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Capsaicin potentiates 1,25-dihydoxyvitamin D_3- and all-*trans* retinoic acid-induced differentiation of human promyelocytic leukemia HL-60 cells

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Abstract

Human promyelocytic leukemia HL-60 cells are differentiated into monocytic or granulocytic lineage when treated with 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] or all-*trans* retinoic acid, respectively. In this study, the effect of capsaicin, an active component of the red pepper of the genus *Capsocum*, on cell differentiation was investigated in a HL-60 cell culture system. Treatment of HL-60 cells with 5–30 μg/ml capsaicin for 72 h inhibited cell proliferation and induced a small increase in cell differentiation. Interestingly, synergistic induction of HL-60 cell differentiation was observed when capsaicin was combined with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM all-*trans* retinoic acid. Flow cytometric analysis indicated that combinations of 1,25-$(OH)_2D_3$ and capsaicin stimulated differentiation predominantly to monocytes whereas combinations of all-*trans* retinoic acid and capsaicin stimulated differentiation predominantly to granulocytes. Capsaicin enhanced protein kinase C activity in 1,25-$(OH)_2D_3$- and all-*trans* retinoic acid-treated HL-60 cells. In addition, inhibitors for protein kinase C [bisindolylmaleimide (GF-109203X), chelerythrine, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7)] and an inhibitor for extracellular signal-regulated kinase [2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD-098059)] significantly inhibited HL-60 cell differentiation induced by capsaicin in combination with either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid. These results indicate that capsaicin potentiates 1,25-$(OH)_2D_3$- or all-*trans* retinoic acid-induced HL-60 cell differentiation and that both protein kinase C and extracellular signal-regulated kinase are involved in the cell differentiation synergistically enhanced by capsaicin.

Keywords: Capsaicin; Cell differentiation; Protein kinase C; HL-60 cell

1. Introduction

Capsaicin, a homovanillic acid derivative (8-methyl-*N*-vanillyl-6-nonenamide), is an active component of the red pepper of the genus *Capsicum* (Holzer, 1994) and has been used in humans for topical treatment of cluster headache, herpes zoster, and vasomotor rhinitis (Fusco et al., 1994; Marabini et al., 1991). In vitro capsaicin modulates cellular growth, collagenase synthesis, and prostaglandin secretion from rheumatoid arthritis synoviocytes (Matucci-Cerinic et al., 1990). Capsaicin has also been shown to be immunomodulatory, as indicated by its ability to modulate lymphocyte proliferation, antibody production, and neutrophil chemotaxis (Nilsson and Ahlstedt, 1988; Nilsson et al., 1991). In addition, capsaicin induces mitochondrial swelling (Chiba et al., 1986), inhibits NADH oxidase, induces apoptosis of transformed cells (Morre et al., 1995), stimulates adenylate cyclase (Jancso and Wollemann, 1977), activates protein kinase C (Harvey et al., 1995), and inhibits superoxide anion generation (Joe and Lokesh, 1994). Recent studies reveal substantial anti-genotoxic and anti-carcinogenic effects of capsaicin, and suggest that capsaicin is an important dietary phytochemical with potential chemopreventive activity (Surh, 1999).

Human promyelocytic leukemia HL60 cells are differentiated into monocytic lineage or granulocytic lineage when treated with 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] or all-*trans* retinoic acid (Breitman et al., 1980; Tanaka et al., 1983), respectively. This has been employed

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as an excellent model system for studying cellular differentiation in vitro. Several studies provide evidence that activation of protein kinase C is necessary for differentiation of HL-60 cells, especially along the monocytic pathway. Continuous treatment of HL-60 cells with 1,25-$(OH)_2D_3$ increased protein kinase C levels and cell differentiation (Pan et al., 1997), effects which were significantly inhibited by protein kinase C inhibitors (Martell et al., 1987, 1988) or protein kinase C antisense constructs (Gamard et al., 1994; Simpson et al., 1998). Treatment with retinoic acids also induced cell differentiation with the increased levels of protein kinase C (Wu et al., 1989).

We now investigated the effect of capsaicin on cellular differentiation in the human promyelocytic leukemia HL-60 cell culture system. We also investigated the effects of combinations of capsaicin with 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid on HL-60 cell differentiation. 1,25-$(OH)_2D_3$ and all-*trans* retinoic acid were chosen for this study because they have been widely used endogenous stimulators of differentiation in HL-60 cells. In addition, all-*trans* retinoic acid is widely used clinically for the treatment of acute promyelocytic leukemia (Chen et al., 1991), and analogues of vitamin D_3 including 1,25-$(OH)_2D_3$ are used clinically for the treatment of psoriasis (Orfanos et al., 1987).

