Role of Intracellular Calcium Ion in Human Promyelocytic Leukemia HL-60 Cell Differentiation¹

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ABSTRACT

The relationship between calcium ions and the differentiation of human promyelocytic leukemia HL-60 cells was investigated. Proliferation of HL-60 cells incubated in calcium-free medium was inhibited without cell differentiation. On the other hand, incubation with 100 μM verapamil markedly inhibited cell proliferation and caused slight cell differentiation into monocytes. Both calcium-free medium and 100 μM verapamil enhanced HL-60 cell differentiation after treatment with 1 nM 1α,25-dihydroxyvitamin D₃, 1 nM β-all-trans-retinoic acid, or 0.75% dimethyl sulfoxide. However, no enhancement was obtained by treatment with 1 nM 12-O-tetradecanoylphorbol-13-acetate.

The free cytosolic calcium concentration was measured by the intracellularly trappable fluorescent calcium indicator, quin 2. The increase of intracellular calcium induced by 250 nM ionomycin was completely blocked by 100 μM verapamil in calcium-free medium, suggesting that the high concentration of verapamil (100 μM) blocks the intracellular calcium mobilization in HL-60 cells.

Therefore, the enhancing effect of calcium deprivation or verapamil of HL-60 cell differentiation seemed to be closely related to the inhibition of intracellular calcium mobilization. This speculation is supported by the finding that 50 μM 8-(N,N-diethylylamino)octyl-3,4,5-trimethoxybenzoate, an intracellular calcium antagonist, also enhanced HL-60 cell differentiation induced by 1α,25-dihydroxyvitamin D₃, β-all-trans-retinoic acid, or dimethyl sulfoxide.

INTRODUCTION

The calcium ion has been proposed as one of the key components for cellular and enzyme functions (1-3). Recently, the role of calcium has been extended to the regulatory mechanisms of some kinds of cell functions as a second messenger. For example, studies on the changes of intracellular free calcium measured by the intracellularly trappable fluorescent indicator, quin 2 (4-8), show a rapid increase of intracellular calcium in lymphocytes treated with mitogenic lectins such as phytohemagglutinin and concanavalin A (5); in human fibroblasts treated with platelet-derived growth factor, epidermal growth factor, and serum (6); in rat pituitary cells treated with thyrotropin-releasing hormone (7); and in human platelet aggregation treated with platelet-activating factor and thrombin (8). In addition, several studies suggest that calcium is involved in cell differentiation (9-13).

To elucidate the role of calcium in cell differentiation, we compared the HL-60 cell differentiation induced by several compounds such as 1,25(OH)₂D₃ (14), RA (15), DMSO (16), and TPA (17) in 0.6 mM standard calcium medium with that in calcium-free medium or in the presence of the calcium antagonist, verapamil, or TMB-8 (18-21). The effect of calcium deprivation or verapamil on the elevation of intracellular calcium by ionomycin was also investigated by the quin 2 method in HL-60 cells.

MATERIALS AND METHODS

Chemicals. 1,25(OH)₂D₃ (1 nM) and verapamil dissolved in ethanol were kind gifts from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan) and Eisai Co., Ltd. (Tokyo, Japan), respectively. RA, DMSO, TPA, quin 2-tetra(acetoxymethyl) ester, NBT, α-naphthyl acetate, and TMB-8 were obtained from Sigma Chemical Co. (St. Louis, MO). Ionomycin was obtained from Calbiochem. These stock solutions were stored at −20°C. Before use, each stock solution was diluted with the culture medium to the required concentration and the final concentration of ethanol was not more than 0.5%, which had no effect on HL-60 cell proliferation and differentiation. Calcium-free RPMI 1640 was obtained from Wakenyaku Co., Ltd. (Kyoto, Japan).

