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Calcium Ionophore, A23187, Induces Commitment to Differentiation But Inhibits the Subsequent Expression of Erythroid Genes in Murine Erythroleukemia Cells

By Jack O. Hensold, George Dubyak, and David E. Housman

Murine erythroleukemia (MEL) cells are a useful model for studying the processes that regulate erythroid differentiation because exposure of these cells to a variety of chemical inducing agents results in expression of erythroid-specific genes and the resultant loss of cellular immortality. Previously it has been suggested that the calcium ionophore, A23187, has effects on the early cellular events that lead to the commitment of these cells to differentiation, but was not in itself sufficient to induce differentiation. We demonstrate here that A23187, as well as another calcium ionophore, ionomycin, are capable of inducing commitment to differentiation. Unlike other inducing agents, continual exposure to A23187 inhibits transcription of the erythroid-specific genes,

β -globin and Band 3. This effect is not attributable to an increase in cytosolic calcium concentration, because cells induced by ionomycin produce normal amounts of hemoglobin. These effects of A23187 on MEL cells confirm that commitment to differentiation is a distinct event from the subsequent transcriptional activation of erythroid genes. The ability of both ionophores to induce commitment to differentiation suggests that an increase in cytosolic calcium can trigger commitment to differentiation. These agents should prove useful in investigating the cellular processes that are responsible for commitment to differentiation.

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FRRIEND VIRUS-TRANSFORMED murine erythroleukemia (MEL) cells are a useful model system for studying growth and differentiation in the erythroid lineage. These malignant pronormoblasts grow indefinitely in culture, but when treated with dimethyl sulfoxide (DMSO) or a variety of other inducing agents (eg, hexamethylene bisacetamide, hypoxanthine, X-irradiation) undergo terminal differentiation with full expression of the erythroid phenotype, as well as a limited capacity for growth.^{1,2} Inducing agents act to trigger the normal pathways of differentiation, because continual exposure to an inducer is not necessary for complete expression of the erythroid phenotype.³ Cells that have undergone this triggering event are said to be "committed" to differentiation. Committed cells can be detected in cultures by subcloning into semi-solid medium without inducers and determining the phenotype of the resulting colonies after 4 days of growth. Colonies arising from committed cells are fully hemoglobinized and less than 32 cells in size (reflecting a limited potential for growth), while cells that have not committed have an unlimited growth capacity (colonies greater than 64 cells in size) with an erythroblastic morphology.³

Following inducer exposure, a latent period of 8 to 12 hours occurs before the cells begin to commit to differentiate. During this time, changes occur in the cells that are

necessary for the subsequent occurrence of commitment.³⁻⁵ Although these early changes are reversible,^{4,5} commitment results in the irreversible loss of cellular immortality and the concomitant expression of the erythroid phenotype. Changes in cytosolic calcium concentration have been suggested to play a role in producing these early changes.⁶⁻⁸ In cells induced by DMSO, both EGTA and procaine block commitment to differentiation, and this block is reversed by the addition of excess calcium to the medium.⁶⁻⁸ Further, a brief (1 hour) exposure of MEL cells to the calcium ionophore, A23187, was shown to abolish the latent period that occurred with subsequent exposure to DMSO.^{6,9} More recently though, direct measurements have shown that cytosolic calcium concentration decreases slightly when cells are exposed to DMSO.¹⁰ In contrast, for MEL cells induced to differentiate by the β -chain of the peptide hormone inhibin, an increase in cytosolic calcium concentration was measured.¹¹ Thus, the significance of these changes for erythroid differentiation remains uncertain.

Although A23187 induces cells to traverse the latent period, this agent has not been reported to be capable by itself of inducing commitment to differentiation. We demonstrate here that A23187, as well as a chemically distinct calcium ionophore, ionomycin, can induce commitment to differentiation. However, these agents have different effects on the expression of the erythroid phenotype. Whereas the committed colonies that arise from A23187-treated cells are fully hemoglobinized, cells grown continually in this agent do not express elevated levels of β -globin or Band 3 transcripts. Rather, despite increasing numbers of cells committed to differentiation, these transcripts only begin to accumulate when the cells are placed in medium free of the ionophore. A23187 also blocks hemoglobinization of cells that have previously become committed to differentiate in DMSO. Thus, A23187 triggers commitment to differentiation while inhibiting the subsequent expression of the differentiated erythroid phenotype. This effect is not seen with ionomycin, which induces commitment and erythroid gene expression in a manner similar to previously described inducing agents. The similar effect of these ionophores on commitment suggests that an increase in intracellular calcium concentration can trigger differentiation of MEL cells. The differing effect of these agents on erythroid gene

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expression suggests that A23187 inhibits these latter processes through a mechanism other than an increase in cytosolic calcium concentration.

MATERIALS AND METHODS

MEL cells were subclones of 745-PC4-B1-2A- (15, 17, 17f, 17j, 17i, and 19) that had been selected for rapid inducibility in DMSO. These subclones responded similarly to each other when exposed to inducers. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with either 12% or 15% fetal bovine serum (FBS; Armour Pharmaceutical Co, Kankakee, IL) and 2 mmol/L L-glutamine, at densities to maintain log phase of growth (0.5 to 10×10^5 cells/mL). Cell growth and differentiation in DMSO (1.5% vol/vol) were similar under these conditions. Because of significant binding of the ionophores to serum proteins, concentrations of these agents were standardized for serum concentration as indicated in the text. A23187 and 4-bromo-A23187 were dissolved in DMSO at concentrations of 1 mg/mL. Ionomycin was dissolved in DMSO at a concentration of 1 mmol/L. Experiments demonstrated that at these final solvent concentrations (<0.15%), DMSO had no effect on MEL growth or differentiation.

