Volume 236, number 2, 401–405 FEB 06265 August 1988

Differentiation-associated changes of cation-transport activities in myeloid leukemic cell lines

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Received 8 June 1988; revised version received 14 July 1988

Induction of differentiation in HL-60 and U-937 leukemic cell lines, resulted in 1.5–10-fold increase in $^{45}Ca^{2+}$ uptake. The increased $^{45}Ca^{2+}$ uptake in the differentiating cells was inhibited by verapamil, cromolyn and amiloride. Elevation in Ca^{2+} uptake in differentiating cells was also demonstrated using the fluorescent probe, fura-2 acetoxymethyl ester. The increased $^{45}Ca^{2+}$ uptake was accompanied by a decrease in ouabain-insensitive and -sensitive $^{86}Rb^{+}$ uptake. Furthermore, correlation between the changes in these activities was observed. Modulation of extracellular pH affected differentiation: higher pH increased the extent of differentiation.

Ca^{2+}; $^{86}Rb^{+}$ transport; Retinoic acid; (HL-60 leukemic cell, U-937 leukemic cell)

1. INTRODUCTION

Differentiation of leukemic cell lines is accompanied by morphological, biochemical and functional changes [1]. There are controversial reports regarding the role of calcium in the differentiation process. Requirement of extracellular Ca^{2+} for differentiation of MEL and U-937 cell lines was demonstrated [2–4]. Increased Ca^{2+} uptake, associated with maturation of erythroid cells was shown [5,6]. On the other hand, there are reports suggesting that Ca^{2+} is not required for differentiation of HL-60 and M-1 cell lines [7,8]. Furthermore, no change in Ca^{2+} uptake could be observed in MEL cells [9]. Changes in uptake activities of monovalent cations, were also reported to occur during the differentiation of myeloid cell lines. Decreased Na^{+}/K^{+}-ATPase activity [5,9,10] and increased Na^{+}/H^{+}-antiporter activity [12,13] were demonstrated.

In this report we describe changes in uptake of Ca^{2+} and Rb^{+}, accompanying differentiation, and the effect of pH on the differentiation.

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Abbreviations: MEL, murine erythroleukemia cell line; NBT, nitro-blue tetrazolium; PMA, phorbol 12-myristate-13-acetate; $[Ca^{2+}]_i$, cytosolic free calcium concentration; fura-2AM, fura-2 acetoxymethyl ester

2. MATERIALS AND METHODS

$^{45}Ca^{2+}$ (10–40 mCi/mg) and $^{86}Rb^{+}$ (1–2 mCi/mg) were from Amersham. All other chemicals were purchased from Sigma, if not otherwise indicated. Fura-2AM was purchased from Molecular Probs Inc., Oregon. Culture media (RMPI-1640), fetal calf serum, L-glutamine, and a mixture of penicillin, streptomycin and Nystatin were purchased from Beth-Haemek Cell Culture, Israel. The cell lines HL-60 and U-937 were kindly provided by Dr M. Rubinstein (The Weizmann Institute of Science). Cells were grown in RMPI-1640 and 10% fetal calf serum, supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 12.5 U/ml Nystatin, at 37°C in a humidified 5% CO_2 incubator. Viability was determined by Trypan-blue exclusion (>95%). Cell differentiation was evaluated by PMA-stimulated NBT reduction activity [1] and is illustrated in the inset to the figures.

The buffers used for Ca^{2+} uptake were PBS supplemented with 10 mM glucose and 0.5 mM $MgCl_2$ (A) and the same plus 1 mM $CaCl_2$ (B). The buffer used for Rb^{+} uptake contained 140 mM NaCl, 8 mM Na_2PO_4, 0.13 mM NaH_2PO_4, 10 mM glucose and 0.5 mM $MgCl_2$ (C).

