Calcium Influx in Induced Differentiation of Murine Erythroleukemia Cells

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Murine erythroleukemia cells (MELC) have served as a model for examining the regulation of erythroid differentiation. However, the role of Ca$^{2+}$ in the signal transduction pathways regulating differentiation remains unclear. To begin to address this uncertainty we have characterized the regulation of cytoplasmic Ca$^{2+}$ and the possible role of calcium channels during induced differentiation in MELC.

The erythroid colony-forming unit (CFU-e) and can grow in appropriate culture conditions. They can be induced to terminal differentiation using HMBA. We found that HMBA stimulated Ca$^{2+}$ influx within 3 to 6 minutes and that Ca$^{2+}$ entry was required but not sufficient for MELC growth and differentiation. Nifedipine (1 to 10 µmol/L), a calcium channel antagonist, blocked HMBA-induced Ca$^{2+}$ influx and inhibited differentiation by ~60%. Depolarization of the MELC membrane did not induce Ca$^{2+}$ influx and whole-cell patch-clamp recordings failed to detect a voltage-activated Ca$^{2+}$ current, suggesting that MELC do not express detectable levels of a functional voltage-dependent calcium channel (VDCC). However, a cDNA probe encoding a portion of the α1 subunit of the cardiac VDCC detected an ~8-kb mRNA on Northern blots of total MELC RNA. Taken together, these data show that Ca$^{2+}$ influx is an early event associated with HMBA-induced differentiation in MELC. Blockade of this calcium influx inhibits induced differentiation, and a voltage-insensitive dihydropyridine-sensitive calcium channel may be involved in Ca$^{2+}$ influx in MELC.

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MATERIALS AND METHODS

MELC culture. MELC lines (a gift from Dr Victoria Richon, Memorial Sloan-Kettering Cancer Center, New York, NY) were maintained in α-minimal essential medium (α-MEM) containing 10% (vol/vol) fetal calf serum (Melloni 1988). Cultures were initiated with an inoculum of 10^6 cells/mL; HMBA (Sigma) was added to cultures at a final concentration of 5 µmol/L and dimethyl sulfoxide (DMSO from Sigma, also an inducer of MELC differentiation) was added at a final concentration of 1.5%.

Commitment assay. Commitment to differentiation, characterized by irreversible induction of differentiation and limited cell division (small colony size), was assayed by subcloning aliquots of cells removed from suspension culture into semisolid medium without inducers and determination of the phenotype of the resulting colonies after 4 days of growth. Colonies arising from committed cells were small (<64 cells) and stained positive for hemoglobin using a benzidine reaction in acetic acid. Each experiment was repeated at least three times; error bars in Fig 1 through 4 represent standard deviations from the mean. Controls with 70% ethanol alone had no effects on cell growth, differentiation or Ca$^{2+}$ flux. Viability was assessed by trypan blue exclusion test in which nonviable cells take up the dye and turn blue.

Characterization of changes in intracellular Ca$^{2+}$ with Fluo-3. Measurements of changes in cytosolic Ca$^{2+}$ in single MELC were performed using a Zeiss inverted microscope (IM 35) equipped with a 40X oil-immersion objective, mounted with a photometer head (PMQII) and MAC 1000 controller equipped with two filter wheels.

