Synergistic Effect of 1,25-Dihydroxyvitamin D₃ and Retinoic Acid in Inducing U937 Cell Differentiation

Yoshihisa Tanaka, Masaaki Shima, Kanji Yamaoka, Shintaro Okada, and Yoshiki Seino*

Department of Pediatrics, Osaka University School of Medicine, Fukushima-ku, Osaka 553, Japan
*Department of Pediatrics, University of Okayama School of Medicine, Shikata-cho, Okayama 700, Japan

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Summary We examined the effects of retinoic acid (RA), 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃), and its synthetic analogue, 22-oxa-1,25-(OH)₂D₃, on differentiation of U937 cells by studying the cellular growth, surface marker expression and cytosolic free Ca²⁺ concentration ([Ca²⁺]). RA inhibited cellular growth but did not induce expression of Mo2 (CD14), a monocyte/macrophage specific surface marker. To the contrary, 1,25-(OH)₂D₃ did not inhibit cellular growth, but increased CD14-positive cells. Simultaneous addition of 1,25-(OH)₂D₃ and RA had no additive effect on cellular growth inhibition or CD14 expression. With regard to [Ca²⁺], however, 5 days' incubation with either of them increased the basal [Ca²⁺], level and induced U937 cells to respond to formyl-methionyl-leucyl-phenylalanine (FMLP). When the cells were incubated with both 10⁻⁶ M RA and 10⁻⁸ M 1,25-(OH)₂D₃, basal [Ca²⁺], was higher and FMLP caused a greater increase in [Ca²⁺], than when only RA or 1,25-(OH)₂D₃ was added. These data suggest that RA and 1,25-(OH)₂D₃ induce monocytoid differentiation in U937 cells through different pathways and act synergistically in the differentiation process. The 22-oxa-1,25-(OH)₂D₃ induced CD14 expression, basal [Ca²⁺], increase and [Ca²⁺] response to FMLP, but did not cause cellular growth inhibition in U937 cells, and in these points, 22-oxa-1,25-(OH)₂D₃ exhibited no significantly different effects from 1,25-(OH)₂D₃. Thus, 22-oxa-1,25-(OH)₂D₃ has the same potent activity as 1,25-(OH)₂D₃ in inducing differentiation of U937 cells.

Key Words 1,25-(OH)₂D₃, retinoic acid, intracellular calcium, U937, cell differentiation, synergistic effect

The human monoblast-like cell line, U937, derived from a histiocytic lymphoma cell (J), has served as a model for monocyte-macrophage differentiation. Its
differentiation can be induced by a variety of agents, including retinoic acid (RA) (2), 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$) (3–5) and γ-interferon (6, 7), resulting in morphological changes (8), increased expression of monocytic cell surface markers and acquisition of various cellular functions, such as phagocytosis and reduction of nitroblue tetrabzoium (NBT).

Differentiated U937 cells are known to acquire the ability to synthesize several monocytic membrane proteins, including FMLP receptors (6, 9). In neutrophils, chemotactic factor induces locomotion, lysosomal enzyme secretion, superoxide generation and aggregation. In addition, an increase in [Ca$^{2+}$], is thought to serve as a signal to activate these subsequent physiological responses (10). Recently, 22-oxa-1,25-(OH)$_2$D$_3$, a synthetic analogue of 1,25-(OH)$_2$D$_3$, has been reported to have a potent effect in inducing differentiation on HL-60 cells, but weak hypercalcemic action in vivo (11). Abe et al. suggested the possibility that the immunoregulating activity of vitamin D compounds can be separated structurally from their hypercalcemic action (12). The objectives of the present study were to investigate the interaction of 1,25-(OH)$_2$D$_3$ and RA in inducing U937 differentiation and to measure the basal [Ca$^{2+}$] and FMLP-stimulated increase in [Ca$^{2+}$] after differentiation induced by these agents. Moreover, we compared these effects of 1,25-(OH)$_2$D$_3$ and 22-oxa-1,25-(OH)$_2$D$_3$ on U937 cells.