2. Materials and methods

2.1. Materials

HL-60 cell line was originally obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA). Capsaicin, 1,25-dihydroxyvitamin D_3, all-*trans* retinoic acid, Giemsa staining solution, ethanol, methanol-free paraformaldehyde and all other reagents were purchased from the Sigma (St. Louis, MO, USA). Bisindolylmaleimide (GF-109203X), chelerythrine, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) and 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD-098059) were purchased from the Tocris Cookson (UK).

2.2. Preparation of differentiation inducers

Stock solutions of 1 mM 1,25-$(OH)_2D_3$ and 1 mM all-*trans* retinoic acid were dissolved in absolute ethanol (Hayman, UK) and stored at -70°C. Capsaicin was also dissolved in ethanol to make a stock solution of 40 mg/ml. The solutions were diluted at least 1000-fold into the growth medium such that the final concentration of ethanol had no effect on the differentiation and proliferation of HL-60 cells. All manipulations were performed in subdued light.

2.3. Determination of cell viability and proliferation

Cell viability was determined by the trypan blue exclusion assay as previously described (Coligan et al., 1995). Viability was calculated as the percentage of live cells in the total cell population. Cell proliferation was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) assay. In brief, after each treatment, 10 μl of MTT (5 mg/ml) was added to each well in 96-well plates. After incubation for 4 h at 37°C, the crystals of viable cells were dissolved with 100 μl of 0.04 N HCl in isopropanol. The absorbance of each well was then read at 540 nm using a kinetic microplate reader.

2.4. Immunofluorescent staining and cytofluorometric measurements

Quantitative immunofluorescence measurements were performed in an Epic V flow cytofluorograph (Coulter Electronics, Hialeah, FL, USA) equipped with a multiparameter data acquisition and display system (MDADS) as previously described (Kim et al., 1995). Briefly, a single cell suspension was collected from the various cultures and washed twice with ice-cold phosphate buffered saline (PBS, pH 7.4). Afterwards, fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 or anti-human CD11b monoclonal antibodies (Becton Dickinson, San Jose, CA, USA) were added, followed by incubation at 4°C for 1 h. After incubation, the cells were washed with PBS and were fixed in PBS containing 1% paraformaldehyde, and cytofluorometric analysis was performed. Background staining was determined by substituting cells stained with FITC-conjugated isotype control monoclonal antibody for cells stained with the specific antibodies. One parameter fluorescence histograms were generated by analyzing at least 1×10^4 cells.

2.5. Determination of cell differentiation

HL-60 cell differentiation was assessed by the nitroblue tetrazolium reduction assay as previously described (Collins et al., 1979). This assay is based on the ability of phagocytic cells to produce superoxide upon stimulation with tissue plasminogen activator. For this assay, 2×10^5 cells were harvested by centrifugation and incubated with an equal volume of 0.2% nitroblue tetrazolium dissolved in PBS containing 1 ng/ml of freshly diluted plasminogen activator at 37°C for 30 min in the dark. Cytospin slides were prepared and were examined for blue–black nitroblue diformazan deposits, indicative of a plasminogen activator-stimulated respiratory burst. At least 200 cells were assessed for each experiment.

2.6. Protein kinase C activity assay

Protein kinase C activity was determined using a commercially available kit (Gibco BRL, Rockville, MD, USA)

as previously described (Yasuda et al., 1990). Briefly, HL-60 cells were homogenized in extraction buffer containing 20 mM Tris (pH 7.5), 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.5% Triton X-100, 25 μg/ml leupeptin and 25 μg/ml aprotinin. These crude extracts in the extraction buffer were partially purified with a diethylaminoethyl (DEAE)-Sephacel column (Sigma) and eluted with buffer containing 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol and 0.2 M NaCl. Subsequently, each sample was incubated with lipid, peptide, and protein kinase C substrates containing [γ-^{32}P]ATP for 5 min at 30°C. The terminated reaction mixture was pipetted onto a phosphocellulose disc and quantified by scintillation counting.

2.7. Statistical analysis

Student's *t*-test and one-way analysis of variance (ANOVA) were used to determine the statistical significance of differences between values for various experimental and control groups. *P*-values < 0.05 were considered significant.

