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Ionic Regulation of MEL Cell Commitment

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A key event in the initiation of the dimethyl sulfoxide (DMSO)-induced program of murine erythroleukemia (MEL) cell differentiation is a rise in the level of cytoplasmic calcium ions. Our interest in the present study is whether other inducers of the terminal erythroid differentiation program also act via a calcium-dependent pathway. Inhibition of calcium transport has been found to prevent the induction of MEL cell commitment by DMSO, butyric acid (BA), or hypoxanthine (HX). Enhancement of the calcium flux rate with A23187 or elevation of cytoplasmic calcium levels with FCCP stimulates the kinetics of commitment in response to all three inducers. These results suggest that of the inducers we have tested (DMSO, BA, and HX), all three act to initiate commitment via a common mechanism which involves modulation of cytoplasmic calcium levels.

Key words: DMSO, murine erythroleukemia, butyric acid, hypoxanthine, cytoplasmic calcium

The ability of MEL cells to undergo a program of terminal erythroid differentiation in response to an inductive stimulus has made this a valuable in vitro model for studying erythroid differentiation. By exposing cells to an inducer of differentiation, such as DMSO, then cloning cells in the absence of the inductive signal, we have shown that cells become irreversibly committed to the terminal differentiation pathway in a stochastic fashion [1]. Commitment to terminal differentiation is normally preceded by a lag period of about 9–12 hr [1]. Recently, we have demonstrated that over the course of this lag, changes in ion fluxes across the plasma membrane occur which play a key role in regulating commitment to terminal differentiation [2–4]. In particular, an increase in the uptake and levels of cytoplasmic calcium ions has been found to be crucial for the initiation of the commitment process.

Several lines of evidence support the important role of calcium in regulating the process of MEL cell differentiation. Inhibition of calcium transport by amiloride will

Abbreviations Used: MEL, murine erythroleukemia; EGTA, ethylene glycol bis (β-aminoethyl ether)-N, N, N'-tetraacetic acid; $DiOC_6$, 3,3'-dihexyloxacarbocyanine iodide; FCCP, carbonyl cyanine p-trifluoromethoxy-phenyl hydrazone.

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prevent MEL cells from executing the commitment process [2]. Commitment can also be inhibited by chelation of calcium in the culture medium by EGTA [3,5]. Increasing the rate of calcium flux by treatment of cells with the calcium ionophore A23187 causes cells to undergo the commitment process without the 12-hr lag period normally observed after treatment with DMSO alone [4].

These results suggest that the lag period represents the time required for intracellular calcium levels to rise above a threshold level. A rise in calcium levels above this threshold would act as a signal for the initiation of the commitment process. Consistent with this hypothesis is the recent observation that in response to DMSO, MEL cells exhibit a significant decrease in the level of mitochondria-associated fluorescence of a probe sensitive to membrane potential, $DiOC_6$ [6]. This result suggests that the intracellular level of calcium ions critical for the initiation of MEL cell differentiation may be regulated by changes in mitochondrial membrane potential.

In the present study, we have extended these observations to include two other inducers of MEL cell differentiation, butyric acid (BA), and hypoxanthine (HX). Induction with these agents is sensitive to both amiloride and EGTA, reduces the level of mitochondria associated $DiOC_6$ fluorescence, and in concert with A23187 or FCCP erases the lag period prior to commitment. These results suggest that a common feature of MEL cells in response to challenge with several independent inducing agents is a critical increase in cytoplasmic calcium levels required for commitment.

MATERIALS AND METHODS

Cell Lines and Conditions

Cell line 745 was originally obtained by Dr C. Friend. A subclone of this cell line is 745-PC-4. All cultures were maintained in α medium lacking nucleosides and supplemented with 13% fetal calf serum (FCS, GIBCO). Cell density was maintained between 2×10^4 and 5×10^5 cells/ml to maintain continuous logarithmic growth. Cell counts were performed with an automatic cell counter (Coulter Counter Model ZBI, Coulter Electronics). Plasma culture was carried out as described previously [1,2,6]. Briefly, 100–200 cells were plated in 0.1 ml clots in microtiter wells (Linbro Scientific, Hamden, Connecticut) which had been sterilized by ultraviolet radiation. Cells were always plated in the absence of inducers or drugs. The clots were incubated at 37°C in a humidified CO_2 atmosphere. Clots were transferred to microscope slides after 90–100 hr of culture, dehydrated with filter paper, fixed in glutaraldehyde, stained in benzidine, counter-stained in hematoxylin, and covered with permount and a coverslip. Scoring of colony type was performed at a magnification of $\times$ 100.

