

In order to compare the effects of emptying the intracellular Ca^{2+} stores on the subsequent changes in $[Ca^{2+}]_i$ and Mn^{2+} entry, the protocol depicted in Figure 2 was used. In populations of both differentiated and undifferentiated cells, depletion of the intracellular Ca^{2+} stores by thapsigargin evoked an increase in $[Ca^{2+}]_i$ that rapidly reversed after removal of extracellular Ca^{2+} (Figure 2). Changes in $[Ca^{2+}]_i$ after removal of extracellular Ca^{2+} from cells that had not been treated with thapsigargin were insignificant ($\leqslant$ 4 nM). The peak amplitudes of the capacitative Ca^{2+} signals, determined after restoration of extracellular Ca^{2+} to populations of cells in which the intracellular stores had been emptied by thapsigargin, were almost 2-fold greater in undifferentiated cells than in differentiated cells (Figure 2). The converse was true of capacitative Mn^{2+} entry, which was more than 2-fold greater in differentiated relative to undifferentiated cells (Figure 2). Similar results were obtained when ATP (50 μM) was used to empty the intracellular Ca^{2+} stores: Mn^{2+} entry was increased to 149 ± 20 % ($n = 5$) and 386 ± 52 % ($n = 10$) of its

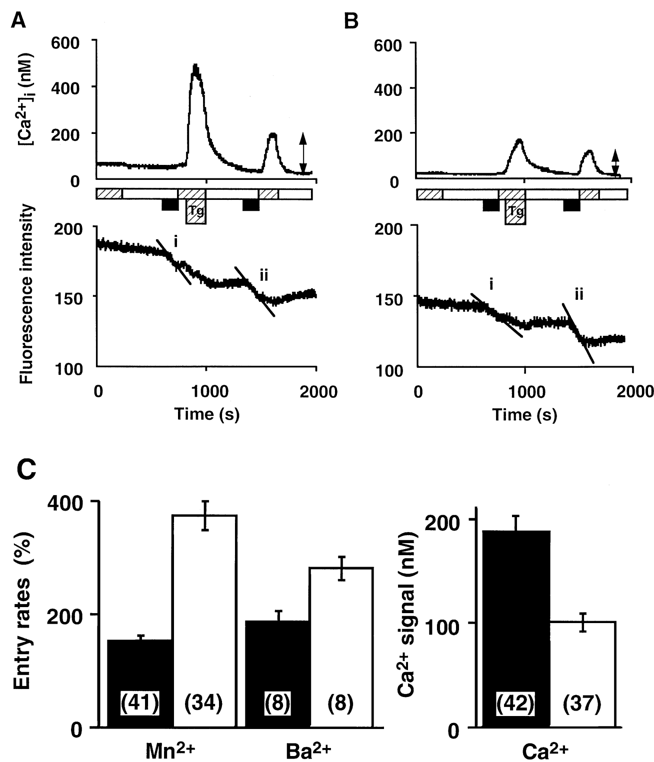

Figure 2 Capacitative Ca^{2+}, Mn^{2+} and Ba^{2+} entry in undifferentiated and differentiated BC_3H1 cells

(**A** and **B**) Populations of fura-2-loaded undifferentiated (**A**) or differentiated (**B**) cells were perfused with HBS (hatched bars) or Ca^{2+}-free HBS (open bars) and exposed to thapsigargin (Tg, 2 μM) for the periods shown to empty their intracellular Ca^{2+} stores. Mn^{2+} (50 μM) was added to the Ca^{2+}-free HBS for the periods shown by solid bars. The magnitude of capacitative Ca^{2+} entry (upper panels, double-headed arrows) was assessed from the decrease in $[Ca^{2+}]_i$ after removal of extracellular Ca^{2+}. Capacitative Mn^{2+} entry was expressed as the rate of fluorescence quench ($\lambda_{ex} = 359$ nm) after store emptying (ii) relative to that recorded in unstimulated cells (i). Each trace is representative of 34–42 similar traces. (**C**) Results from experiments similar to those shown in panels (**A**) and (**B**) are summarized. Rates of Mn^{2+} and Ba^{2+} entry (% of basal rates) and the magnitude of capacitative Ca^{2+} entry in undifferentiated (solid bars) and differentiated (open bars) cells were determined using the methods described above. Results are the means ± S.E.M. of the number of independent determinations shown within each bar.