MATERIALS AND METHODS

1. Cells and cell culture. U937 cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Japan) supplemented with 10% fetal calf serum (HyClone, Logan, UT) at 37°C in a humidified atmosphere of 95% air and 5% CO$_2$. 1,25-(OH)$_2$D$_3$ (Teijin Institute for Bio-medical Research, Tokyo, Japan), 22-oxa-1,25-(OH)$_2$D$_3$ (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) and RA (Sigma Chemical Co., St. Louis, MO) were dissolved in absolute ethanol and added to the culture medium. The ethanol concentration in the test cultures and the controls did not exceed 0.1% (10). Cells were counted every 2 days, and viability was determined by trypan blue exclusion. Cell viability of both the control and treated cells was similar and more than 95%.

2. Monoclonal antibody. The binding of the monocyte/macrophage specific monoclonal antibody, anti-Mo2 (Coulter Immunology, Hialeah, FL), to cell surface antigens (CD14) was determined by indirect immunofluorescence with flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA). CD14 is a surface marker whose expression is mainly restricted to the monocyte macrophage series (13). Control and treated cells ($10^6$ cells) were incubated with the monoclonal antibody for 30 min at 4°C. The cells were washed three times with ice cold phosphate-buffered saline (PBS, pH 7.4) containing 0.1% bovine serum albumin (BSA, Boehringer, Mannheim, W. Germany) and 0.1% sodium azide (Wako Pure Chemical Industries, Osaka, Japan). For indirect immunofluorescence, they were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse J. Nutr. Sci. Vitaminol.
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IgG (Becton Dickinson, Mountain View, CA) for 30 min at 4°C. After washing, the cells were analyzed by flow cytometry.

3. Measurement of cytosolic free calcium concentration. After 5 days in culture, U937 cells were detached by gently pipetting, resuspended in RPMI 1640 containing 0.1% BSA and incubated with 1 μM acetoxymethyl ester of fura-2 (fura-2 AM, Dojindo Laboratories, Kumamoto, Japan) for 20 min at 37°C. After removal of the dye, the cells were incubated for an additional 10 min in serum-free medium, rinsed twice and stored in Krebs-Ringer bicarbonate glucose buffer (KRBG, 105 mM NaCl, 5 mM KCl, 26 mM NaHCO3, 10 mM sodium acetate, 1 mM/1 mM Na2HPO4/NaH2PO4, 1 mM MgCl2, 1.8 mM CaCl2, 8.3 mM d-glucose) containing 0.1% BSA at 4°C until use. Omission of Ca2+ from KRBG buffer was achieved by addition of 1 mM EGTA to the Ca2+-free KRBG buffer. Cells (10⁷) were placed in a quartz cuvette at 37°C. Fluorescence was monitored in a Hitachi 650-40 spectrometer fitted with a magnetic stirrer and thermostatted cuvette holder (excitation wavelength, 335 nm; emission wavelength, 495 nm). Following each experiment, Fmax and Fmin were determined after lysing the cells by addition of 0.25% Triton X-100 (Wako Pure Chemical Industries, Osaka, Japan) in the presence of extracellular Ca2+, and by adding 1 mM MnCl2 after cell lysis, respectively. Cells were equilibrated for 5 min prior to FMLP (Peptide Institute, Inc., Osaka, Japan) addition until the medium temperature was stabilized. Each assay was completed within 15 min. Extracellular dye leakage was negligible since addition of 1 mM MnCl2 caused no change in fluorescence 15 min after the start of assay. Autofluorescence and fluorescence of any of the additives were negligible. [Ca2+]i values were calculated as described by Tsien et al. with some modifications (14). Cell sizes were not changed by incubation with RA, 1,25-(OH)2D3 or 22-oxa-1,25-(OH)2D3, analyzing with a FACScan. Data are presented as mean ± SEM. Statistical analysis was done by the t-test.