3. Results

3.1. Effect of capsaicin on HL-60 cell proliferation and differentiation

HL-60 cells were seeded at a density of 2×10^5 cells/ml, and the cells were treated with medium alone or with 5–30 μg/ml capsaicin for 72 h. Cell proliferation for each group was determined as described in Section 2. Treatment of HL-60 cells with 5 μg/ml capsaicin had no effect on cell proliferation. Treatment of the cells with more than 5 μg/ml capsaicin for 72 h significantly inhibited cell proliferation (Fig. 1A).

Next, the effect of capsaicin on HL-60 cell differentiation was assessed with the nitroblue tetrazolium reduction assay. As shown in Fig. 1B, incubation with more than 15 μg/ml capsaicin induced cell differentiation with slight increases as the concentration increased. However, incubation of the cells with medium alone or with less than 10 μg/ml capsaicin had little or no effect on cell differentiation. The results indicated that capsaicin was a very weak inducer of differentiation in HL-60 cells. Staining the cells with Giemsa revealed little or no effect of capsaicin on cell morphology (data not shown).

3.2. Synergistic effect of capsaicin on 1,25-$(OH)_2D_3$- and all-trans retinoic acid-induced HL-60 cell differentiation

To determine the effect of capsaicin on 1,25-$(OH)_2D_3$- and all-*trans* retinoic acid-induced cell differentiation, HL-

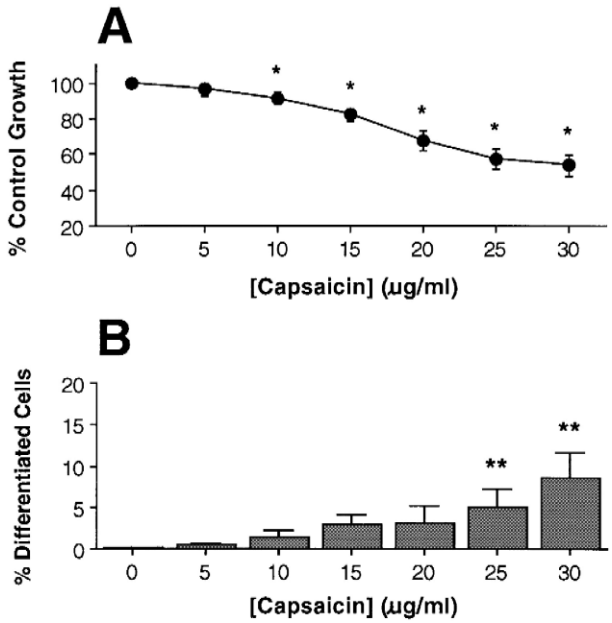

Fig. 1. Effect of capsaicin on HL-60 cell proliferation and differentiation. HL-60 cells were treated for 72 h with medium alone or 5–30 μg/ml capsaicin. Cell proliferation was measured as described in Section 2, and is represented as the percentage for each treated group relative to the untreated control group (A). Aliquots were removed and cell differentiation was determined by the nitroblue tetrazolium reduction assay (B). Each value represents the means ± S.D. of triplicate determinations from one representative experiment. Each experiment was repeated at least twice with similar results. $^{*}P < 0.05$, compared with an untreated group. $^{**}P < 0.01$, compared with an untreated group.

60 cells were treated with capsaicin alone or in combination with either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid, and cellular differentiation was assessed by the nitroblue tetrazolium reduction assay. As shown in Fig. 2A, capsaicin synergistically potentiated 1,25-$(OH)_2D_3$- and all-*trans* retinoic acid-induced HL-60 cell differentiation. For example, the observed effect of 5 nM 1,25-$(OH)_2D_3$ in combination with 20 μg/ml capsaicin (60.7% differentiated cells) was greater than the sum of the effects of the individual treatments (24.6% differentiated cells). Similar synergistic effects of capsaicin and all-*trans* retinoic acid were observed. The effect of 50 nM all-*trans* retinoic acid in combination with 20 μg/ml capsaicin (53.6% differentiated cells) was also greater than the sum of the effects of the individual treatments (23.2% differentiated cells).

As shown in Fig. 2B, cell proliferation was somewhat reduced by exposure of HL-60 cells to 5 nM 1,25-$(OH)_2D_3$ or 50 nM all-*trans* retinoic acid (13.5% and 11.4% reduction, respectively). The combined treatment of HL-60 cells with capsaicin together with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM all-*trans* retinoic acid for 72 h had a greater inhibitory effect on cell proliferation than the treatment with a single compound. The combined effect on cell proliferation was additive, not synergistic.