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for dual excitation and emission fluorescent dyes (Ludl Electronic Products, Scarsdale NY). Data analysis was with SCOPE software (Kinetek, Yonkers, NY). The following filters were used: (1) Fluo-3, excitation filter 485DF22, emission filter 530DF30, dichroic beam splitter 505DRLP (all from Omega Optics Inc, Brattleboro, VT). Coverslips (no. 0-0; Biophisica Technologies, Baltimore, MD) were washed in nitric acid and coated with sterile polylysine (>300,000 molecular weight [MW]). MELC in exponential growth phase at 5 \times 10^5/mL were added to coverslips and incubated for 1 to 2 hours at 37°C. Fluo-3 (4 \text{ pmol/L}) was added in Earl's Balanced Salt Solution with HEPES (EBSS-H, bicarbonate is replaced with 26 mmol/L HEPES, pH 7.4 + 0.1% bovine serum albumin [BSA], Sigma) and cells incubated for 30 minutes at 37°C. Cells were washed once with EBSS-H and incubated for 30 minutes at 37°C. Coverslips were mounted on a microscope tissue chamber (Biophisica Technologies). Cells were constantly perfused with solutions at a rate of 2 to 3 mL/min and the drug to be studied was added to the perfusate. HMBA was dissolved directly in cell culture medium (α-MEM) and other drugs were dissolved in 70% ethanol. Controls with 70% ethanol alone had no effects on cell growth, differentiation, or Ca^{2+} flux. At the conclusion of each trial, maximal fluorescence (F_{max}) was obtained by perfusing the cell with 3 \text{ pmol/L} digitonin for 5 minutes in Ca^{2+} containing saline solution (145 mmol/L NaCl, 5 mmol/L CaCl_2, 5 mmol/L HEPES, pH = 7.4) followed by a 5-minute perfusion in Ca^{2+} free saline solution (saline solution in which 5 mmol/L CaCl_2 was replaced by 5 mmol/L EGTA) to obtain minimal fluorescence (F_{min}, autofluorescence). Single cells were selected under red illumination and the photometer reading area confined to the selected cell using a squared field diaphragm. Cells were illuminated using a xenon arc light source (alternating 100-fold) for 200 milliseconds every 3 seconds, and emitted fluorescence samples were recorded and stored on computer for further analysis. Only cells giving a maximal response to ionophore (≥fivefold increase in fluorescence signal in 5 mmol/L Ca^{2+} over minimal response in 5 mmol/L EGTA) at the conclusion of each experiment were considered viable. Reagents used included Fluo-3 (Molecular Probes), HMBA (Sigma), calcium ionophore A23187 (CalBiochem), ionomycin (CalBiochem), pluronic F-127 (Molecular Probes), >300,000 MW poly-D-lysine (Sigma).
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Fig 3. (A) Bay K 8644 has no effect on commitment to differentiation in MELC. MELC were cultured in α-MEM with HMBA (5 mmol/L) in the presence Bay K 8644 (1 μmol/L) or with Bay K 8644 alone with no effect on commitment to differentiation. (B) Bay K 8644 does not inhibit cell growth. For the cultures in (A) cell number was determined at 12, 24, 48, and 72 hours after plating and cell viability assessed by trypan blue exclusion. Bay K 8644 did not effect cell growth. There were no significant differences in cell viability among the cultures (data not shown).

Analysis of the percentile increase in fluorescence (ΔF/F) was as follows: the autofluorescence, Fmin, was subtracted from the fluorescence recorded for each of the samples (F) to yield F1. Baseline fluorescence, F0, was then derived by averaging 10 to 20 samples after a stable baseline was achieved immediately before application of the first agent in each trial. Each data point, ΔF/F, was calculated as follows: ΔF/F = (F1 - F0)/F0 and plotted against time in minutes.

To obtain [Ca2+], the equation [Ca2+] = kd × [F1/Fmax - F] was used. kd is the dissociation constant for Ca2+-bound Fluo-3 which equals 400 nmol/L.15,16 F1, F0, and Fmax were as defined above.

cDNA probes. A cDNA probe encoding a portion of the α1 subunit of the cardiac VDCC was synthesized as follows: Two synthetic oligonucleotide primers (antisense and sense) were designed based on the sequence of the α1 subunit of the rabbit skeletal muscle dihydropyridine receptor; an antisense oligonucleotide (5'-AGATGGTGTCGACCATGATGAGGGCGAACA-3', corresponding to nucleotides 3595-3625, except that two conservative changes in the nucleotide sequence were made: a T to C switch at nucleotide 3616 and a G to C switch at nucleotide 3613 to create an SphI site without changing the deduced amino acid sequence) and a