Cells and Culture. Human promyelocytic leukemia HL-60 cells were maintained in the constant exponential growth in Falsen tissue culture flasks containing RPMI 1640 supplemented with 10% fetal calf serum. When the cells were incubated with differentiation-inducing compounds, serum-free RPMI 1640 supplemented with insulin (5 μg/ml) and transferrin (5 μg/ml) was used. The calcium concentration was adjusted to the desired concentration by the addition of an appropriate volume of 2 M CaCl₂ solution. The standard serum-free RPMI 1640 contained 0.6 mM calcium. The cells in their logarithmic phase were washed twice in calcium/magnesium-free phosphate-buffered saline, resuspended in 3 ml of the above serum-free medium with an appropriate concentration of calcium, and treated with various compounds. When the cells were treated with verapamil or TMB-8, the cells were suspended in the standard calcium medium unless otherwise indicated. Cell number was assessed by the standard procedure of leukocyte counting using a hemocytometer, and cell viability was judged by the ability of cells to exclude 0.025% trypan blue.

Assessment of Differentiation. The extent of cell differentiation was assessed by NBT reduction ability (22) and NSE activity (23). The former is used to observe cell differentiation into both granulocyte and monocyte/macrophage lineage and the latter into the monocyte/macrophage lineage. The percentage of NBT-positive cells was quantitated as described previously (16). NSE activity was determined colorimetrically by modification of the method of Li et al. (24), using α-naphthyl acetate as a substrate. At least 600 cells were counted.

Quin 2 Loading and Fluorescence Measurement. The concentration of intracellular calcium was monitored with quin 2 as described elsewhere (4, 6, 7). Fluorescence was continuously recorded at an excitation wavelength of 339 nm (5 nm slit) and at an emission wavelength of 492 nm (10 nm slit) at 37°C with an RF-500LC spectrofluorimeter (Shimadzu Co., Ltd., Kyoto, Japan). The changes in the intracellular calcium concentration were calculated according to the formula

\[ [\text{Intracellular calcium concentration}] (\text{nm}) = \frac{F_{\text{max}} - F}{F_{\text{max}} - F_{\text{min}}} \times 115 \]

The observed fluorescence value was shown by F and quin 2 was calibrated by the addition of 0.1% Triton X for F_{\text{max}} and 20 nm Tris base and 5 mM ethyleneglycol bist(α-aminoethylether)-N,N′,N,N′-tetraacetic acid for F_{\text{min}}. Analysis of Statistical Significance. Results are shown as mean ± SD of more than three determinations by Student's t test.

RESULTS

Effect of Calcium Deprivation and of Verapamil on HL-60 Cell Proliferation and Differentiation. As shown in Fig. 1A, HL-
60 cell proliferation was inhibited with the reduction of calcium concentration in the culture medium. The cell proliferation was completely inhibited in calcium-free medium, although the cell viability always continued to be more than 70%.

As shown in Fig. 1B, verapamil inhibited cell proliferation dose dependently. By treatment with 100 μM verapamil, cell proliferation was markedly inhibited without reducing cell viability and 150 μM verapamil showed strong cytotoxicity.

When the cells were incubated in calcium-free medium for 4 days, the kinetic experiments showed that the percentages and absolute numbers of NBT- and NSE-positive cells increased negligibly (Table 1). On the other hand, in the presence of 100 μM verapamil, slight but statistically significant increases in the percentages and the absolute numbers of both positive cells were seen on day 4 (Table 1).

These results suggest that differentiation of HL-60 cells was induced by a high concentration of verapamil but not by calcium deprivation alone.

Effects of Calcium Deprivation and of Verapamil on HL-60 Cell Differentiation Induced by 1,25(OH)2D3. The addition of 1 nM 1,25(OH)2D3 to the standard calcium medium caused a slight increase in both NBT- and NSE-positive cells on day 4 (Table 1). The optimal concentration of 1,25(OH)2D3 for the differentiation of HL-60 cells used in this study was approximately 100 nM as described previously (25) and the above concentration was 100 times less than the optimal concentration. By calcium deprivation, the percentages of NBT-positive cells induced by 1 nM 1,25(OH)2D3 increased about 11 times and NSE activity increased about 17 times compared with those treated with 1 nM 1,25(OH)2D3 in the standard calcium medium (Table 1). As shown in Fig. 2, A and B, both the percentages and the absolute numbers of NBT-positive cells induced by 1 nM 1,25(OH)2D3 increased with the reduction of calcium concentration in the culture medium and the same increase of NSE-positive cells was also observed (data not shown).