Chemicals

Amiloride was a gift from Merck, Sharp and Dohme. A23187 was provided by Eli Lilly Co., Indianapolis, Indiana. Ouabain, monensin, FCCP, and EGTA were from Sigma. $DiOC_6$ was from Eastman Kodak.

Fluorescence Measurements

The accumulation of $DiOC_6$ into MEL cells was measured according to the method described previously by Shapiro et al [7] and Levenson et al [6]. MEL cells were adjusted to a density of 5×10^5 cells/ml in α medium lacking FCS, and 10 μl of a 5 μM solution of $DiOC_6$ in ethanol was added per milliliter of cells. Cells and

TABLE I. Effect of Amiloride and EGTA on MEL Cell Differentiation*

Inducer (48 hr)	Inhibitor	% Committed colonies
DMSO (1.5%)	None	47
DMSO (1.5%)	Amiloride	1
DMSO (1.5%)	EGTA (2.7 mM)	2
Butyric acid (1 mM)	None	41
Butyric acid (1 mM)	Amiloride	0
Butyric acid (1 mM)	EGTA	1
Hypoxanthine (500 μg/ml)	None	46
Hypoxanthine (500 μg/ml)	Amiloride	2
Hypoxanthine (500 μg/ml)	EGTA	2

*MEL cells were grown in liquid culture with the additions specified above. After 48 hr of treatment, an aliquot of cells was removed from each culture and plated in plasma culture in the absence of inducers or inhibitors. Plasma clot procedure and determination of the proportion of B^+ colonies was as described in Materials and Methods.

dye were allowed to equilibrate for 15 min at 37°C. After incubation, samples were analyzed employing an SLM spectrophotometer (SLM Co, Urbana, Illinois) with excitation at 490 nm and emission at 507 nm.

RESULTS

Initial studies in our laboratory demonstrated that inhibition of calcium transport with amiloride [2,7] or chelation of calcium in the culture medium with EGTA [3,4] were extremely effective inhibitors of DMSO-induced MEL cell differentiation. Here we have examined the effect of amiloride and EGTA on BA- and HX-induced MEL cell differentiation. As shown in Table I, it is clear that as reported by Leder and Leder [8], treatment of MEL cells with 1 mM butyric acid is effective in causing commitment to terminal erythroid differentiation. However, treatment with 10 μg/ml amiloride or 2.7 mM EGTA are extremely effective in blocking the induction of differentiation caused by butyric acid. Similarly, treatment of MEL cells with 500 μg/ml hypoxanthine causes 46% of the cells to become committed during the treatment period. Hypoxanthine treated cells, however, are also blocked from undergoing the differentiation process by the addition of amiloride or EGTA. These results suggest that DMSO, BA, and HX cause induction of MEL cells by a pathway involving a calcium dependent process.

To further explore the relationship between inducer action and alterations in cytoplasmic calcium levels, we have examined the effect of DMSO, BA, and HX on the level of $DiOC_6$ fluorescence. Our previous data indicate that the intracellular level of $DiOC_6$ fluorescence may serve as an indicator of mitochondrial membrane potential, and that the level of mitochondria-associated $DiOC_6$ fluorescence decreases in response to DMSO [6]. These results are consistent with the data in other systems which suggest that depolarization of the mitochondrial membrane leads to an increase in cytoplasmic Ca^{2+} levels [9].