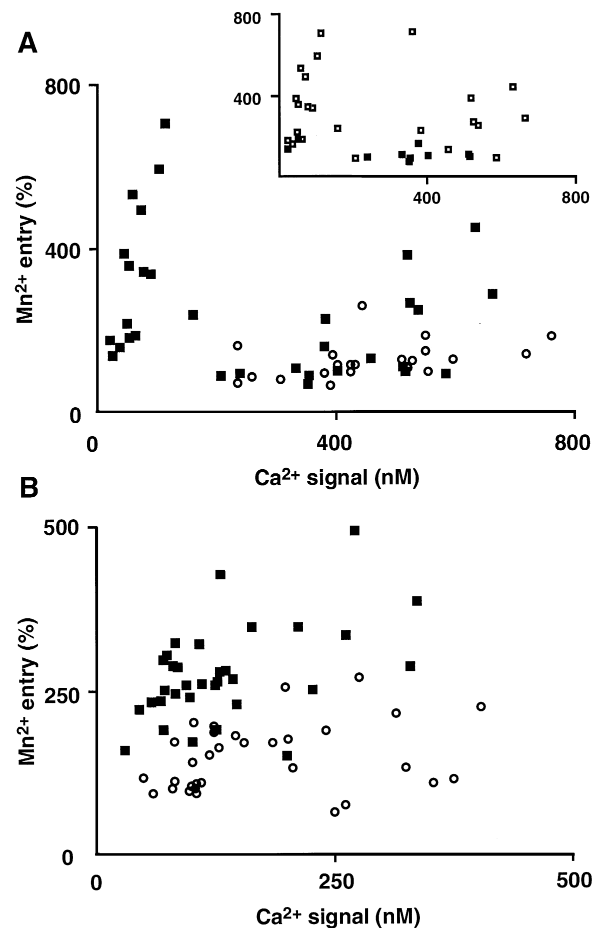

Figure 3 Comparison of capacitative Ca^{2+} and Mn^{2+} entry in undifferentiated and differentiated BC_3H1 cells

Experiments similar to those shown in Figure 2 were used to determine capacitative Ca^{2+} or Mn^{2+} entry in undifferentiated (○) or differentiated (■) cells. Each point represents the results from Ca^{2+} and Mn^{2+} entry measurements in a single cell (**A**) or population of cells (**B**). The inset to (**A**) shows the same results as those in the main panel, but only cells from differentiated populations are shown and categorized according to whether they were caffeine-sensitive (□) or-insensitive (■). The results demonstrate that caffeine-sensitivity of the intracellular stores is more tightly correlated with the behaviour of the capacitative pathway than with the condition under which the cells were cultured.

basal rate in undifferentiated and differentiated cells, respectively. When caffeine (10 mM) and ryanodine (5 μM) were used to empty the stores of differentiated cells, the stimulated rate of Mn^{2+} entry (300 ± 69 %, $n = 4$) was also similar to that evoked by ATP or thapsigargin (374 ± 26 %, $n = 34$). These results from cell populations, suggesting a discrepancy between capacitative Ca^{2+} and Mn^{2+} signals in undifferentiated and differentiated cells, were confirmed by single-cell analyses.

In single cells, the peak amplitudes of the capacitative Ca^{2+} signals (see above) were 458 ± 31 nM ($n = 21$) and 272 ± 37 nM ($n = 33$), and the stimulated rates of Mn^{2+} entry were 106 ± 7 % ($n = 39$) and 265 ± 32 % ($n = 33$) of their basal rates in undifferentiated and differentiated cells respectively. Although our analyses of single cells and cell populations provide different estimates of the changes in $[Ca^{2+}]_i$ (Figures 2 and 3), the discrepancy is likely to result from the different problems associated with accurately calibrating fluorescence ratios under the different experimental conditions [29]. Figure 3 illustrates the significantly different ($P < 0.01$, Mann–Whitney U test) relationships between capacitative Mn^{2+} and Ca^{2+} signals in undifferentiated and differentiated BC_3H1 cells. The different patterns of capacitative Ca^{2+} and Mn^{2+} entry in undifferentiated and differentiated cells are unlikely to have been influenced by differential expression of voltage-operated Ca^{2+} channels, because there were no detectable changes in $[Ca^{2+}]_i$ after depolarization with increased extracellular K^+ (75 mM) or in the presence of BAY K 8644 (1 μM) (results not shown).