Simultaneous treatment with 1 nM 1,25(OH)2D3 and 100 μM verapamil in the standard calcium medium increased the percentages of NBT-positive cells about 12 times and the percent-

Fig. 1. Effect of various concentrations of calcium (A) and verapamil (B) in the culture medium on HL-60 cell proliferation. The viabilities determined by trypan blue dye exclusion were always more than 70%. In A, calcium concentration in the medium was adjusted to 0.6 mM (●), 0.1 mM (○), 0.04 mM (△), or 0 mM (□). In B, verapamil concentration added in the medium was 0 mM (●), 25 μM (○), 50 μM (△), 100 μM (□), and 150 μM (×). Bars, SD.

Table 1. Effects of Inducers on NBT- and NSE-Positive Cells in HL-60 Cells in the Standard Calcium Medium at 0.6 mM

<table>
<thead>
<tr>
<th>Inducer</th>
<th>NBT-positive cells</th>
<th>NSE-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.2 ± 1.4</td>
<td>9.4 ± 0.4</td>
</tr>
<tr>
<td>1,25(OH)2D3</td>
<td>24.0 ± 1.6</td>
<td>10.2 ± 0.3</td>
</tr>
<tr>
<td>1,25(OH)2D3</td>
<td>24.3 ± 1.6</td>
<td>10.3 ± 0.3</td>
</tr>
<tr>
<td>1,25(OH)2D3</td>
<td>24.0 ± 1.6</td>
<td>10.4 ± 0.4</td>
</tr>
</tbody>
</table>

*P < 0.05 in comparison with the absolute number of NBT- or NSE-positive cells treated with the same inducers without verapamil addition in the standard calcium medium.
Ionomycin-induced Changes of Intracellular Calcium Concentration in HL-60 Cells and the Blockage of Intracellular Calcium Mobilization by Verapamil. By the addition of 250 nM ionomycin, the concentration of intracellular calcium concentration was elevated from the resting level, 131 ± 19 nM (n = 10) in 1 mM calcium medium and 82 ± 10 nM (n = 5) in calcium-free medium to 244 ± 39 nM (n = 5) and 136 ± 18 nM (n = 5), respectively. In 1 mM calcium medium, ionomycin-induced intracellular calcium elevation was decreased to about 40% by 10 μM verapamil and to about 17% by 100 μM verapamil. However, in calcium-free medium, the complete inhibition was observed by 100 μM verapamil, although not by 10 μM verapamil. Thus, a higher concentration (100 μM) of verapamil inhibited the mobilization of intracellular calcium.

Treatment with 100 nM 1,25(OH)2D3, 100 nM RA, 1.5% DMSO, or 100 nM TPA did not produce any immediate change of intracellular calcium by the quin 2 method (data not shown).

Effect of TMB-8 and of Ionomycin on HL-60 Cell Proliferation and Differentiation. As shown in Fig. 3, HL-60 cell proliferation was not affected by incubation with less than 50 μM TMB-8. When the cells were incubated with 100 μM TMB-8, little cell proliferation was observed, but cell viability was always more than 85%.

As shown in Fig. 4, both the percentages and the absolute numbers of NBT-positive cells were increased dose dependently.
by treatment with various concentrations of TMB-8 without any inducer. The results obtained suggest that TMB-8 itself can induced HL-60 cell differentiation.

By simultaneous treatment with 1 nm 1,25(OH)2D3 and less than 50 µM TMB-8, both the percentages and the absolute numbers of NBT-positive cells were enhanced with increased TMB-8 concentration, but by treatment with 100 µM TMB-8, the above increase in the absolute number of NBT-positive cells reached a plateau level (Fig. 4). As shown in Table 2, the percentages and the absolute numbers of NBT-positive cells induced by 0.1 nm 1,25(OH)2D3, 1 nm RA, or 0.75% DMSO were also increased by simultaneous treatment with 50 µM TMB-8.