An experiment in which the level of mitochondria associated $DiOC_6$ fluorescence has been measured in inducer treated cells is shown in Table II. Treatment of MEL cells with DMSO, BA, or HX causes a reduction in the relative fluorescence of

TABLE II. Relationship Between Inducer Treatment and Mitochondrial Membrane Potential*

Inducer (24 hr)	Inhibitor (24 hr)	% B$^+$ Colonies	Fluorescence/cell
None	None	0	100
None	Amiloride	0	100
None	EGTA (2.7 mM)	1	95
1.5% DMSO	—	17	68
1.5% DMSO	Amiloride	0	92
1.5% DMSO	EGTA	0	91
1 mM Butyric acid	—	15	69
1 mM Butyric acid	Amiloride	0	96
1 mM Butyric acid	EGTA	0	92
500 μg/ml hypoxanthine	—	19	65
500 μg/ml hypoxanthine	Amiloride	2	93
500 μg/ml hypoxanthine	EGTA	1	89

*MEL cells were grown in liquid culture under the conditions described above. At 24 hr, an aliquot of cells was removed from each culture and split in half. One aliquot of cells was plated in plasma culture as described in Table I to assess the differentiation status of each culture. The accumulation of the fluorescent dye $DiOC_6$ in the second aliquot was measured according to the procedure described in Materials and Methods.

TABLE III. Time Course of $DiOC_6$ Fluorescence During Differentiation*

Inducer	Time of treatment	Fluorescence/cell	% B$^+$ Colonies
Control	9 hrs	100	0
Control	14 hrs	100	0
1.5% DMSO	9 hrs	92	1
1.5% DMSO	14 hrs	71	8
1 mM BA	9 hrs	94	0
1 mM BA	14 hrs	74	7
500 μg/ml HX	9 hrs	95	2
500 μg/ml HX	14 hrs	69	11

*MEL cells were grown in liquid culture in the presence of 1.5% DMSO, 1 mM BA, or 500 μg/ml HX. At the times indicated, aliquots of cells were removed from each culture and plated in plasma clots as described in Materials and Methods. The accumulation of the fluorescent dye $DiOC_6$ in each sample was measured as in Table II.

$DiOC_6$ per cell of 30–35% 24 hr after addition of inducer. However, the addition of either amiloride or EGTA to any of the inducer-treated cultures blocks the reduction in probe fluorescence. These results support the view that all three inducers—DMSO, BA and HX—cause changes in MEL cell mitochondria which may be important in the regulation of cytoplasmic calcium levels.

We have previously shown that in response to DMSO, the change in the level of mitochondria associated $DiOC_6$ fluorescence is correlated with the end of the lag period, very close to the time of the commitment event [6]. An experiment which extends this observation is shown in Table III. Here we have measured the level of $DiOC_6$ fluorescence as a function of time after addition of inducer. The data clearly demonstrate that no significant decrease in $DiOC_6$ fluorescence occurs after 9 hr after treatment with DMSO, BA, or HX. The 9-hr time point is within the lag period for all three inducers and no significant increase in committed cells is observed at that time. However, after 14 hr of treatment with DMSO, BA, or HX, a decrease of 25–

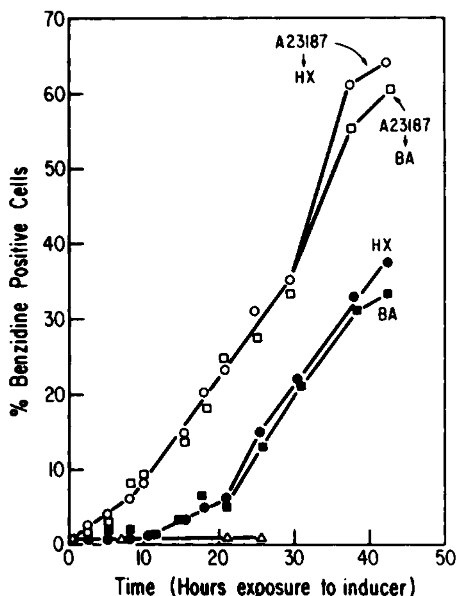

Fig. 1. Effect of A23187 on the lag period. Murine erythroleukemia (MEL) cells were treated with 1 μg/ml A23187 for 1 hr. A23187 was then removed and cells were grown in 1 mM butyric acid (BA, □—□) or 500 μg/ml hypoxanthine (HX, ○—○) for the remainder of the experiment. A control culture was grown continuously in 1 mM BA (■—■) or 500 μg/ml HX (●—●). A final culture was treated continuously with 1 μg/ml A23187 (△—△). At the times indicated, aliquots of cells were removed from liquid culture and plated in plasma clots. The proportion of B^+ colonies was determined as in Materials and Methods.