Commitment to differentiation was assessed as described by Gusella et al.³ Briefly, following exposure to inducer, aliquots of cells were washed and plated in inducer-free plasma clot culture at a clonal density of 2000 cells/mL. After 4 days of growth, the clots were fixed with glutaraldehyde, stained with benzidine, and counterstained with hematoxylin. The percentage of colonies that reacted with benzidine (an indication of hemoglobin accumulation) was determined on triplicate samples of 0.1-mL plasma clot cultures. Cloning efficiencies were calculated by dividing the number of colonies in each clot by 200 (the number of cells plated per 0.1-mL plasma clot) and multiplying by 100.

For determination of growth rate, cells were maintained at concentrations that ensured log phase growth of control cells (ie, 0.5 to 10×10^5 cells/mL). Cells were diluted as necessary with prewarmed medium to maintain these densities. Cell counts were performed with a Coulter Counter (Model Z_p, Coulter Electronics, Hialeah, FL) and absolute cell number calculated by considering the previous dilutions.

Cell cycle profiles were determined as described by Crissman and Steinkamp.¹² Briefly, cells in log phase of growth were exposed to inducer and at various times following inducer exposure, aliquots of cells were removed from culture, washed with ice-cold phosphate-buffered saline (PBS), and fixed with ice-cold 70% ethanol. Before analysis, cells were treated with RNase A (200 μ g/mL) to reduce fluorescence from RNA, then stained with propidium iodide (10 μ g/mL). DNA-bound propidium iodide was excited at a wavelength of 480 nm in a Cytofluorograf System 50 (Ortho Diagnostic Systems, Raritan, NJ) and cellular DNA content was determined by fluorescence at a wavelength of 630 nm. Cell cycle distribution was calculated using an Ortho 2150 System computer and the 2150 Program Constant method.

Relative rates of DNA synthesis were determined by pulse-labeling with tritiated thymidine (³H-TdR). ³H-TdR (10 μ Ci/mL) was added to cultures of cells for 30 minutes. Incorporation into DNA was terminated by the addition of 5 vol of ice-cold PBS followed by two washes in PBS. Cells were lysed and DNA precipitated by the addition of ice-cold 10% trichloroacetic (TCA) acid. Lysed cell pellets were incubated for 30 minutes on ice before collection by filtration on Whatman (Maidstone, England) GF/C glass fiber filters. The filters were washed extensively with 5% TCA, with a final wash in ice-cold 70% ethanol. Incorporated radioactivity was determined by scintillation counting in a Packard (Downers Grove, IL) Tri-carb Liquid Scintillation Spectrometer.

For determination of mRNA levels, cytoplasmic RNA was extracted from cells by the method of Favalaro et al,¹³ except that cell lysis was performed in 5 mmol/L Tris (pH 7.4), 2.5 mmol/L MgCl₂, 1.5 mmol/L KCl, 1% Triton X-100 (Sigma, St Louis, MO), and 0.5% deoxycholate and the nuclei pelleted in Eppendorf centrifuge tubes by a 60-second spin at 10,000g. Extracted RNA was quantitated by absorbance at 260 nm and separated by electrophoresis in 1.2% agarose/20 mmol/L 3-[N-Morpholino]propanesulfonic acid (MOPS)/2.2 mol/L formaldehyde gels. Following electrophoresis, the RNA was transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) by Northern blotting. Transferred RNA was hybridized at 42°C in 5X SSPE, 5X Denhardt's solution, 50% formamide, and sheared salmon sperm DNA (100 μ g/mL) with the indicated cloned fragments of DNA that had been labeled with [³²P]-dCTP in the manner described by Feinberg and Vogelstein.¹⁴ The cloned DNA fragments used in these experiments and the nuclear run-off experiments (see below) were a 2.2-kb fragment of the mouse β -globin gene,¹⁵ a 600-bp cDNA encoding mouse actin¹⁶ and a 3.4-kb cDNA fragment encoding the murine erythrocyte Band 3 protein.¹⁷ The Northern blot hybridization results were standardized for ribosomal RNA loaded by rehybridizing all the filters with a cloned fragment of the human 18S rRNA gene.¹⁸

Nuclear run-off transcriptions were performed as described by Greenberg and Ziff.¹⁹ An excess amount of linearized plasmid DNA was bound to nitrocellulose and hybridized for 48 hours with standardized amounts of ³²P-UTP-labeled run-off transcripts. In addition to the cloned DNA fragments indicated above plasmids containing a human hsp90 cDNA,²⁰ a hamster GRP78 cDNA,²¹ and a murine hsc70 cDNA,²² were also used in these experiments. Relative transcription rates were internally standardized to levels of actin transcripts because previous experiments had demonstrated that the relative mRNA abundance and transcription rate of this gene was unaltered during MEL cell differentiation.