Fig 4. (A) Calcium ionophores alone do not induce MELC to commitment to differentiation. MELC were cultured in α-MEM with either 1 μmol/L ionomycin, or 1.7 μmol/L A23187. No induced commitment to differentiation was observed. Similar data were obtained when MELC were cultured with (in μmol/L) 0.2, 0.4, 0.6, 0.8 of either ionomycin or A23187 (data not shown). (B) Calcium ionophores are toxic to MELC. For the cultures in (A) cell number was determined at 12, 24, 48, and 72 hours after plating and cell viability assessed by trypan blue exclusion. Both ionomycin and A23187 significantly decreased cell growth. There was also a significant decrease in cell viability in cultures with either ionomycin (1 μmol/L) or A23187 (1.7 μmol/L) in both cases greater than 90% of the cells died within 48 hours (data not shown).
sense oligonucleotide (5'-CCACGCTCCTGCAATTCA-3'), corresponding to nucleotides 3030-3045). The antisense primer was synthesized as first-strand cDNA from 1 μg of rat cardiac muscle total RNA using avian myeloblastosis virus reverse transcriptase (Life Sciences, FL). Both primers were then used in the polymerase chain reaction (PCR) to amplify a 570-bp cDNA. Conditions for PCR were: 94°C × 1 minute, 50°C × 2 minutes, 74°C × 1 minute. Otherwise, first-strand synthesis and PCR amplification were performed as described previously.14 The resulting cDNA, CDHPR-1, was subcloned into the SalI and PstI sites of pGEM3Z (Promega) and sequenced using dideoxy chain termination method15 and an ABI Model 373 sequencer. This PCR-generated cDNA probe used for Northern blot analyses was uniformly labeled with random primers, Klenow and α32P dCTP, to a specific activity of 10^9 cpm/μg.

RNA preparation and Northern blot analysis. Total RNA was prepared from MELC using standard guanidinium-thiocyanate lysis buffer and centrifugation over a cesium chloride cushion. Northern blot analysis was performed as described previously.16 RNA was electrophoretically size separated on formaldehyde/agarose gels and prepared from MELC using standard guanidinium-thiocyanate lysis media. Using Fluo-3 loaded MELC we found that Bay K 8644 (0.2 mg/mL) to 1 mg/mL) or ionomycin (0.2 μmol/L to 1 μmol/L). At low doses (0.2 to 0.4 μmol/L) no effect on cell growth (data not shown) or differentiation (Fig 4A) was observed. At higher doses (0.6 to 1.0 μmol/L) a 12-hour or greater exposure to either calcium ionophore caused cell death (as determined by trypan blue staining) in a dose-dependent manner, representative experiments are shown (Fig 4B). These results contrast with published reports17 that both A23187 (~1 μmol/L) and ionomycin (~1 μmol/L) induced differentiation to an extent comparable with that of dimethyl sulfoxide. When low doses of A23187 (0.2 mg/mL) and ionomycin (0.2 μmol/L) or Bay K 8644 (1 to 10 μmol/L) were added immediately before a dose of HMBA (1 mmol/L), which by itself did not stimulate differentiation, commitment to differentiation was observed (80% benzidine-positive colonies after 48 hours), indicating that the ionophores and Bay K 8644 could potentiate the effects of HMBA.

RESULTS

Ca2+ influx is required for HMBA-induced differentiation in MELC. We sought to determine whether influx of extracellular Ca2+ was required for HMBA-induced differentiation in MELC. MELC were cultured with 5 mmol/L HMBA which induced 80% of the cell population to be committed to terminal differentiation within 48 hours (Fig 1A). Using the Ca2+-sensitive fluorescent dye Fluo-3 we determined that HMBA induced an increase in cytoplasmatic Ca2+ (see below and Fig 5). Low [Ca2+] (<10 μmol/L Ca2+, α-MEM with 2.7 mmol/L EGTA) in the medium inhibited cell growth and HMBA-induced MELC differentiation (Fig 1, A and B).

To further characterize the requirement of Ca2+ entry during HMBA-induced differentiation and to determine if this requirement was secondary to growth inhibition (caused by low [Ca2+]), MELC were cultured with HMBA (5 mmol/L) and the calcium channel blocker nifedipine (which blocks HMBA-induced rise in intracellular Ca2+, see below and Fig 5C). Nifedipine is a dihydropyridine which at low concentration (1 to 10 μmol/L) blocks voltage-activated calcium channels. HMBA-induced differentiation was inhibited greater than 60% by 10 μmol/L nifedipine at 48 hours (Fig 2A). Unlike low [Ca2+] plus EGTA, nifedipine (10 μmol/L) did not inhibit cell growth (Fig 2B). Inhibition (>60%) of HMBA-induced differentiation was also seen with 1 μmol/L nifedipine. Using the fluorescent Ca2+-sensitive dye Fluo-3 we found that nifedipine (10 μmol/L) blocked HMBA-induced Ca2+ influx in MELC (see below and Fig 5C). These data indicate that HMBA-induced Ca2+ entry in MELC is dihydropyridine sensitive.