RESULTS

Cellular differentiation was assessed by two related phenomena: (a) inhibition of cell growth, and (b) acquisition of a specific marker. After the addition of 10⁻⁶ M RA, there was a maximal decrease in the rate of cell growth. Morphologically, RA-treated cells developed pseudopodia and adhered to the culture dishes. Addition of 10⁻⁸ M 1,25-(OH)2D3 or 10⁻⁸ M 22-oxa-1,25-(OH)2D3 also caused similar morphological changes but showed no significant effect on cellular proliferation in comparison with untreated control. There was no significant difference in growth inhibition between addition of 10⁻⁶ M RA alone and addition of both 10⁻⁸ M 1,25-(OH)2D3 and 10⁻⁶ M RA (Fig. 1).

In order to evaluate the differentiation of U937 cells, CD14 expression was investigated by using flow cytometry. CD14 is known to be expressed little in undifferentiated U937 cells but expressed highly in mature monocytes (13). In our study, in fact, CD14 was expressed in only a minority of the control, uninduced U
Fig. 1. Effects of 1,25-(OH)2D3, 22-oxa-1,25-(OH)2D3 and RA on growth of U937 cells. The culture dishes were plated with 1 × 10^5 cells/ml on Day zero in the absence and presence of the following concentration of RA, 1,25-(OH)2D3, or 22-oxa-1,25-(OH)2D3: 10^{-6} M RA; 10^{-8} M 1,25-(OH)2D3; 10^{-8} M 22-oxa-1,25-(OH)2D3; 10^{-6} M RA and 10^{-8} M 1,25-(OH)2D3. The number of cells (means±SEM) was determined as described in Materials and Methods (n=3-4). Cellular growth was significantly inhibited when incubated with 10^{-6} M RA (p<0.01) and both 10^{-8} M 1,25-(OH)2D3 and 10^{-8} M 22-oxa-1,25-(OH)2D3 did not inhibit the cellular growth. *p<0.01 significant difference compared with the number of control cells after 6 days' incubation (unpaired t-test).

937 cells. The percentage of U937 cells positive for CD14 increased as a result of culture with 10^{-8} M 1,25-(OH)2D3. The 22-oxa-1,25-(OH)2D3 also increased CD14-positive cells. Conversely, incubation with 10^{-6} M RA did not increase the expression of CD14. When the cells were incubated with both 10^{-8} M 1,25-(OH)2D3 and 10^{-6} M RA, the pattern of fluorescence was similar to that obtained by incubation with 10^{-8} M 1,25-(OH)2D3 alone (Fig. 2). Non-specific binding of the monoclonal antibody was negligible since there were few positive cells after incubation with FITC-conjugated goat anti-mouse IgG alone.

The cytosolic free Ca^{2+} concentration ([Ca^{2+}]) in untreated U937 cells was found to be 112.2±8.5 nM. Treatment with 10^{-6} M RA for 5 days increased the basal [Ca^{2+}] to 185.3±5.7 nM (p<0.01). The 10^{-8} M 1,25-(OH)2D3 and 10^{-8} M 22-oxa-1,25-(OH)2D3 increased the basal [Ca^{2+}] to 143.8±10.0 nM (p<0.05) and 135.8±14.4 nM (p<0.05), respectively. RA, 1,25-(OH)2D3 and 22-oxa-1,25-(OH)2D3 caused dose-dependent increase in basal [Ca^{2+}] in U937 cells (Fig. 3).

FMLP stimulated a dose-dependent increase in [Ca^{2+}] in differentiated cells, as shown in Fig. 4. The [Ca^{2+}] level peaked within 10 s, followed by a slow decline towards the base line for 5 min. Addition of 10^{-6} M FMLP increased the [Ca^{2+}].