Free cytosolic calcium concentrations were determined as previously described.²³ Briefly, cells were removed from growth medium, washed, and resuspended in buffer containing 5 mmol/L glucose and 12% FBS. The serum was added to ensure that measurements were performed at similar effective concentrations of ionomycin and 4-bromo-A23187 as those present in the growth medium. Cells were loaded with fura2-AM ester (Molecular Probes, Eugene, OR) and fura2 fluorescence (339-nm excitation/500-nm emission) determined as previously described.²³ Calcium-dependent fura2 fluorescence was calibrated using standard techniques^{24,25} after lysis of the cells with digitonin. To determine the effect of prolonged incubations on cytosolic calcium, cells were exposed to inducers for the times described in the text, then pelleted and resuspended in one-half of the supernatant medium and loaded with fura2 acetoxymethyl ester (AM) for 30 minutes at 37°C. The cells were then pelleted and resuspended in the remaining half volume of medium and cytosolic calcium determined as above.

RESULTS

A23187 induces commitment to differentiation of MEL cells. Previous work in this laboratory had demonstrated that prior exposure of MEL cells to the calcium ionophore, A23187 (1 μ g/mL), abolished the normally occurring latent period of 10 to 12 hours that precedes the onset of commitment to differentiation in DMSO-exposed cells.⁶ To further evaluate the cellular changes that occurred during this time preceding differentiation, we sought to determine the changes in gene expression that took place in MEL cells following A23187 exposure. However, in initial experiments

it was noted that, following overnight exposure of the cells to A23187 (1 $\mu\text{g}/\text{mL}$, 15% FBS), committed colonies began to appear. Because this result suggested that the ionophore not only shortened the latent period but was capable of inducing commitment to differentiation, this effect was examined more closely. MEL cells were exposed to either DMSO or A23187 (1 $\mu\text{g}/\text{mL}$) for up to 48 hours, then subcloned into inducer-free plasma clot cultures and commitment determined as previously described.³ The results of this experiment (Fig 1A) demonstrated that DMSO and A23187 induce commitment to differentiation with similar kinetics. However, relative to DMSO, A23187 produced a slightly higher percentage of committed colonies at early times of exposure (by 9 hours, 2.4% *v* 1.5%), and a lower percentage of committed colonies with prolonged exposure times (by 48 hours, 71% *v* 87%). This data demonstrated that A23187 was at least as efficient at inducing the metabolic changes that occur during the latent period as DMSO and that the ionophore was sufficient by itself to induce commitment of MEL cells to differentiation.

To determine the optimal dose of A23187 for inducing commitment to differentiation, MEL cells were exposed to varying concentrations of A23187 for 48 hours, and the percentage of committed cells was determined. The results of this experiment are indicated in Fig 1B. Concentrations below 0.5 $\mu\text{g}/\text{mL}$ had no detectable effect on growth or differentiation. As ionophore concentration was increased, up to 1 $\mu\text{g}/\text{mL}$, an increased percentage of committed cells was observed. For the dose ranges and times examined here toxicity was not evident, as demonstrated by the cloning efficiencies indicated at the bottom of Fig 1. However,

concentrations of over 1 $\mu\text{g}/\text{mL}$ were associated with toxicity, and this was occasionally evidenced at a concentration of 1 $\mu\text{g}/\text{mL}$, particularly with prolonged incubations. Thus, this agent is similar to previously described inducing agents in having a maximal effect on differentiation just below the concentration where toxicity is manifested.² In subsequent experiments, an A23187 concentration of 0.9 $\mu\text{g}/\text{mL}$, in 15% FBS, or a comparable dose of 0.72 $\mu\text{g}/\text{mL}$ in 12% FBS was used.

A23187 and DMSO have different effects on growth of MEL cells. While these experiments demonstrated that the ionophore functioned as a complete inducer of differentiation, differences were noted in cells grown in A23187 compared with those grown in DMSO. While the growth rate in DMSO was equivalent to that of control cells for up to 2 to 3 days, cells cultured with A23187 rapidly ceased to grow. In addition, despite high percentages of committed cells in cultures induced with either DMSO or A23187, hemoglobin synthesis appeared to be minimal in A23187-exposed cells as judged by the color of the cell pellets. Therefore, we investigated these differences more carefully.

Growth rates were compared in cells treated with DMSO or A23187 and in untreated cells. As the growth curves in Fig 2A demonstrate, normal growth rates persisted in all cells for the first 12 hours of inducer exposure. After this, cell number in the A23187-treated cultures did not change, while cells grown in DMSO continued to grow at rates identical to control for up to 72 hours. As previously shown, with longer DMSO exposures a gradual slowing of the growth rate occurs (not shown).