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00145793/88/$3.50

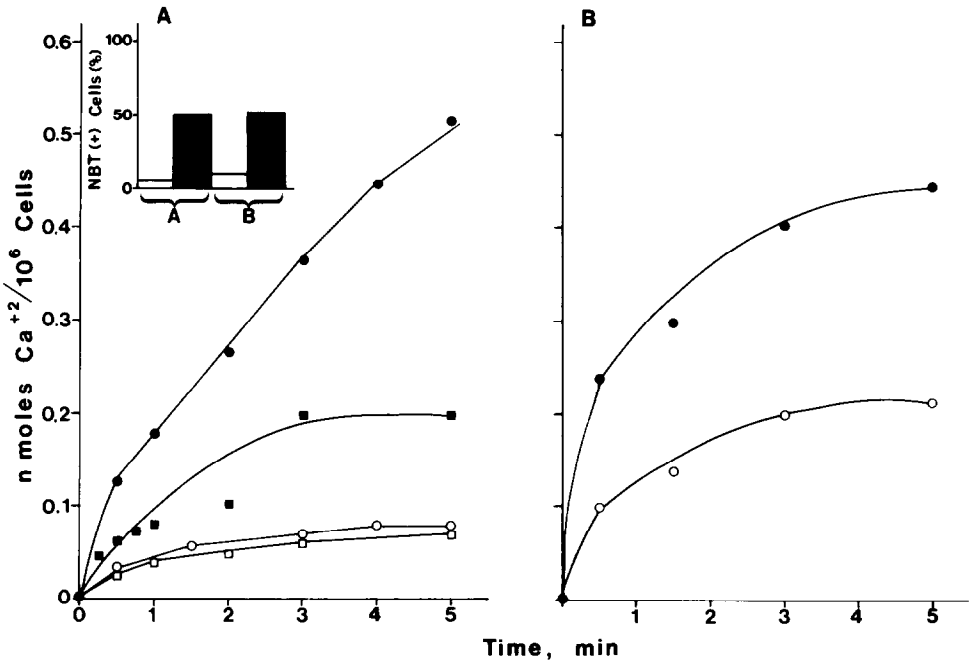

Fig.1. $^{45}Ca^{2+}$ uptake in HL-60 (A) and U-937 (B) cells measured at 4°C (○,●) and at 25°C (□,■) in cells grown for 48 h without (○,□) and with 5×10^{-6} M retinoic acid (●,■). Inset: NBT reduction activity, control (blank bars) and differentiating cells (black bars).

2.1. *Measurement of $^{45}Ca^{2+}$ uptake*

Cells were centrifuged at 1000 rpm for 5 min and resuspended in buffer A to 2×10^6 cells/ml. Unless otherwise indicated, $^{45}Ca^{2+}$ uptake was conducted at 4°C and was initiated by addition of 0.2 mM $^{45}CaCl$ (100 μCi/μmol). Samples were analyzed using separation on Tris-Dowex columns as described [14].