Ionomycin at 250 nm did not affect either the HL-60 cell proliferation or cell differentiation induced by the optimal concentration of 1,25(OH)2D3 (100 nm) in the calcium standard medium and also did not inhibit significantly the enhancing effect of calcium deprivation or verapamil on HL-60 cell differentiation induced by 1 nm 1,25(OH)2D3 (data not shown).

**DISCUSSION**

Recently, there have been several controversial reports on the effect of calcium on cell differentiation. The addition of ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetate acid was reported to suppress the differentiation of the human monocytic leukemia U 937 cells induced by 1,25(OH)2D3 (9). The incubation in low calcium medium and the treatment with verapamil affected murine myeloid leukemia M1 cell proliferation rather than cell differentiation induced by 1,25(OH)2D3 (10). Levenson et al. (11) reported that an essential early event in the DMSO-induced differentiation of Friend erythroleukemia cells was an increase in intracellular calcium, but more recently Falletto and Macara (12) reported a small but significant decrease of cytosolic calcium by treatment of Friend cells with DMSO using the fluorescent indicator quin 2.

In the present study, we showed that both calcium deprivation and verapamil addition in the medium enhanced the differentiation of HL-60 cells induced by 1,25(OH)2D3, RA, or DMSO (Table 1). By using the quin 2 method, we also demonstrated that in HL-60 cells, calcium deprivation or verapamil inhibited an increase of intracellular calcium induced by ionomycin. Particularly, a higher concentration (100 µM) of verapamil, which markedly enhanced HL-60 cell differentiation, seemed to inhibit calcium mobilization from intracellular storage. These results are consistent with the reports on the inhibitory effect of calcium-deprived medium or a higher concentration of verapamil on intracellular calcium mobilization in human platelets (8) and in human neutrophils (26). Furthermore, the present results showed that TMB-8, which blocks the mobilization of intracellular calcium by various stimuli (18-21), also enhanced HL-60 cell differentiation induced by 1,25(OH)2D3, RA, or DMSO (Table 2). These results suggest that the inhibition of intracellular calcium mobilization by calcium deprivation or calcium antagonists plays an important role in the enhancement of HL-60 cell differentiation induced by various compounds. However, contrary to our expectation, HL-60 cell differentiation induced by 1,25(OH)2D3 was not affected by a transient elevation of intracellular calcium concentration with ionomycin either in the standard calcium medium or in calcium-free medium, although calcium ionophore A23187 was reported to block the differentiation of U937 cells induced by 1,25(OH)2D3 (13). The precise reason for this remains to be clarified.

We also found that TPA-induced HL-60 cell differentiation was not enhanced by calcium deprivation, verapamil, or TMB-8 (data for TMB-8 not shown) for an unknown reason.

Although verapamil itself appeared to differentiate HL-60 cells into a monocyte lineage, simultaneous treatment with calcium deprivation or verapamil and an inducer such as RA and DMSO yielded granulocytes and these enhancing effects exceeded the additive effects of each treatment. These results suggest that the direction of cell differentiation enhanced by calcium deprivation or calcium antagonists depends on the added inducers and that these enhancing effects seem to be nonspecific and trans-acting. Recently, it has been reported that the increase of intracellular calcium mobilization appears necessary for the activation of cell proliferation (1-6) and that calcium-activated and phospholipid-dependent protein kinase C plays an important role in a signal transduction of cell proliferation (27-31). Both the inhibition of intracellular calcium mobilization and several inhibitors of protein kinase C such as alkyllysophospholipids (32), trifluoperazine (33), chlorpromazine (data not shown), and also verapamil (33, 34) seem to enhance HL-60 cell differentiation (35, 36). Thus, the inhibition of the cell proliferation-related process may enhance the probability of HL-60 cells to differentiate (37, 38).

From the above results, a close relationship between the inhibition of intracellular calcium mobilization and cell differentiation seems to be evident in HL-60 cells, although the possibility cannot be excluded that verapamil or TMB-8 has a differentiation-inducing ability, independent of the calcium-related process, such as an increase of intracellular cyclic AMP (39, 40).

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