Exposure of MEL cells to DMSO results in a transient arrest of cells in the G_1 phase of the cell cycle.^{26,27} Previous experiments had demonstrated that for these clones, the G_1 arrest occurred at approximately 7 to 9 hours of DMSO exposure.²⁸ To determine if the ionophore's effect on growth rate was to prolong this normally occurring G_1 arrest, cell-cycle profiles were determined on MEL cells following increasing time of exposure to A23187. As demonstrated in Fig 2B, a small decrease in the percentage of cells in S phase occurred following exposure to A23187. However, significant alterations in the cell-cycle distribution did not occur until 49 hours, a time when a majority of the cells had become committed to differentiation (see Fig 1). This late accumulation of cells in G_1 has also been observed in DMSO-exposed cells as well.²⁹ It was evident from this data though that the change in growth rate that occurred after 12 hours of A23187 exposure was not caused by an arrest in a particular phase of the cell cycle. Therefore, it is likely that the transit time through all phases of the cell cycle must be slowed. To confirm that the rate of DNA synthesis at 24 hours was indeed slowed despite a large percentage of cells still in S phase, MEL cells were pulse-labeled with ^3H -TdR and the incorporation of label into trichloroacetic acid-insoluble material in control and A23187-exposed cells was determined. This experiment (Fig 2C) demonstrated an 87% reduction in thymidine incorporation following 24 hours of A23187 exposure. This finding confirmed that transit through S phase of the cell cycle was indeed slowed

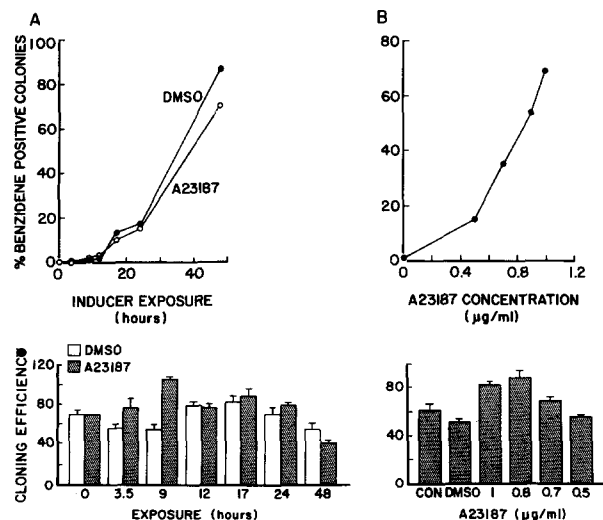


Fig 1. (A) A23187 and DMSO induce commitment to differentiation with similar kinetics. MEL cells were exposed to either DMSO (1.5% vol/vol) (●) or A23187 (1.0 $\mu\text{g}/\text{mL}$) (○) for the times indicated. The cells were then washed, and seeded at clonal density in inducer-free plasma clot culture. After 4 days of growth the percentage of committed cells was determined by staining the resulting colonies with benzidene. (B) A23187 dose-response curve. MEL cells were exposed for 48 hours to A23187 at the concentrations indicated. Commitment to differentiation was determined as above. Cloning efficiencies are indicated in the bar graphs at the bottom of the figure.