To further characterize the requirement for Ca2+ during induced differentiation in MELC, the dihydropyridine calcium channel agonist Bay K 8644 was added to the culture media. Using Fluo-3 loaded MELC we found that Bay K 8644 (1 μmol/L) induced Ca2+ influx in MELC comparable to that seen with 5 mmol/L HMBA (see below and Fig 7A). Bay K 8644 (1 μmol/L) alone did not induce commitment to terminal differentiation (Fig 3A). Bay K 8644 (1 μmol/L) did not accelerate the time course for HMBA-induced differentiation in MELC (Fig 3A). Bay K 8644 (1 μmol/L) did not significantly inhibit cell growth (Fig 3B).

The calcium ionophores A23187 and ionomycin were examined for their effects on MELC differentiation (Fig 4A). MELC were exposed to varying concentrations of either A23187 (0.2 mg/mL to 1 mg/mL) or ionomycin (0.2 μmol/L to 1 μmol/L). At low doses (0.2 to 0.4 μmol/L) no effect on cell growth (data not shown) or differentiation (Fig 4A) was observed. At higher doses (0.6 to 1.0 μmol/L) a 12-hour or greater exposure to either calcium ionophore caused cell death (as determined by trypan blue staining) in a dose-dependent manner, representative experiments are shown (Fig 4B). These results contrast with published reports17 that both A23187 (~1 μmol/L) and ionomycin (~1 μmol/L) induced differentiation to an extent comparable with that of dimethyl sulfoxide. When low doses of A23187 (0.2 mg/mL) and ionomycin (0.2 μmol/L) or Bay K 8644 (1 to 10 μmol/L) were added immediately before a dose of HMBA (1 mmol/L), which by itself did not stimulate differentiation, commitment to differentiation was observed (80% benzidine-positive colonies after 48 hours), indicating that the ionophores and Bay K 8644 could potentiate the effects of HMBA.

HMBA and Bay K 8644 evoke Ca2+ influx in MELC. The observation that Ca2+ entry was required for growth and induced differentiation led us to study changes in intracellular Ca2+ level in MELC loaded with the Ca2+ sensitive fluorescent dye, Fluo-3. MELCs demonstrated an increase in fluorescence intensity that peaked approximately 3 to 6 minutes after addition of HMBA (5 mmol/L) to the perfusate (Fig 5A). Sixty-eight percent of cells tested showed a mean increase in fluorescence intensity of 87% ± 10% (n = 10). In some cells (~40%) the HMBA-induced increase in [Ca2+]i, was sustained for more than 10 minutes, despite removal of HMBA. A similar increase in fluorescence intensity was seen when cells were treated with 1.5% DMSO or with sodium butyrate (1 mmol/L), both inducers of MELC differentiation.18 Removal of Ca2+ from the perfusate (Fig 5B) completely eliminated the HMBA-induced Ca2+ influx in all cells tested (n = 6), demonstrating that the source of increased cytoplasmatic Ca2+ induced by HMBA was influx of Ca2+ rather than intracellular Ca2+ release.