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Fig. 2. Representative flow cytometric analysis of anti-Mo 2 antibody binding. Reactivity of the monoclonal antibody with U937 cells grown in the presence of vehicle, 10⁻⁸M 1,25-(OH)₂D₃, 10⁻⁸M RA, 10⁻⁸M 22-oxa-1,25-(OH)₂D₃ or both 10⁻⁸M 1,25-(OH)₂D₃ and 10⁻⁶M RA for 5 days. The cultured cells were assayed by indirect immunofluorescence using a FACScan as described in Materials and Methods. 10⁻⁸M 1,25-(OH)₂D₃ and 10⁻⁸M 22-oxa-1,25-(OH)₂D₃ increased Mo2-positive cells, but 10⁻⁶M RA did not induce Mo2 expression. Incubation with both 1,25-(OH)₂D₃ and RA did not have different effect from 1,25-(OH)₂D₃ alone.

Fig. 3. Effects of various concentration of 1,25-(OH)₂D₃, 22-oxa-1,25-(OH)₂D₃, or RA on basal [Ca²⁺], and [Ca²⁺] response to FMLP. U937 cells were preincubated with various concentration of 1,25-(OH)₂D₃, 22-oxa-1,25-(OH)₂D₃, or RA for 5 days. [Ca²⁺] values were measured under basal condition (□) and after stimulation with 10⁻⁶M FMLP (□), using fura-2 as described in Materials and Methods. Each basal [Ca²⁺] of U937 cells incubated with 10⁻⁸M 1,25-(OH)₂D₃ (n=6), 10⁻⁸M 22-oxa-1,25-(OH)₂D₃ (n=4) or 10⁻⁶M RA (n=25) were higher than that of control (n=5; *p<0.05, **p<0.01). FMLP increased [Ca²⁺] from the basal level in U937 cells treated with 10⁻⁸M 1,25-(OH)₂D₃ (p<0.01), 10⁻⁸M 22-oxa-1,25-(OH)₂D₃ (p<0.05), 10⁻⁶M RA (p<0.01). Each column represents the mean±SEM.
Fig. 4. FMLP increased \([\text{Ca}^{2+}]_i\) of U937 cells dose-dependently. Representative recording of \([\text{Ca}^{2+}]_i\) of U937 cells were treated with \(10^{-6} \text{M RA}\) for 5 days. The cells were loaded with fura-2 and suspended in KRBG buffer containing 1.8 mM Ca\(^{2+}\). Each concentration of FMLP was added at the arrow.

from 185.3±5.7 nM to 298.8±17.7 nM (p<0.01) within 10 s in \(10^{-6} \text{M RA}\)-treated cells. The \(10^{-8} \text{M} 1,25-(\text{OH})_2\text{D}_3\)-treated cells also responded to FMLP, and \([\text{Ca}^{2+}]_i\) was increased from 143.8±10.0 nM to 210.3±14.2 nM (p<0.01). The \(10^{-8} \text{M} 22\text{-oxa-1,25-(OH)}_2\text{D}_3\)-treated cells also showed a \([\text{Ca}^{2+}]_i\) increase to 184.0±33.4 nM (p<0.05) in response to FMLP (Fig. 3). In the case of incubation with both \(10^{-6} \text{M RA}\) and \(10^{-8} \text{M} 1,25-(\text{OH})_2\text{D}_3\), the basal \([\text{Ca}^{2+}]_i\) was increased to 265.8±10.8 nM, and a marked increase in \([\text{Ca}^{2+}]_i\) was observed (774.5±95.2 nM, p<0.01) on stimulation with FMLP; the \([\text{Ca}^{2+}]_i\) level then decreased more slowly to the basal level, with a prolonged increase in \([\text{Ca}^{2+}]_i\), persisting more than 10 min (Fig. 5).