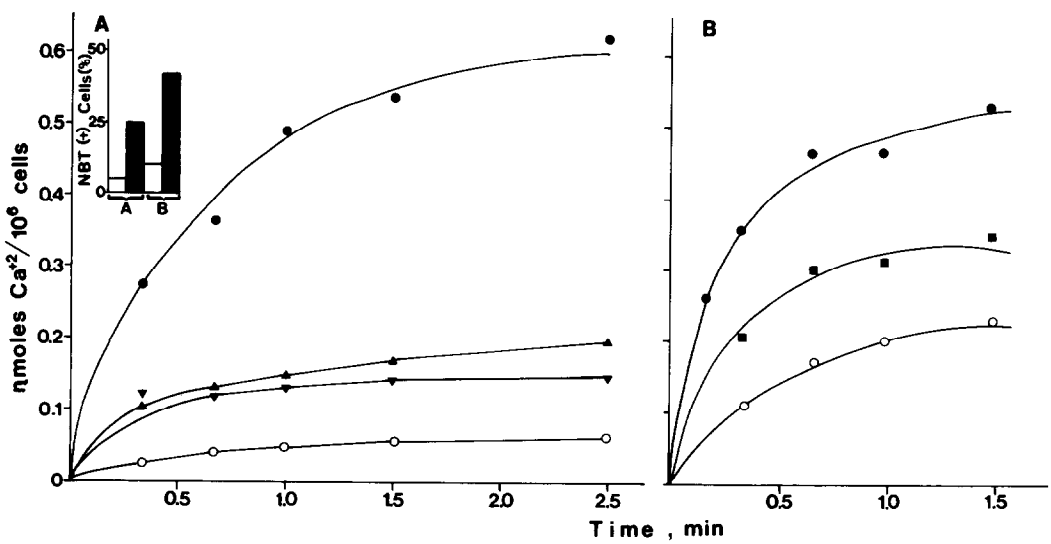

Fig.2. The effect of verapamil, cromolyn and amiloride on $^{45}Ca^{2+}$ uptake of differentiating HL-60 cells. Cells were grown for 48 h in the absence (○) and presence of 5×10^{-6} M retinoic acid (●,▲,▼,■). Prior to the uptake assay the differentiating cells were incubated for 30 min at room temperature in the absence (●) and presence of verapamil 100 μM (▲), cromolyn 500 μM (▼) and amiloride 50 μM (■). (A) and (B) represent two independent experiments. Inset: As in fig.1.

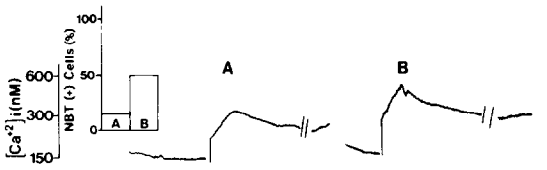

Fig.3. Increased $[Ca^{2+}]_i$ in undifferentiated and differentiating U-937 cells. Cells were grown for 48 h in the absence (A) and presence of 5×10^{-6} M retinoic acid (B). Changes in $[Ca^{2+}]_i$ were measured upon addition of 1 mM $CaCl_2$ to fura-2-loaded cells at 37°C. Inset: As in fig.1.

2.2. *Measurement of $^{86}Rb^+$ uptake*

2×10^6 cells/ml were resuspended in buffer C. The uptake at 37°C was initiated by addition of 2 mM ^{86}RbCl (4–6 mCi/mmol), samples were analyzed using Tris-Dowex columns.

2.3. *Fluorescence measurements*

Suspensions of 10^7 cells/ml in buffer B, were incubated with 1 μM fura-2AM for 40 min at 37°C. Loading by the fluorescent probe was terminated by 10-fold dilution, centrifugation and resuspension in buffer B. Fluorescence measurements (340 nm excitation, 500 nm emission) were conducted using a Perkin Elmer MPF44B spectrofluorometer. Cells at 8×10^5/ml, in buffer A containing 80 μM $CaCl_2$, were preequilibrated for 5 min at 37°C under continuous stirring. The changes in fluorescence upon addition of 1 mM $CaCl_2$ were recorded. The $[Ca^{2+}]_i$ was calculated from the maximal (in the presence of 0.2% Triton X-100) and minimal (in the presence of 5 mM EGTA) fluorescence levels as described [15].

3. RESULTS

Growth of the promyelocytic HL-60 and the monoblastic U-937 cell lines, for 48 h, in the presence of retinoic acid, resulted in an elevation of Ca^{2+} uptake activity. The increased uptake in the two cell lines was expressed in an enhanced rate of influx, and a higher steady-state level (fig.1A and B). Enhanced Ca^{2+} uptake at 4°C as well as at 25°C was observed (fig.1A). The extent of increase, which was higher at the lower temperature, is likely to result from decreased excretion and mobilization into organelles of cytoplasmic Ca^{2+} [14]. The elevated Ca^{2+} uptake was inhibited, up to 70% by Ca^{2+} antagonists verapamil (100 μM) and cromolyn (500 μM), amiloride (50 μM) caused an inhibition up to 40% (fig.2).