A23187-INDUCED COMMITMENT OF MEL CELLS

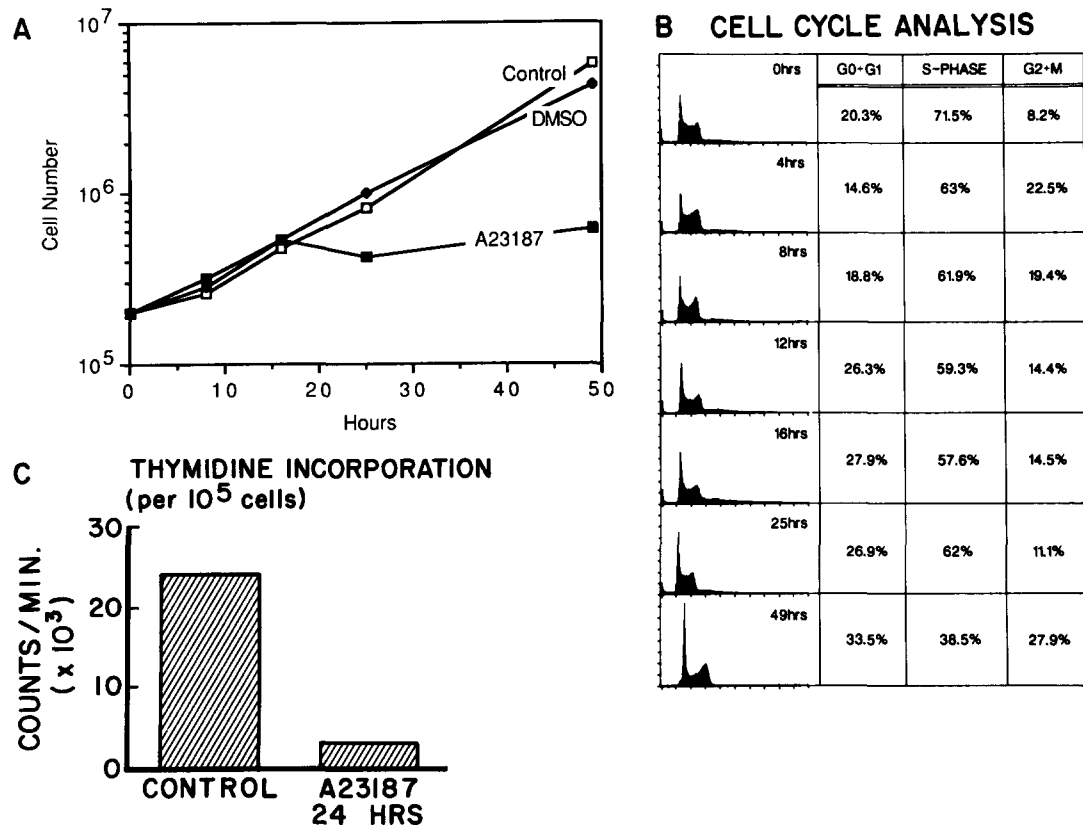


Fig 2. A23187 produces cell cycle slowing of MEL cells. (A) Growth rate of MEL cells exposed to either DMSO or A23187. Cells were grown in medium with DMSO (1.5% vol/vol) (●), A23187 (0.9 μ g/mL) (■) or without inducers (□) and diluted with prewarmed medium to maintain densities between 1 and 10×10^5 and the dilutions figured in the final calculation of cell number. Cell counts were determined using a Coulter Counter (model Z_r). (B) Cell-cycle profiles following exposure to A23187. DNA content of MEL cells exposed to A23187 for the times indicated was determined by fluorescence of propidium bound to DNA. The left-most peak in the figure represents (G0 + G1 cells (2 N amount of DNA), the right-most peak represents G2 + M cells (4 N amount of DNA), and the area between the two peaks represents cells in S phase (between 2 N and 4 N amount of DNA). The percentage of cells in these cell-cycle phases was determined as described in Materials and Methods. (C) DNA synthesis rates in control cells and in cells exposed to A23187. The relative rate of DNA synthesis in MEL cells exposed to A23187 was determined by labeling the cells for 30 minutes with [³H]-TdR, and determining the incorporation of labeled [³H]-TdR into TCA acid-insoluble material. Following the labeling period, cells were lysed in ice-cold 10% TCA and the precipitates collected on Whatman GF/C filters. The results were standardized for cell number.

by exposure to A23187. Thus, in contrast to cells induced in DMSO, A23187 slows MEL cell growth rate and the transit time through all stages of the cell cycle is affected.

A23187 inhibits expression of β -globin and band 3 genes in committed MEL cells. As noted above, cells induced to differentiate in A23187 did not synthesize significant amounts of hemoglobin (as assessed by lack of red color of the cell pellet). Because the rate of commitment to differentiation was similar in DMSO and A23187-exposed cells, and because both inducers produced committed colonies which reacted with benzidine (after 4 days of growth in inducer-free medium) this suggested that continual exposure to A23187 had an inhibitory effect on accumulation of hemoglobin. A decrease in protein synthetic rate occurs in MEL cells following exposure to inducers of differentiation.³⁰ Because this effect is more pronounced in A23187-exposed cells (data not shown), the failure to accumulate significant amounts of hemoglobin might reflect a decrease in translation of globin mRNA in A23187-induced cells. In such a case, globin mRNA would increase in parallel with commit-

ment, but the cells would remain poorly hemoglobinized. Therefore, levels of β -globin message were determined in cells exposed to DMSO and A23187 by hybridization of Northern-blotted RNA with a ³²P-labeled β -globin DNA fragment. Preliminary experiments demonstrated that β -globin mRNA did not increase in cells treated with A23187 for up to 60 hours, a time when a significant increase in this message had occurred in DMSO-treated cells (see text below and Fig 3A). A similar effect was also seen for accumulation of Band 3 mRNA (data not shown). These results implied that, despite inducing commitment to differentiation, A23187 inhibited erythroid gene expression. To determine if this was the case, MEL cells were exposed to either DMSO or A23187 for 54 hours to induce commitment in a high percentage of the cells. Cell cultures were then split and maintained in the presence or absence of inducer for an additional 18 hours. β -globin mRNA levels were determined as above. As shown in Fig 3A, in the continued presence of A23187, β -globin mRNA level does not significantly increase relative to control cells. However,