Populations of differentiated and undifferentiated cells are neither morphologically nor functionally homogeneous. From single-cell analyses, for example, although $\geqslant 80$ % of cells responded to ATP, a fraction ($\leqslant 20$ %) of cells within the undifferentiated population responded to caffeine and a similar proportion of cells from the differentiated population failed to respond to caffeine. Within differentiated cell populations, those single cells that failed to respond to caffeine were also similar to undifferentiated cells in the behaviour of their capacitative pathways in that large Ca^{2+} signals were accompanied by small

Mn^{2+} signals (Figure 3A, inset). Likewise, with populations of undifferentiated cells, the few cells (4/21) that gave detectable increases in $[Ca^{2+}]_i$ after addition of caffeine included the three cells that had the highest rates of capacitative Mn^{2+} entry (results not shown). These results, which suggest that intracellular Ca^{2+} stores and Ca^{2+} entry pathways change in parallel during differentiation, indicate that differentiation *per se*, and not the different culture conditions used to achieve it, cause the change in the properties of the capacitative pathway in BC_3H1 cells.

Thapsigargin treatment increased Ba^{2+} entry into undifferentiated cells to $187 \pm 20\%$ ($n = 8$) of its basal rate and to $282 \pm 20\%$ ($n = 8$) in differentiated cells (Figure 2C).

Our assessment of capacitative Ca^{2+} entry reflects the balance between Ca^{2+} entry and the activities of Ca^{2+} extrusion mechanisms, whereas our experiments with Ba^{2+} and Mn^{2+} provide unidirectional measurements of their influx. In order to establish whether different rates of Ca^{2+} extrusion could have undermined our comparison of capacitative Ca^{2+} entry in differentiated and undifferentiated cells, we compared the ability of the two cell types to recover from an elevated $[Ca^{2+}]_i$. Rapid injection of Ca^{2+}-free HBS containing bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N′*,*N′*-tetra-acetic acid (BAPTA) (5 mM) into the cuvette containing the cells in which capacitative Ca^{2+} entry had been activated, allowed the free $[Ca^{2+}]$ of the bathing medium to be reduced from 1.5 mM to ~ 0 mM within 1 s. The fall in $[Ca^{2+}]_i$ recorded immediately after chelation of extracellular Ca^{2+}, reflecting the rate of Ca^{2+} extrusion from the cytosol, occurred with the same half-times in both undifferentiated (21 ± 1 s, $n = 6$) and differentiated (23 ± 2 s, $n = 7$) cells. When the fall in $[Ca^{2+}]_i$ was measured from exactly the same initial $[Ca^{2+}]_i$ in each cell type (170 nM), the half-times were 23 ± 1 s ($n = 6$) and 23 ± 2 s ($n = 7$) in undifferentiated and differentiated cells respectively.

The different relationships between capacitative Ca^{2+} and Mn^{2+} signals in the two cell types are not a consequence of them having loaded to differing extents with fura 2 because the average fura 2 loading of both cell types (assessed by the initial fluorescence intensity at $\lambda_{ex} = 359$ nm) was similar. Furthermore, although individual cells differed by up to 5-fold in their fura 2 loading, there was no correlation, in either cell type, between fura 2 loading and either the basal or stimulated rates of Mn^{2+} entry, or between the basal rate of Mn^{2+} entry and the fold increase in Mn^{2+} entry after depletion of intracellular Ca^{2+} stores (results not shown).

DISCUSSION

Our results (Figure 1) confirm previous studies in which serum-deprivation of BC_3H1 cells was shown to cause them to differentiate into smooth-muscle-like cells [23] that were responsive to activators of ryanodine receptors, but unresponsive to histamine [24]. The expression of functional ryanodine receptors after differentiation is consistent with the appearance of both mRNA for [30], and immunoreactivity to (J. M. MacKrill, personal communication), type-1 ryanodine receptors. Although the loss of responsiveness to histamine was previously ascribed to a loss of $InsP_3$ receptors [24], our results (Figure 1) show that differentiated cells retain the ability to respond to ATP and that after permeabilization they remain capable of releasing Ca^{2+} in response to $InsP_3$. We conclude that as BC_3H1 cells differentiate, they express functional ryanodine receptors, they continue to express $InsP_3$ receptors, but they lose functional histamine receptors.