The fluorescent probe, fura-2 which binds specifically cytoplasmic Ca^{2+}, was utilized to measure cellular Ca^{2+} concentration. The recorded basal values of $[Ca^{2+}]_i$ (in nM) in undifferentiated cells were 158.8 ± 11.2 ($n = 3$) in HL-60 and 145.5 ± 16.4 ($n = 9$) in U-937. Following 48 h of growth in the presence of 5×10^{-6} M retinoic acid, the basal values of $[Ca^{2+}]_i$ were 159.2 ± 12.0 ($n = 4$) in HL-60 and 134.4 ± 20.0 ($n = 5$) in U-937. The similarity of the above measured $[Ca^{2+}]_i$ values

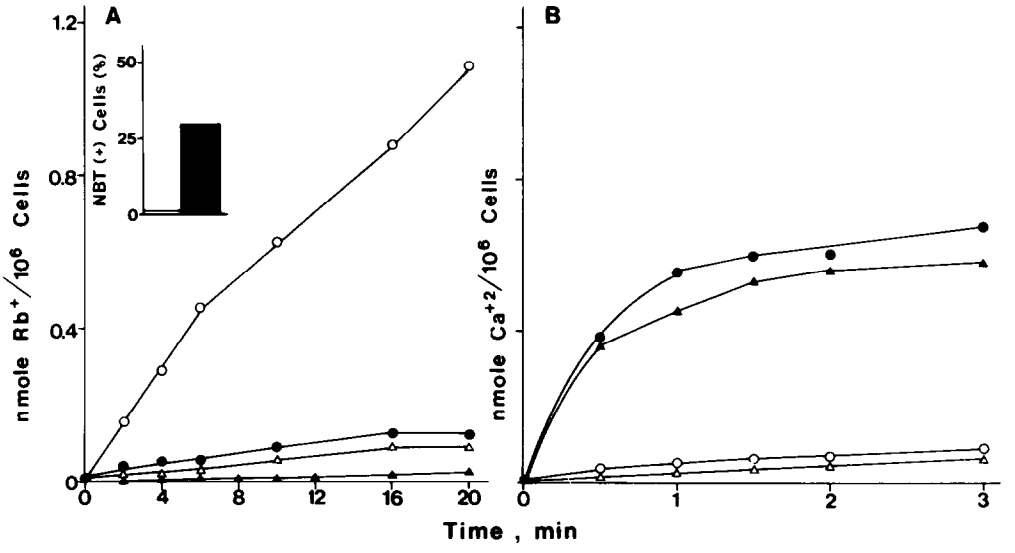

Fig.4. $^{86}Rb^+$ (A) and $^{45}Ca^{2+}$ (B) uptake in HL-60 cells. Cells were grown for 48 h without (○, △) and with 5×10^{-6} M retinoic acid (●, ▲). The effect of ouabain was examined by preincubating the cells for 30 min at 37°C in the absence (○, ●) and presence (△, ▲) of ouabain 10 μM prior to the uptake assay. Inset: As in fig.1.

strongly suggests that the basal $[Ca^{2+}]_i$ in these cells does not change upon differentiation.

Fig.3 demonstrates a representative experiment, in which the changes in $[Ca^{2+}]_i$ were recorded upon addition of 1 mM $CaCl_2$ to undifferentiated and differentiating U-937 cells suspended in low Ca^{2+} buffer (80 μM). The extent of increase in $[Ca^{2+}]_i$ was higher in the differentiating cells, 1.7-fold in undifferentiated compared with 3.5-fold in differentiated cells. Similar results were obtained with the HL-60 cell line (not shown). Verapamil at 100 μM, inhibited up to 70% of the increased $[Ca^{2+}]_i$. These results are consistent with the results obtained by measurement of $^{45}Ca^{2+}$ uptake (figs 1 and 2) and strongly suggest that the increase in cytosolic Ca^{2+} was due to uptake of extracellular Ca^{2+}.

Potassium transport was measured by ouabain-insensitive and -sensitive (via Na^+/K^+-ATPase) $^{86}Rb^+$ uptake. Fig.4 demonstrates that while the $^{86}Rb^+$ uptake decreased 10-fold (A), $^{45}Ca^{2+}$ uptake increased 10-fold (B). The extent of decrease in $^{86}Rb^+$ uptake correlated with the increase in $^{45}Ca^{2+}$ uptake in the differentiating cells (based on several independent experiments). Ouabain, 10 μM, inhibited 88% of the $^{86}Rb^+$-uptake activity in differentiating and undifferentiated cells. The ouabain-insensitive activity also decreased about 10-fold in differentiating cells. Ouabain had a negligible effect (up to 10% inhibition) on $^{45}Ca^{2+}$ uptake in undifferentiated and differentiated cells.