31% is observed in $DiOC_6$ fluorescence levels. This result suggests that changes occur in MEL cell mitochondria at or near the end of the lag period, regardless of the inducer used.

These results lead to the prediction that if cytoplasmic calcium levels rise above a threshold prior to the addition of inducer then the lag period can be eliminated. Our previous data suggest that following a brief exposure of cells to the calcium ionophore A23187 (to increase Ca^{2+} flux rate) or the proton ionophore FCCP (to cause release of mitochondrial calcium stores) cells initiate commitment to terminal differentiation with no lag upon addition of DMSO [4,6]. As shown in Figure 1, a 1-hr exposure to A23187 followed by induction with BA or HX serves to eliminate the lag period. Similarly, as presented in Figure 2, a 1-hr exposure to FCCP followed by BA or HX treatment also eliminates the lag. These results are consistent with the view that a common effect of DMSO, BA, or HX on the differentiation of MEL cells is to cause an increase in cytoplasmic calcium ions which is crucial for the initiation of the commitment process.

DISCUSSION

A central unresolved issue in the analysis of MEL cell differentiation is the identification of the target or targets of inducer action. A significant limitation in the

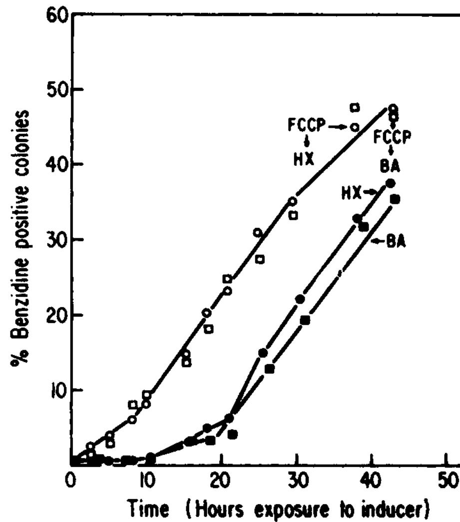

Fig. 2. Effect of FCCP on the lag period. MEL cells were treated with 10 μg/ml FCCP for 1 hr. FCCP was removed and cells were grown in 1 mM butyric acid (□—□) or 500 μg/ml hypoxanthine (○—○) for the remainder of the experiment. A control was grown continuously in 1 mM BA (■—■) or 500 μg/ml HX (●—●). The proportion of B^+ colonies was determined at the indicated times, as described in Figure 1.

analysis of this problem has been that the program of MEL cell differentiation is elicited by a remarkably diverse range of molecules which differ from one another both structurally and (presumably) mechanistically [10]. Interestingly, the response of individual MEL cells to challenge with several independent inducers indicates that in all cases, a lag period of at least 9 hr occurs followed by a stochastic commitment to the terminal differentiation program [11,12]. Furthermore, when cells treated with one inducer are shifted to treatment with a second inducer during the lag period, no measurable delay is observed in the kinetics of commitment of the cells [12]. This experiment strongly suggests that the three inducers tested (DMSO, BA, and HX) appear to induce commitment by initiating the same series of events during the lag period.

The hypothesis that DMSO, butyric acid, and hypoxanthine seem to initiate MEL cell commitment via a common mechanism is further substantiated by the results presented here. This conclusion is based on the following results: (1) Inhibition of calcium transport by amiloride or chelation of calcium in the culture medium by EGTA prevents the induction of MEL cell differentiation by DMSO, BA, or HX. 2) All three inducers cause a reduction in the level of mitochondria-associated $DiOC_6$ fluorescence. The decrease in the level of $DiOC_6$ fluorescence always occurs at a point very close to the initiation of commitment. 3) Amiloride and EGTA prevent the decrease in $DiOC_6$ fluorescence caused by DMSO, BA, or HX. This result supports the idea that MEL cell mitochondria may play an important role in the regulation of cytoplasmic calcium levels. 4) Following a brief (1-hr) exposure to A23187 or FCCP, MEL cells can initiate the commitment process with no lag upon exposure to either DMSO, HX, or BA.