In common with many other cell types [3–5], emptying of the intracellular Ca^{2+} stores with either thapsigargin or Ca^{2+}-mobilizing agonists stimulates capacitative Ca^{2+} entry in both undifferentiated and differentiated BC_3H1 cells (Figure 2). In permeabilized differentiated cells, caffeine released $\sim 45\%$ of the Ca^{2+} stores, $InsP_3$ released $\sim 65\%$, and thapsigargin released $> 90\%$ of the stores; this is consistent with the results from intact cells where ATP evoked greater Ca^{2+} release than caffeine. Yet the rates of capacitative Mn^{2+} entry evoked by each were indistinguishable at $300 \pm 69\%$ (caffeine), $386 \pm 52\%$ (ATP) and $374 \pm 26\%$ (thapsigargin) of the basal rates. These results suggest that intracellular Ca^{2+} stores differ in their abilities to stimulate the capacitative pathway, with stores expressing ryanodine receptors being more effective than those expressing $InsP_3$ receptors, and both being more effective than stores that lack the receptors. Alternatively, incomplete emptying ($\geqslant 45\%$) of the stores may be capable of fully activating the capacitative pathway.

Since the activities of the Ca^{2+} extrusion mechanisms are the same in undifferentiated and differentiated cells, our use of $[Ca^{2+}]_i$ to assess capacitative Ca^{2+} entry (a steady-state measurement) provides a reliable means of comparing capacitative Ca^{2+} entry in the two cell types. However, the relative magnitudes of capacitative Ca^{2+}, Ba^{2+} and Mn^{2+} entry differ substantially between the two cell types: in undifferentiated cells, large Ca^{2+} signals are accompanied by relatively small Ba^{2+} and Mn^{2+} signals, whereas the converse is true of differentiated cells (Figures 2 and 3).

Because Mn^{2+} binds with such high affinity to fura 2 ($K_d \sim 3$ nM) [31], the fractional rate of quench of fura 2 could depend upon the intracellular concentration of fura 2 if fura 2 were to comprise a substantial fraction of the intracellular Mn^{2+}-binding sites. However, although variable between individual cells, the average fura 2 loading of differentiated and undifferentiated cells was similar, and within single cells from each population there was no correlation between fura 2 loading and rates of basal or stimulated Mn^{2+} quenching. Nor could differential fura 2 loading in the two cell types account for the different rates of capacitative Ba^{2+} entry, because Ba^{2+} ($K_d \sim 1.4$ μM) has much lower affinity than Ca^{2+} ($K_d = 135$ nM) for fura 2 [32]. Finally, since unstimulated rates of Mn^{2+} entry into undifferentiated and differentiated populations of cells were similar, our expression of capacitative Mn^{2+} entry as a fraction of its basal rate cannot have contributed to the discrepancy. The different capacitative Ba^{2+} and Mn^{2+} signals recorded from undifferentiated and differentiated cells (Figure 2C) therefore reflect the different rates at which these two bivalent cations permeate the capacitative pathways of the two cell types.

We conclude that during differentiation of BC_3H1 cells to a smooth-muscle-like phenotype, the intracellular Ca^{2+} stores express type-1 ryanodine receptors and the capacitative Ca^{2+} entry pathway changes its permeation properties to become less selective for Ca^{2+} over Ba^{2+} and Mn^{2+}. Allosteric regulation of ryanodine receptors by ryanodine causes substantial changes in their permeation properties [33], and under the influence of atrial natriuretic peptide cardiac Na^+ channels conduct Ca^{2+} [34]. The changes in ion selectivity of the capacitative pathway after differentiation of BC_3H1 cells may result from a similar modulation of the permeation properties of a single pathway, or they may result from expression of a different capacitative pathway. Either mechanism would suggest that the capacitative Ca^{2+} entry pathway is subject to more complex regulation than previously supposed.

This work was supported by grants from the MRC and the Wellcome Trust. C. W. T. is a Lister Institute Fellow. L. M. B. was supported by a studentship from the MRC. D. A. P. was supported by the University of Newcastle Outside Studies Programme Committee.