However, in the absence of extracellular Ca\(^{2+}\) by addition of 1 mM EGTA to Ca\(^{2+}\)-free KRBG buffer, FMLP did not increase \([\text{Ca}^{2+}]_i\) of U937 cells induced differentiation by either \(1,25-(\text{OH})_2\text{D}_3\) or RA (Fig. 5). In U937 cells treated with both \(1,25-(\text{OH})_2\text{D}_3\) and RA, FMLP caused a small peak of \([\text{Ca}^{2+}]_i\), and showed a short duration of the sustained phase, in spite of chelation of extracellular Ca\(^{2+}\). Omission of extracellular Ca\(^{2+}\) decreased the peak of \([\text{Ca}^{2+}]_i\) upon addition of \(10^{-6} \text{M FMLP}\). The increase in \([\text{Ca}^{2+}]_i\) stimulated by FMLP might be derived from both mobilization from the intracellular Ca\(^{2+}\) store and Ca\(^{2+}\) influx.

Treatment with combination of various concentrations of \(1,25-(\text{OH})_2\text{D}_3\) and RA increased the basal \([\text{Ca}^{2+}]_i\), and resulted in a further rise in \([\text{Ca}^{2+}]_i\) induced by FMLP (Figs. 6, 7). The basal \([\text{Ca}^{2+}]_i\) level of cells treated with both \(10^{-6} \text{M RA}\) and \(10^{-8} \text{M} 1,25-(\text{OH})_2\text{D}_3\) was higher than the levels of cells treated with either of them. Synergistic effect was observed on the basal \([\text{Ca}^{2+}]_i\) level. \(\Delta [\text{Ca}^{2+}]_i\) ([\(\Delta [\text{Ca}^{2+}]_i\) after stimulation with FMLP – basal \([\text{Ca}^{2+}]_i\)] of U937 cells treated with \(10^{-6} \text{M RA}\) was greater than that treated with \(10^{-8} \text{M} 1,25-(\text{OH})_2\text{D}_3\). Although, \(10^{-7} \text{M RA}\) had no effect on \(\Delta [\text{Ca}^{2+}]_i\), after stimulation by FMLP (Fig. 7), \(1,25-(\text{OH})_2\text{D}_3\) enhanced the effect of RA on \(\Delta [\text{Ca}^{2+}]_i\), more than expected by additive effect of \(1,25-(\text{OH})_2\text{D}_3\) (Fig. 8). In addition to the basal \([\text{Ca}^{2+}]_i\), RA and \(1,25-(\text{OH})_2\text{D}_3\) also exhibited a synergistic effect on \(\Delta [\text{Ca}^{2+}]_i\) after FMLP stimulation.

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Fig. 5. Effects of FMLP on [Ca2+]i or fluorescence intensity in U937 cells suspended with Ca2+-containing buffer or Ca2+-free buffer. Representative recording of [Ca2+]i of U937 cells treated with 10^-6 M RA, 10^-8 M 1,25-(OH)2D3, 10^-6 M RA and 10^-8 M 1,25-(OH)2D3 for 5 days. 10^-6 M FMLP was added at the arrow. (A) Cells were suspended in KRBG buffer containing 1.8 mM CaCl2. FMLP increased [Ca2+]i level in the cells treated with RA, 1,25-(OH)2D3, and both of them. (B) Effect of omission of extracellular Ca2+ on [Ca2+]i transients induced by FMLP. Cells were suspended with Ca2+-free KRBG buffer containing 1 mM EGTA. Response to FMLP was not observed in control, RA-, or 1,25-(OH)2D3-treated cells. But, only when the cells were incubated with both 10^-6 M RA and 10^-8 M 1,25-(OH)2D3, change in fluorescence was observed on stimulation with FMLP.