The differentiation of HL-60 cells evaluated by NBT reduction activity, was affected by extracellular pH. Increase in extracellular pH was associated with increase in differentiation (fig.5).

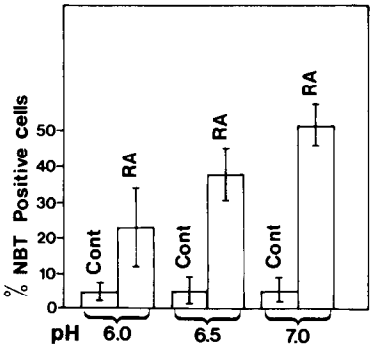

Fig.5. The effect of extracellular pH on NBT reduction activity. HL-60 cells were grown for 48 h in the absence (cont) and presence of 5×10^{-6} M retinoic acid (RA) in pH buffered medium.

4. DISCUSSION

Increased rate of uptake and elevated steady-state level of Ca^{2+} in differentiating myeloid leukemic cell lines, was demonstrated employing two distinct methods. The kinetics of $^{45}Ca^{2+}$ uptake reflects increased V_i and V_{max}, resulting in higher levels of $^{45}Ca^{2+}$ in the cells. Measurements of $[Ca^{2+}]_i$ with fura-2, demonstrated that the influx of extracellular Ca^{2+} caused a greater increase in $[Ca^{2+}]_i$ in differentiating compared with undifferentiated cells. The increased Ca^{2+} uptake, measured by the two methods, was only partially inhibited by Ca^{2+} antagonists, suggesting that more than one transport mechanism is involved. Despite the higher Ca^{2+}-uptake activity, no differences between the basal levels of $[Ca^{2+}]_i$ in undifferentiated and differentiating cells were observed. This suggests that the mechanisms for Ca^{2+} excretion and mobilization into intracellular organelles are activated as well as the pathways for Ca^{2+} entry. The increased Ca^{2+}-uptake activity in differentiating cells might be an expression of 'transport system maturation' necessary for the function of the mature cells.

Changes in transport activity of monovalent cations during maturation were demonstrated. Decrease in the Na^+/K^+-ATPase activity in differentiating leukemic cells was observed [10,11]. Regulation of this activity on the transcriptional level was reported [16]. In this report we have demonstrated an inverse correlation between Ca^{2+}- and K^+-uptake activities. Inhibition of Na^+/K^+-ATPase could possibly account for the operation in the reverse mode of the Na^+/Ca^{2+}-antiporter, i.e. Na^+ efflux and a Ca^{2+} influx [5,17,18]. Despite the inverse correlation between the two activities, inhibition of Na^+/K^+-ATPase in undifferentiated cells by ouabain, did not increase Ca^{2+} uptake, suggesting that the two activities are coordinately regulated. Since K^+-uptake activity in differentiating cells decreased, increased Ca^{2+}-uptake activity due to non-specific membrane permeabilization could be excluded.

In this report we have shown that the extent of differentiation can be modulated by external pH.

Activation of the Na^+/H^+-exchanger in differentiating HL-60 cells was demonstrated [12,13]. Moreover, acidic extracellular pH was reported to reduce the Na^+/H^+-exchange activity in HL-60 cells [13]. Therefore, it is assumed that the lower extent of differentiation at acidic extracellular pH could be attributed to reduced Na^+/H^+ exchange. Multiple changes in cations transport activities occurring during differentiation of leukemic cell lines were reported. The sequence by which they occur and their role in differentiation remain to be studied.

Acknowledgements: The authors acknowledge the kind help of Dr N. Shaklai and Dr Z. Keinan. This work was partially supported by a grant from the Maurits Van-Beets Fund for Leukemia Research.

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