REFERENCES

1 Berridge, M. J. (1993) Nature (London) **361**, 315–325
2 Taylor, C. W. and Richardson, A. (1991) Pharmacol. Ther. **51**, 97–137
3 Putney, Jr., J. W. (1986) Cell Calcium **7**, 1–12
4 Putney, Jr., J. W. (1990) Cell Calcium **11**, 611–624
5 Putney, Jr., J. W. and Bird, G.St.J. (1993) Endocr. Rev. **14**, 610–631
6 Randriamampita, C. and Tsien, R. Y. (1993) Nature (London) **364**, 809–814
7 Kim, H. Y., Thomas, D. and Hanley, M. R. (1995) J. Biol. Chem. **270**, 9706–9708
8 Gilon, P., Bird, G.St.J., Bian, X., Yakel, J. L. and Putney, Jr., J. W. (1995) J. Biol. Chem. **270**, 8050–8055
9 Morgan, A. J. and Jacob, R. (1994) Biochem. J. **300**, 665–672
10 Takemura, H., Hughes, A. R., Thastrup, O. and Putney, Jr., J. W. (1989) J. Biol. Chem. **264**, 12266–12271
11 Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. and Dawson, A. P. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2466–2470
12 Hoth, M. and Penner, R. (1992) Nature (London) **355**, 353–356
13 Parekh, A. B., Terlau, H. and Stühmer, W. (1993) Nature (London) **364**, 814–818
14 Hallam, T. J. and Rink, T. J. (1985) FEBS Lett. **186**, 175–179
15 Penner, R., Fasolato, C. and Hoth, M. (1993) Curr. Opin. Neurobiol. **3**, 368–374
16 Kass, G. E. N., Webb, D.-L., Chow, S. C., Llopis, J. and Berggren, P.-O. (1994) Biochem. J. **302**, 5–9
17 Glennon, M. C., Bird, G.St.J., Kwan, C.-Y. and Putney, Jr., J. W. (1992) J. Biol. Chem. **267**, 8230–8233
18 Merritt, J. E. and Hallam, T. J. (1988) J. Biol. Chem. **263**, 6161–6164
19 Mertz, L. M., Baum, B. J. and Ambudkar, I. S. (1990) J. Biol. Chem. **265**, 14822–14827
20 Lückhoff, A. and Clapham, D. E. (1992) Nature (London) **355**, 356–358
21 Byron, K. L. and Taylor, C. W. (1995) J. Physiol. (London) **485**, 455–468
22 Schubert, D., Harris, A. J., Devine, C. E. and Heineman, S. (1974) J. Cell Biol. **61**, 398–413
23 Spizz, G., Roman, D., Strauss, A. and Olson, E. N. (1986) J. Biol. Chem. **261**, 9483–9488
24 De Smedt, H., Parys, J. B., Missiaen, L. and Borghgraef, R. (1991) Biochem. J. **273**, 219–224
25 Byron, K. L. and Taylor, C. W. (1993) J. Biol. Chem. **268**, 6945–6952
26 Hargreaves, A. C., Lummis, S. C. R. and Taylor, C. W. (1994) Mol. Pharmacol. **46**, 1120–1128
27 Palade, P., Dettbarn, C., Brunder, D., Stein, P. and Hals, G. (1989) J. Bioenerg. Biomembr. **21**, 295–320
28 Richardson, A. and Taylor, C. W. (1993) J. Biol. Chem. **268**, 11528–11533
29 Poenie, M. (1992) in Neuromethods. Vol. 20: Intracellular Messengers (Boulton, A. A., Baker, G. B. and Taylor, C. W., eds.), pp. 129–174, Humana Press Inc, Totawa, NJ
30 Ambler, S. K. and Taylor, P. (1986) J. Biol. Chem. **261**, 5866–5871
31 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
32 Kwan, C.-Y. and Putney, Jr., J. W. (1990) J. Biol. Chem. **265**, 678–684
33 Lindsay, A. R. G., Tinker, A. and Williams, A. J. (1994) J. Gen. Physiol. **124**, 425–447
34 Sorbera, L. A. and Morad, M. (1990) Science **247**, 969–972

Received 23 November 1995/6 February 1996; accepted 14 February 1996