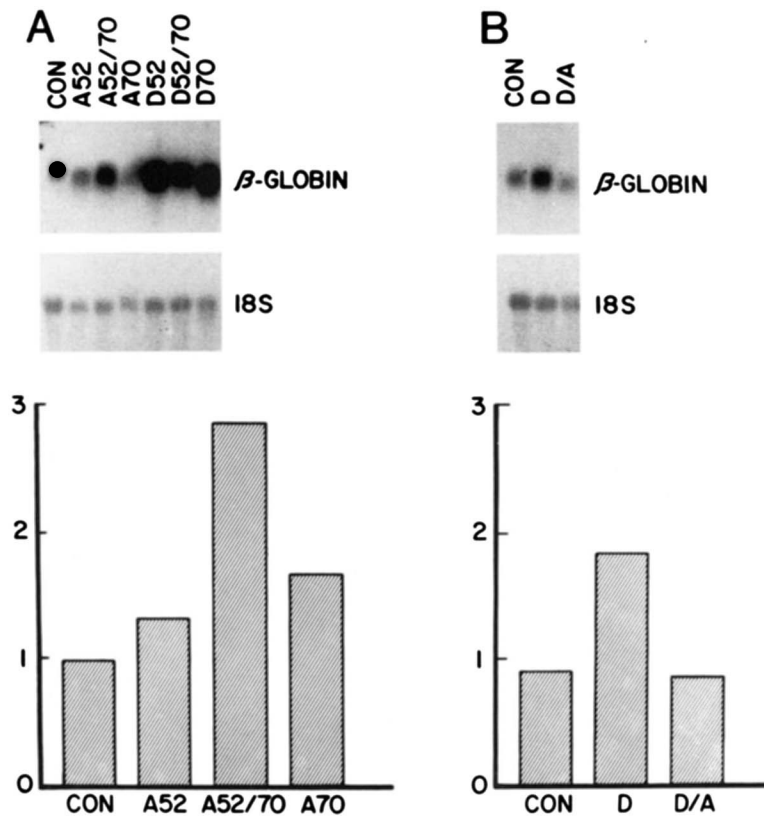


Fig 3. A23187 inhibits accumulation of β -globin mRNA in committed MEL cells. (A) β -globin mRNA only accumulates in MEL cells induced to differentiate in A23187 when removed from the inducer. MEL cells were exposed to either A23187 (0.9 μ g/mL) or DMSO (1.5% vol/vol) for 52 hours (A52 and D52). The cultures were then split and for the following 18 hours maintained in medium either with inducer (A70 and D70) or without inducer (A52/70 and D52/70). Cytoplasmic RNA was extracted from the cells at the indicated times and β -globin mRNA levels determined by blot hybridization with a [32 P]-labeled mouse β -globin gene fragment. β -globin mRNA levels were quantitated by densitometry and internally standardized to the amount of 18S rRNA loaded/lane and is indicated in the graph below the blots. Quantitation of signal in the DMSO lanes was not attempted because of saturation of the film. (B) A23187 inhibits accumulation of β -globin mRNA in cells induced to differentiate in DMSO. MEL cells were induced in DMSO (1.5% vol/vol) for 26 hours, then split and grown in either DMSO alone, or in both DMSO (1.5%) and A23187 (0.9 μ g/mL) for the ensuing 18 hours. β -globin mRNA levels were determined and quantitated as above.

by 18 hours after withdrawal of the ionophore, this message has accumulated nearly threefold. In contrast, cells induced in DMSO accumulated high levels of β -globin message in the continual presence of the inducer. The extent of this increase could not be quantitated by densitometry of these gels, because at exposure times sufficient for detection of β -globin mRNA in control cells, the signal from β -globin mRNA from the DMSO-exposed cells had saturated the film. The inhibitory effects of the ionophore on β -globin mRNA accumulation also extended to cells that had been induced in DMSO. As shown in Fig 3B, cells that had been induced by exposure to DMSO for 26 hours and then were incubated in both A23187 and DMSO accumulated significantly less β -globin mRNA than those maintained exclusively in DMSO. This effect was not because of a toxic effect of coinubation with both agents, because cell growth following removal of the agents was similar for cells exposed to either DMSO or DMSO and A23187 (data not shown).

The high level of β -globin mRNA accumulation in cells induced with DMSO is the result of an increase in the rate of transcription of the gene,^{31,32} as well as the stability of the β -globin message.³³ To determine if the inhibitory effects of the ionophore on expression of β -globin and Band 3 occurred at the transcriptional or posttranscriptional level, the relative transcription rates for these two genes were determined by nuclear run-off assay. Transcription rates were determined following 60 hours of exposure to DMSO or A23187, a time when significant accumulation of both β -globin and Band 3 message had occurred in DMSO-

induced cells. These results are shown in Fig 4. Following 60 hours of DMSO exposure, the rate of β -globin transcription had increased 15-fold. At similar times of A23187 exposure, β -globin transcripts had not changed relative to control cells. A similar effect was detected for Band 3 gene transcription. This effect on the transcription rates of β -globin and Band 3 genes was specific, because the transcription of actin, GRP78, HSP90, and hsc70 transcripts persisted in cells exposed to A23187. These experiments demonstrated that, although A23187 induces commitment of MEL cells, it can block the subsequent transcriptional activation of at least two genes whose expression is characteristic of terminal erythroid differentiation.

Ionomycin induces both commitment and β -globin gene expression in MEL cells. The ability of A23187 to induce commitment to differentiation suggested that a decrease in cytosolic calcium (previously reported to occur with DMSO) was not necessary for commitment. However, because the effects of A23187 on cation permeability are not selective for calcium, it is possible that A23187-induced commitment is mediated by other effects of this agent. Therefore, we investigated the effects of ionomycin on MEL cell commitment and erythroid gene expression. This agent has a more selective effect on membrane permeability to calcium than does A23187.³⁴ The ability of this agent to induce differentiation was determined by a 48-hour exposure to a range of concentrations as previously done for A23187. As demonstrated in Fig 5A, within the same molar range of concentrations as A23187 (0.7 μ g/mL A23187 is 1.36 μ mol/L),