DISCUSSION

This study shows that RA and 1,25-(OH)2D3 have different effects on the differentiation of U937 cells. Differentiation of U937 cells induced by 1,25-(OH)2D3 was not associated with reduced cellular growth but with expression of CD14. On the other hand, RA inhibited cellular proliferation but did not induce expression of CD14. Bhalla et al. (15) also reported that, on the basis of CD14 expression, 1,25-(OH)2D3 induced U937 differentiation but RA did not. They used only CD14 expression and growth inhibition as markers of differentiation. In our study,
Fig. 6. Basal $[\text{Ca}^{2+}]_i$ of U937 cells which were induced differentiation by incubation with various combination of 1,25-(OH)$_2$D$_3$ and RA. Both RA and 1,25-(OH)$_2$D$_3$ caused dose-dependent increase of basal $[\text{Ca}^{2+}]_i$. Addition of both $10^{-8} \text{M}$ 1,25-(OH)$_2$D$_3$ and $10^{-6} \text{M}$ RA ($n=4$) increased the basal $[\text{Ca}^{2+}]_i$ of U937 cells more than expected by additive effect of them. Each column represents the mean±SEM.

Fig. 7. Increments of $[\text{Ca}^{2+}]_i$ from basal level after FMLP stimulation ($\Delta[\text{Ca}^{2+}]_i$). U937 cells were incubated with vehicle or various concentration of RA or 1,25-(OH)$_2$D$_3$, alone or in combination, for 5 days. $[\text{Ca}^{2+}]_i$ levels were calculated as detailed in Materials and Methods. Increments of $[\text{Ca}^{2+}]_i$ after stimulation with FMLP were enhanced when incubated with both 1,25-(OH)$_2$D$_3$ and RA. They acted synergistically from the viewpoint of $[\text{Ca}^{2+}]_i$ response to FMLP. Each column represents the mean±SEM.
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Fig. 8. Δ[Ca2+]i after FMLP stimulation in U937 cells treated with both 10⁻⁷ M RA and various concentration of 1,25-(OH)2D3. Addition of 10⁻⁷ M RA alone did not increase [Ca2+]i, as is also shown in Fig. 7. But incubation with both 10⁻⁷ M RA and 1,25-(OH)2D3 brought more increase of [Ca2+]i after stimulation with FMLP than incubation with 10⁻⁷ M RA alone or 1,25-(OH)2D3 alone. Each column represents the mean±SEM.

Treatment with RA led to increased basal [Ca2+]i levels and an intracellular Ca2+ increase in response to FMLP. Incubation with 1,25-(OH)2D3 or RA induced U937 cells to respond to a chemotactic factor, FMLP. RA and 1,25-(OH)2D3 could induce differentiation of U937 cells, both alone and in combination, from the viewpoints of cellular growth inhibition, CD14 expression and the [Ca2+]i response after stimulation with FMLP, including already-reported cellular functions such as phagocytosis and reduction of NBT (2, 3, 7). In this study, we observed a synergistic effect between RA and 1,25-(OH)2D3 on the basal [Ca2+]i level and [Ca2+]i response to FMLP. The 22-oxa-1,25-(OH)2D3, which has been reported to have little hypercalcemic effect, was found to have the same effect as 1,25-(OH)2D3 of inducing U937 cell differentiation.

1,25-(OH)2D3 receptors have been found in U937 cells; this and the possible presence of RA receptor are the likely mediators of inducing differentiation of U937 cells.

A response to FMLP has been observed in monocytes (16), neutrophils (10), and differentiated HL-60 cells (17). The FMLP-stimulated increase in [Ca2+]i might occur due to two distinct mechanisms: 1) the release of Ca2+ from intracellular storage sites (mobilization), or 2) the influx of extracellular Ca2+ through Ca2+ channels in the plasma membrane (10). Chelation of extracellular Ca2+ with EGTA did not completely prevent the rapid increase in [Ca2+]i, suggesting that mobilization from intracellular stores occurs in differentiated U937 cells. Suppression of the sustained increase in [Ca2+]i by EGTA represented the
contribution of extracellular Ca$^{2+}$ to the elevated cytosolic Ca$^{2+}$. In our study, FMLP did not increase [Ca$^{2+}$]$_i$ in cells treated with either 1,25-(OH)$_2$D$_3$ or RA alone when extracellular Ca$^{2+}$ was chelated.