We examined the effects of calcium channel blockers on HMBA-induced Ca2+ influx in MELC. The inorganic calcium channel blockers nickel (Ni2+, 1 mmol/L, n = 6) and lanthanum (La3+, 1 mmol/L, n = 5) reversibly blocked the HMBA-induced Ca2+ influx (Fig 6, A and B). The organic calcium channel blocker nifedipine (10 μmol/L, n = 4) also inhibited HMBA-induced Ca2+ influx (Fig 5C). This finding suggested that MELC might have a dihydropyridine-sensitive calcium channel. To test this hypothesis we applied the dihydropyridine calcium channel agonist, Bay K 8644, to Fluo-3-loaded MELC (Fig 7A). Bay K 8644 (1 μmol/L, n = 15) induced an increase in intracellular Ca2+ level similar in extent to that of HMBA (94% ± 10%). As with HMBA, Bay K 8644-
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Fig 7. HMBA stimulates Ca²⁺ influx in MELC. Change in fluorescence (ΔF/F, arbitrary units) is plotted versus time (minutes). Recordings were made on single cells loaded with Fluo-3. For each condition, three representative recordings are shown. (A) MELC treated with HMBA (5 mmol/L); (B) MELC treated with HMBA (5 mmol/L) in Ca²⁺ free medium. HMBA evoked an increase in Fluo-3 signal of 87% ± 12% (mean ± SEM in seven experiments) within 3 to 6 minutes (A), which was not observed in Ca²⁺ free medium (B). (C) Nifedipine inhibits HMBA induced Ca²⁺ influx in MELC. MELC cultured with HMBA (5 mmol/L) plus Nifedipine (10 µmol/L) which blocked the HMBA-induced increase in Fluo-3 signal. Horizontal lines indicate duration of perfusion with indicated agent.

induced Ca²⁺ influx could be reversibly attenuated by Ni²⁺ (1 mmol/L, n = 6, Fig 7C) and La³⁺ (1 mmol/L, n = 5, Fig 7D) and was abolished in all cells pretreated with nifedipine (10 µmol/L, n = 9, Fig 7B). These results suggest that a dihydropyridine-sensitive calcium channel is present in MELC that may be activated by HMBA.

Voltage-gated calcium channels are not detected in MELC. Having established that Ca²⁺ influx is important for HMBA-induced MELC growth and differentiation, we sought to further elucidate the mechanism of Ca²⁺ entry. One possibility is that Ca²⁺ entry occurs via a VDCC. We tested the possibility that MELC contain a VDCC by examining the effects of membrane depolarization on Ca²⁺ fluxes and ion currents using both the fluorescent dye Fluo-3 and whole-cell patch-clamp techniques.

No increase in fluorescence intensity was observed (n = 5) on depolarization of the cell membrane by substitution of 80 mmol/L K⁺ for Na⁺ in the perfusate of MELC loaded with Fluo-3.

Whole cell recordings were performed on MELC using the patch-clamp technique. MELC were perfused with EBSS-H and the pipette solution contained 140 mmol/L KCl, 3 mmol/L MgCl₂, 0.02 mmol/L EGTA, 1 mmol/L adenosine triphosphate (ATP), 0.1 mmol/L guanosine triphosphate (GTP), and 5 mmol/L HEPES, pH = 7.2. The mean resting membrane potential was 60 ± 4 mV (n = 13) and the mean
Fig 6. Effects of calcium channel blockers on HMBA stimulated Ca^{2+} influx in MELC. Change in fluorescence (ΔF/F, arbitrary units) is plotted versus time (minutes). Recordings were made on single cells loaded with Fluo-3. For each condition, three representative recordings are shown (A) MELC cultured with HMBA (5 mmol/L) plus nickel (1 mmol/L); (B) MELC cultured with HMBA (5 mmol/L) plus lanthanum (La^{3+}, 1 mmol/L) both of which reversibly inhibit HMBA-induced increase in Fluo-3 signal. Horizontal lines indicate duration of perfusion with indicated agent.

membrane resistance was 0.75 ± 0.14 GΩ (n = 13), measured within the first 2 minutes after breaking the patch membrane. The cell membrane was clamped at −70 mV. To investigate voltage-activated channels the cell membrane was hyperpolarized to −90 mV for 0.5 seconds and then stepped from −90 to +70 mV (at 20 mV intervals, for 0.5 seconds). With Cs^{+} in the recording pipette leak subtraction was performed. Five sequential voltage jumps from −90 mV to +50 mV (leak current) and from −90 mV to +10 mV were averaged and the leak current subtracted. A residual current of 8 to 12 pA remained (n = 5); however, this current was not affected when cells (n = 9) were perfused with Ba^{2+}-NMG solution (110 mmol/L N-methyl-D-glucamine-Cl, 20 mmol/L BaCl_{2}, 5 mmol/L HEPES, 6 mmol/L glucose, 0.1% BSA). These results suggested that no voltage-dependent Ca^{2+} current was detected.