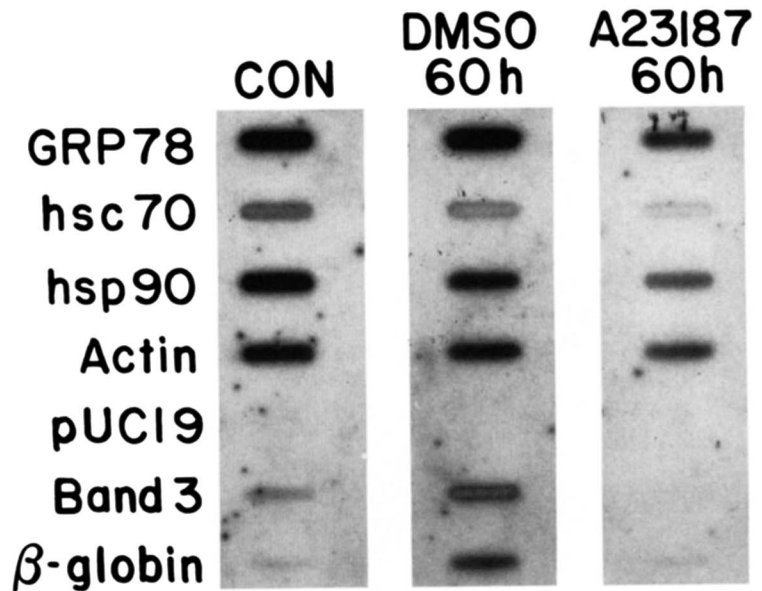


Fig 4. β -globin and Band 3 genes are not transcriptionally activated despite commitment to differentiation of MEL cells in A23187. Relative transcription rates were determined by labeling of run-off transcripts with [32 P]-UTP as described by Greenberg and Ziff.¹⁹ Times of exposure (60 hours) to either DMSO (D) or A23187 (A) before labeling of run-off transcripts are indicated above each blot. The labeled transcripts were hybridized with an excess of linearized plasmid DNA that had been previously bound to nitrocellulose filters. The plasmid DNAs used in these experiments are described in Materials and Methods and include DNA fragments encoding mouse actin, Band 3, β -globin, hsc70, hamster GRP78, and human HSP90. pUC19 plasmid DNA was used as a negative control. The filters were developed by autoradiography.

ionomycin induced commitment to differentiation in a dose-dependent manner. However, in contrast to A23187, cells induced to differentiate in ionomycin accumulate β -globin mRNA at a rate approximately equal to cells induced in DMSO (Fig 5B). Thus, ionomycin is capable of inducing commitment to differentiation and concomitant erythroid gene expression in a manner similar to DMSO and other, previously described inducing agents.

The effects of ionomycin on commitment and erythroid gene expression suggested that a decrease in cytosolic calcium is neither necessary for the occurrence of commitment to differentiation (as previously suggested for DMSO),^{10,35} nor responsible for inhibiting erythroid gene expression (as in cells exposed to A23187). To be certain that the ionophore concentrations used in these experiments were sufficient to increase cytosolic calcium, this parameter was measured in cells exposed to these agents using fura2 fluorescence. Because cytosolic calcium concentration cannot be determined in cells exposed to A23187 (the ionophore has a fluorescent spectra similar to fura2), the 4-bromo-analog of A23187 was used in these studies. This agent has been shown to have similar effects on calcium permeability, but has a different fluorescent spectrum than the parental compound.³⁶ At a concentration of 4-bromo-A23187 of 0.8 μ g/mL (equimolar to 0.72 μ g/mL A23187), cytosolic calcium increased from 155 nmol/L to 475 nmol/L (see Table 1). A similar effect on final cytosolic calcium concentration was also seen at a 4-bromo-A23187 concentration of 0.4 μ g/mL, although the time required to reach this level was longer. Ionomycin at concentrations ranging from 0.5 to 1.5 μ mol/L similarly increased cytosolic calcium, up to 435 nmol/L at the latter concentration. Consistent with previous findings, DMSO produced a small decrease in cytosolic calcium concentration (data not shown).

These results demonstrated that acute exposure to these ionophores resulted in an increase in cytosolic calcium. However, it is possible that the cells rapidly adapt to this

increase and, hence, cytosolic calcium concentrations might not be increased by the time the cells began to commit to differentiate. Therefore, we investigated whether cytosolic calcium concentration remained elevated in cells exposed to the ionophores. To obtain these measurements, cells were grown for either 13 or 48 hours in ionomycin and loaded with fura2 in the same medium. Cytosolic calcium concentration was determined with the cells in their normal growth medium, with ionomycin added. These studies demonstrated that at 13 and 48 hours of ionomycin exposure cytosolic calcium remained elevated, at 399 nmol/L and 416 nmol/L, respectively. Cytosolic calcium concentration in control cells measured under identical conditions was 262 nm. Thus, calcium ionophores induce commitment to differentiation while producing a sustained increase in cytosolic calcium concentration.

DISCUSSION

The data presented in this paper demonstrates that exposure of MEL cells to A23187 is sufficient to induce commitment to differentiation. However, this inducer differs from those previously described because continued exposure to the ionophore, while inducing commitment to differentiation, inhibits expression of at least two genes that are characteristic of differentiated erythroid cells. These findings confirm that commitment to differentiation is a distinct event from the ultimate expression of the erythroid phenotype in inducer-exposed MEL cells. The demonstration that ionomycin can also induce differentiation of MEL cells, but does not inhibit β -globin gene expression, suggests that an increase in cytosolic calcium can trigger commitment and that this increase is not responsible for the inhibition of erythroid gene expression in cells exposed to A23187.