The use of the fluorescence probe $DiOC_6$ to monitor changes in MEL cell mitochondria bears consideration. The alteration in $DiOC_6$ fluorescence levels at the time of MEL cell commitment may reflect depolarization of the mitochondrial membrane. This view is supported by the following results: First, dissipation of plasma membrane potential has virtually no effect on $DiOC_6$ fluorescence, whereas $DiOC_6$ fluorescence is reduced either by treatment of MEL cells with inducers or agents thought to alter mitochondrial membrane potential [6]. Secondly, exposure of both DMSO-treated and untreated cells to the K^+, H^+ ionophore nigericin resulted in an increase in cyanine dye fluorescence. However, the fact that the fluorescence levels in DMSO treated cells were still significantly reduced compared to control cells suggests that the total electrochemical gradient is reduced during MEL cell differentiation [6]. It is possible that the change in cyanine dye fluorescence in response to inducer action could be a reflection of changes in mitochondrial pH rather than mitochondrial membrane potential. While this possibility cannot be ruled out, our attempts to alter intracellular pH levels were found to have no effect on $DiOC_6$ fluorescence or the differentiation process [Levenson R, unpublished results]. This result may merely reflect the fact that $DiOC_6$ is relatively insensitive to small pH changes. Experiments designed to directly assess the role of mitochondrial pH changes during MEL cell differentiation would therefore be of value.

The data presented both here and previously [2,6] suggest a relationship between the regulation of cytoplasmic calcium levels and alterations in mitochondria at the time of MEL cell commitment. It is possible that at the end of the lag period, mitochondria lose their ability to buffer cytoplasmic calcium levels. Cytoplasmic calcium levels would then rise above a threshold necessary to signal the initiation of commitment. Studies carried out in isolated mitochondria support this view. These studies indicate that mitochondrial membrane potential is the driving force for calcium uptake and that the rate of calcium uptake correlates linearly with a reduction in mitochondrial membrane potential [16]. Thus amiloride and EGTA prevent the reduction in mitochondria-associated cyanine dye fluorescence associated with the influx of calcium ions into MEL cells during the lag period.

We have previously shown that FCCP causes release of calcium from MEL cell mitochondria [6]. One possible explanation for increased calcium levels in MEL cells at the time of commitment may be the release of mitochondrial calcium stores. Treatment of digitonin-lysed cells with high Na^+ levels did not cause release of calcium from MEL cell mitochondria [6]. Thus cytoplasmic calcium levels most likely do not rise via Na^+/Ca^{2+} exchange across the mitochondrial membrane. It is also possible that increased cytoplasmic calcium levels could be due to changes in the level of ATP synthesis. This possibility is unlikely, however, for we have found that cellular ATP levels remain virtually unchanged during MEL cell differentiation [Macara IG, unpublished observations].

One of the earliest detectable events in MEL cell differentiation is a decrease in rubidium uptake via the plasma membrane Na^+/K^+-ATPase [13,14]. This observation suggests that the Na^+/K^+-ATPase may be a primary target of inducer action. Recent data from our group [15] suggest that in response to DMSO, calcium entry into MEL cells is regulated by a Na^+/Ca^{2+} antiport system. The reduced activity of the Na^+ pump following DMSO treatment may cause a sufficient increase in cytoplasmic Na^+ levels to reverse the direction of the Na^+/Ca^+ antiport. Calcium would thus enter rather than exit the cell through the antiport system. Amiloride apparently

acts to inhibit DMSO induced MEL cell differentiation by preventing Ca^{2+} influx through the Na^{+}/Ca^{2+} antiport. It will be interesting to analyze whether other inducers also facilitate calcium entry into MEL cells via this transport system.

Our results do not as yet permit us to distinguish between the possibility that DMSO and other inducers act directly on the Na^{+} pump or whether some other cellular structure or process is the primary target of inducer action. The ability to distinguish between these two possibilities should prove crucial in understanding how the initiation of the terminal differentiation program in the MEL cell system is controlled.

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