MELC express an mRNA that hybridizes to the cardiac calcium channel α_1 subunit. We used PCR to isolate a 570-bp rat cardiac calcium channel α_1 subunit cDNA that was greater than 91% homologous at the nucleotide level to the corresponding region (III-S5 to IV-S1) of the α_1 subunit of the rabbit cardiac dihydropyridine receptor. To characterize the expression of calcium channel mRNA in MELC we performed Northern blot analysis at high stringency (65°C, 0.1X SSC) on MELC total RNA using the rat cardiac calcium channel α_1 subunit cDNA as a probe (Fig 8). Although the structure of calcium channels in nonexcitable cells has not yet been reported, we reasoned that such channels might be structurally similar to their counterparts in excitable tissues such as muscle and brain. Indeed, we found that a cardiac calcium channel cDNA probe detected an ~8 kb mRNA (Fig 8) in both proliferating and differentiated MELC.

DISCUSSION

Modulation of cytoplasmic Ca^{2+} concentration is a mechanism common to signal transduction pathways regulating many cellular phenomena. It has been suggested that regulation of cytoplasmic Ca^{2+} may play a role in the signal transduction pathways stimulated by inducers of differentiation in MELC. However, little is known about how cytoplasmic Ca^{2+} is regulated in MELC and whether, for example, these nonexcitable cells have calcium channels that could participate in the flux of Ca^{2+} across the cell membrane. In the present study we have shown that Ca^{2+} entry is necessary but not sufficient for MELC growth and induced differentiation. Moreover, pharmacologic and physiologic data and Northern blot analyses suggest that a voltage-insensitive, receptor-operated calcium channel is expressed in MELC and may be involved in Ca^{2+} influx during induced differentiation.

Regarding Ca^{2+} entry in MELC we have shown that: (1) Ca^{2+} entry is modulated by agents that block or activate dihydropyridine-sensitive calcium channels; (2) Northern blot
Fig 7. BAY K 8644 induces an increase in [Ca^{2+}] in MELC. (A) MELC treated with BAY K 8644 (10 μmol/L); (B) MELC treated with BAY K 8644 (10 μmol/L) plus Nifedipine (10 μmol/L) which blocked the BAY K 8644-induced increase in Fluo-3 signal. Effects of calcium channel blockers on BAY K 8644 stimulated Ca^{2+} influx in MELC. Change in fluorescence (ΔF/F, arbitrary units) is plotted versus time (minutes). Recordings were made on single cells loaded with Fluo-3. For each condition, three representative recordings are shown. (C) MELC treated with Bay K 8644 (1 μmol/L) plus nickel (1 mmol/L). (D) MELC treated with Bay K 8644 (1 μmol/L) plus lanthanum (1 mmol/L). Horizontal lines indicate duration of perfusion with indicated agent.

Analysis indicates the expression of an mRNA that is homologous to the α₁ subunit of the cardiac VDCC. The α₁ subunit of the cardiac VDCC forms a functional calcium channel when expressed in Xenopus oocytes and contains the 24 putative transmembrane segments that are believed to form the Ca^{2+}-conducting channel in the cell membrane. In the present study we have shown that HMBA, DMSO and sodium butyrate (inducers of differentiation) stimulate...
Fig 8. Northern blot analysis of calcium channel mRNA expressed in MELC. Each lane contains total RNA (30 μg) from: lane 1, differentiated MELC (induced with 5 mmol/L HMBA for 48 hours) and lane 2, undifferentiated MELC. Blots were hybridized with a cardiac calcium channel α subunit cDNA probe uniformly labeled with α-32P dCTP (see Materials and Methods) and autoradiographed for 5 days at -80°C with an intensifying screen. An ~8-kb mRNA is identified in MELC which hybridizes to the cardiac calcium channel cDNA probe at high stringency (final washing at 65°C, 0.1x SSC). Positions of the 28s and 18s ribosomal RNAs are indicated for size comparison.