It has been previously demonstrated that a 1-hour exposure to A23187 eradicates the latent period of differentiation in MEL cells subsequently exposed to DMSO.⁶⁹ The

ability of A23187 to induce commitment to differentiation was not detected in these studies. The reason for these differences may be the intrinsic properties of A23187 that influence its potency. A23187 is a hydrophobic molecule that binds to albumin and partitions into cell membranes.³⁷

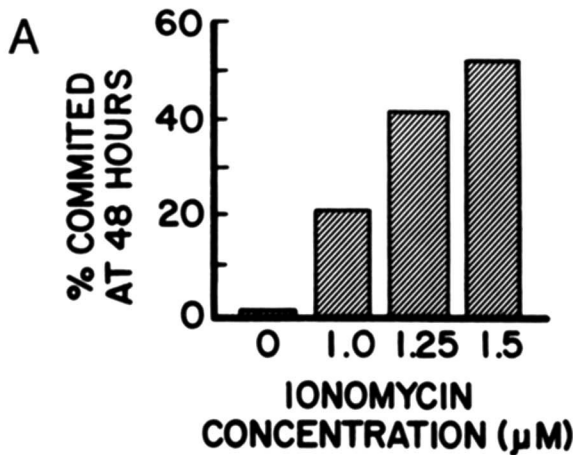


Fig 5. The calcium ionophore, ionomycin, induces both commitment to differentiation and hemoglobinization of MEL cells. (A) Ionomycin induces commitment to differentiation of MEL cells. MEL cells were incubated for 48 hours with ionomycin at the concentrations indicated below the graph. Commitment to differentiation was determined as described in the text. Cloning efficiencies for the ionomycin treated cells (0.5 μg/mL, 94%, 1.0 μg/mL, 71%, 1.5 μg/mL, 100%) were similar to control cells (77%). (B) Ionomycin induces β-globin mRNA accumulation in MEL cells. MEL cells were exposed to DMSO (1.5% vol/vol) or ionomycin (1.5 μmol/L) for 65 hours and cytoplasmic RNA extracted as described in the text. Ten micrograms of RNA was analyzed for β-globin mRNA by Northern blotting and hybridization with a [³²P]-labeled fragment of the mouse β-globin gene.

Table 1. Effects of Calcium Ionophores on Cytosolic Calcium Concentration

Ionophore	Concentration	Cytosolic Calcium
Control	—	155 nmol/L
4-bromo-A23187	0.4 μmol/L	475 nmol/L
	0.8 μmol/L	475 nmol/L
Ionomycin	0.5 μmol/L	380 nmol/L
	1.0 μmol/L	390 nmol/L
	1.5 μmol/L	435 nmol/L

Cells were loaded with fura2 AM in buffered solution containing 12% FCS. Ionophore was added at the concentration indicated and cytosolic calcium concentration determined as described in Materials and Methods.

Because its effective concentration is relative to the concentration of cell membranes in the medium, at low cell concentrations A23187 has greater effects as an ionophore than at higher cell concentrations. Thus, both serum concentration and cell concentration must be standardized in determining the optimal dose. In the previous experiments only brief exposures to A23187 were evaluated because toxicity was noted with prolonged exposures. It is likely that these observations were made under different conditions than those used in the experiments presented here. The observation of commitment in our early experiments likely resulted from the serendipitous occurrence of conditions that produced an effective dose of A23187 with limited toxicity. Using this initial observation as a starting point, optimal inducing conditions were then established as described here.

Previous experiments have demonstrated that inhibition of hemoglobin accumulation by imidazole does not affect commitment to differentiation of MEL cells simultaneously exposed to DMSO.³⁸ Our results confirm those observations and extend them to include Band 3 gene expression, but differ in that the ability to induce commitment and the ability to block hemoglobinization are shared properties of a single agent. A23187 inhibits transcriptional activation of both the β-globin and Band 3 genes, as demonstrated in the nuclear run-off experiments. Thus, commitment to differentiation of these cells is most closely linked to their loss of immortality and represents a distinct event that can be separated from the subsequent transcriptional activation of lineage-specific genes.

The effects of A23187 on transcription of erythroid genes cannot be simply attributed to an increase in cytosolic calcium concentration. Exposure of MEL cells to an equimolar concentration of ionomycin, which produces a similar affect on cytoplasmic calcium concentration (1.5 μmol/L ionomycin/435 nmol/L [Ca²⁺], 1.4 μmol/L 4-bromo-A23187/475 nmol/L [Ca²⁺]), induces commitment but does not inhibit β-globin gene expression. The continued transcription of the actin, hsc70, hsp90, and GRP78 genes suggests that the transcriptional activation of newly induced genes is most sensitive to inhibition by this agent. Our data do not allow us to draw a conclusion as to the mechanism of this effect. However, the ability of ionomycin to induce commitment without inhibiting erythroid gene expression suggests

that the difference in selectivity for cations of these two ionophores may underlie their differing effects on gene expression.

The mechanisms by which agents as diverse as DMSO, hypoxanthine, X-irradiation, and actinomycin D act to induce commitment to differentiation of MEL cells remains unknown. The ability of the calcium ionophores, ionomycin and A23187, to induce commitment to differentiation suggests that an increase in cytosolic calcium concentration may trigger commitment. Although our data does not exclude that the ionophores have a synergistic effect with the low levels of DMSO used to solubilize them, this interpretation would still suggest that calcium has a role in triggering commitment. Early experiments suggested that an increase in cytosolic calcium concentration played a critical role in inducing commitment.^{6,7,9} More recently,

Shibata et al¹¹ have also demonstrated that MEL cells induced to differentiate by the β -subunit of the hormone inhibin increase their cytosolic calcium. In contrast, Arrow and Macara have demonstrated that DMSO decreases cellular calcium levels in MEL cells.¹⁰ Thus, the role of changes in calcium concentration in triggering commitment is uncertain. However, it seems likely that an increase in cytosolic calcium concentration is one possible mechanism that may be used by inducing agents to trigger differentiation.

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