Their basal [Ca$^{2+}$]$_i$ is not high enough to increase the [Ca$^{2+}$]$_i$ level after FMLP stimulation, and the cells treated with both RA and 1,25-(OH)$_2$D$_3$ exhibited a higher basal [Ca$^{2+}$]$_i$ level and could mobilize more Ca$^{2+}$ from intracellular storage even though extracellular Ca$^{2+}$ was chelated.

One possible mechanism by which U937 cells acquire the ability to respond to FMLP is by increasing the number of FMLP receptors in the process of differentiation. Imaizumi et al. (9) and Rao et al. (17) also showed that, on stimulation with 10$^{-6}$ M RA, HL-60 cells acquired the ability to respond to this chemotactic peptide, but the density of the receptors was very low. In U937 cells treated with both 1,25-(OH)$_2$D$_3$ and RA, FMLP induced a more prolonged increase in [Ca$^{2+}$]$_i$ than cells treated with either 1,25-(OH)$_2$D$_3$ or RA. It may be due to a decreased rate of Ca$^{2+}$ efflux or prolonged Ca$^{2+}$ influx in those cells. This phenomenon also reflects a synergistic effect between 1,25-(OH)$_2$D$_3$ and RA. Therefore, it is possible that some changes are induced in the post-receptor signal transduction system in the process of differentiation other than an increase in the number of FMLP receptors.

In addition to the FMLP-stimulated [Ca$^{2+}$]$_i$ increase, we showed that basal [Ca$^{2+}$]$_i$ was increased in a dose-dependent fashion in incubation with 1,25-(OH)$_2$D$_3$ or RA. Hruska et al. (18) also reported that in HL-60 cells, basal [Ca$^{2+}$]$_i$ was increased when incubated with 10$^{-8}$ M 1,25-(OH)$_2$D$_3$ for at least 12 h, and priming of cellular Ca$^{2+}$ stores was induced by 1,25-(OH)$_2$D$_3$ through an increase in Ca$^{2+}$ entry through the plasma membrane. They also reported that differentiation of HL-60 cells by 1,25-(OH)$_2$D$_3$ was Ca$^{2+}$-independent, but expression of responses to regulatory stimuli, as mature cell functions, required priming of cellular Ca$^{2+}$ stores (19). We also showed a correlation between basal [Ca$^{2+}$]$_i$ and FMLP-stimulated [Ca$^{2+}$]$_i$ of U937 cells incubated with 1,25-(OH)$_2$D$_3$ or RA for 5 days; when U937 cells were incubated with a low concentration of 1,25-(OH)$_2$D$_3$ or RA, both the basal [Ca$^{2+}$]$_i$, and FMLP-stimulated [Ca$^{2+}$]$_i$ were low. The FMLP-stimulated increase in [Ca$^{2+}$]$_i$ may be related to Ca$^{2+}$ storage during incubation with RA or 1,25-(OH)$_2$D$_3$ (Figs. 5, 7).

In summary, 1,25-(OH)$_2$D$_3$ and RA were able to induce differentiation both alone and in combination. It is interesting that 22-oxa-1,25-(OH)$_2$D$_3$, which does not show a hypercalcemic effect, exhibits the same effects as 1,25-(OH)$_2$D$_3$ in inducing differentiation of U937 cells. Differentiated U937 cells showed elevation of the basal [Ca$^{2+}$]$_i$ level and acquired a Ca$^{2+}$ signaling system in response to FMLP. In addition, 1,25-(OH)$_2$D$_3$ and RA showed a synergistic effect on increasing [Ca$^{2+}$]$_i$ from basal level after FMLP stimulation. They may act through different pathways and interact with each other.

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