The role of [Ca2+]i during induced differentiation in MELC has been controversial. In agreement with our findings others have shown that Ca2+ influx is an important signal for differentiation in MELC. However, this same group had reported that calcium ionophores (A23187 and ionomycin) alone are capable of inducing MELC differentiation. We found that over a dose range of 0.2 to 6.0 μmol/L of calcium ionophores alone there was no effect on MELC differentiation.

Falleto and Macara incubated MELC with DMSO for 0 to 48 hours before loading with Quin 2 and found a slight decrease in [Ca2+]i from 145 to 120 nmol/L. Unlike our study, the immediate effect (within minutes) of inducer exposure on [Ca2+]i was not examined. Moreover, Falleto and Macara determined [Ca2+]i at 23°C while all of our studies were performed at physiologic temperature (37°C). It is possible that the Ca2+-dependent signaling for MELC differentiation involves two phases, an early rapid HMBA-induced increase in cytoplasmic Ca2+ followed by a return to baseline or even a slight decrease; however, further studies under comparable conditions would be required to confirm this.

Inhibition of Ca2+ entry with nifedipine, at doses (1 to 10 μmol/L) that did not affect cell growth, did inhibit commitment to terminal differentiation. These data indicate that Ca2+ influx is required for progression through the cell cycle, which in turn is required for HMBA-induced commitment to terminal differentiation. This conclusion is consistent with previous evidence that passage through G1 or early S in the presence of inducer is required for terminal differentiation. Blocking Ca2+ entry with EGTA reduces cell growth and inhibits induced differentiation. However, blocking HMBA-induced Ca2+ entry with nifedipine did not decrease cell growth but did inhibit cell differentiation. These data imply that Ca2+ entry, specifically via a dihydropyridine-sensitive calcium channel, is required for induced differentiation in MELC.

Using the Ca2+ indicator, Fluo-3, we found that HMBA-induced Ca2+ entry in MELC demonstrates features characteristic of calcium channels present in excitable cells. These include sensitivity to blockade by both nifedipine and inorganic calcium channel blockers (Ni2+ and La3+). Moreover, the activation of a Ca2+ influx by Bay K 8644 with a similar time course and amplitude to the HMBA effect suggests that a dihydropyridine-sensitive calcium channel in MELC might be involved in the response to HMBA. Bay K 8644 alone had no effects on MELC differentiation.

In the present study voltage-gated calcium channels (L-type) were not detected in MELC using whole cell patch clamp. The presence of a calcium channel with pharmacologic properties similar to the voltage-dependent dihydropyridine receptors of muscle and brain in a cell without detectable voltage-gated calcium channels raises the possibility that MELC have a receptor or second-messenger operated channel. It has been proposed that Ca2+ influx in nonexcitable cells occurs via such receptor- or second messenger-operated channels. Several groups have reported that voltage-clamp depolarization does not evoke inward Ca2+ currents in T or B cells, indicating that calcium channels in T cells may be voltage-insensitive.34-38
As to what the second messenger might be, it has been suggested that inositol 1,4,5-trisphosphate (InsP3) or inositol 1,3,4,5-tetrakisphosphate (InsP4) may activate receptor/second-messenger operated calcium channels in nonexcitable cells. The structure of an InsP3-activated calcium release channel on the endoplasmic reticulum has been characterized in brain and aortic smooth muscle.\(^{18,39,44}\) However, no other InsP\(_1\) or InsP\(_4\)-activated calcium channels have been identified in terms of structure in nonexcitable cells. The potential role of InsP\(_3\) or InsP\(_4\) in activating calcium channels in MELC remains to be elucidated.

The present study provides evidence for the Ca\(^{2+}\)-dependence of induced differentiation in MELC. Ca\(^{2+}\) influx in MELC may be occurring via a non-voltage-dependent, or receptor-operated calcium channel. Further studies are required to determine the complete structure of the calcium channel expressed in MELC and to establish whether it is a pathway for Ca\(^{2+}\) entry during induced differentiation. Furthermore, the downstream effects of raising cytoplasmic Ca\(^{2+}\) concentration have yet to be determined. Regulation of gene expression by Ca\(^{2+}\) could be achieved via Ca\(^{2+}\)-dependent phosphorylation of transcription factors.

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