HL-60 cell differentiation was also assessed by the expression of CD11b (Mac-1) antigen. CD11b is a cell

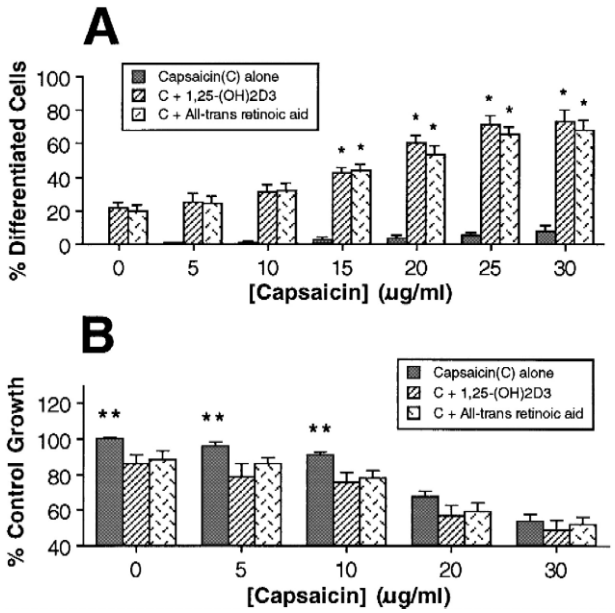

Fig. 2. Synergistic effects of capsaicin on 1,25-$(OH)_2D_3$- and all-*trans* retinoic acid-induced HL-60 cell differentiation. HL-60 cells were treated for 72 h with 0–30 μg/ml of capsaicin alone or in combination with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM all-*trans* retinoic acid. Afterwards, cell differentiation (A) and proliferation (B) were determined as described Section 2. Each value represents the mean ± S.D. of triplicate determinations from one representative experiment. The experiment was repeated more than three times with similar results. $^{*}P < 0.001$, compared with the sum of the individual treatments. $^{**}P < 0.05$, compared with groups treated with capsaicin in combination with either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid.

surface marker for differentiation into either monocytes or granulocytes (Kansas et al., 1990). As shown in Fig. 3, 1,25-$(OH)_2D_3$ and all-*trans* retinoic acid strongly mediated an increase in CD11b expression. Capsaicin synergistically increased the number of CD11b-positive cells when combined with either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid, confirming that capsaicin potentiated 1,25-$(OH)_2D_3$- or all-*trans* retinoic acid-induced HL-60 cell differentiation.

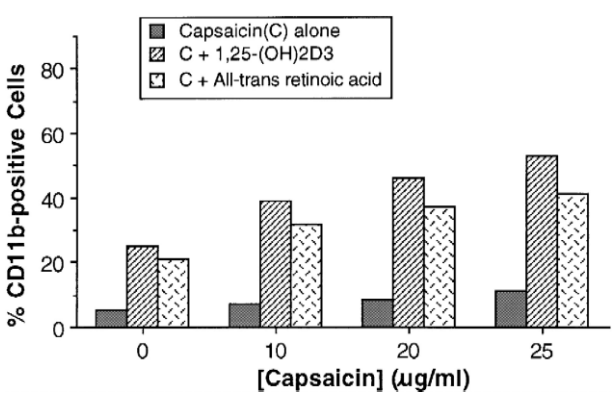

Fig. 3. Effect of capsaicin on 1,25-$(OH)_2D_3$-or all-*trans* retinoic acid-induced HL-60 cell differentiation as measured by CD11b expression. HL-60 cells were exposed to various concentrations of capsaicin for 72 h in the absence or presence of either 5 nM 1,25-$(OH)_2D_3$ or 50 nM all-*trans* retinoic acid. CD11b expression was determined by flow cytometry as described in Section 2; 10,000 cells were analyzed each time. Data are representative of three separate experiments.

3.3. Effects of capsaicin and 1,25-$(OH)_2D_3$ or capsaicin and all-trans retinoic acid on differentiation pathways of HL-60 leukemia cells

To determine the differentiation pathway that HL-60 cells have followed after treatment with capsaicin and 1,25-$(OH)_2D_3$ or with capsaicin and all-*trans* retinoic acid, HL-60 cells were treated with capsaicin alone or in combination with either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid, and flow cytometric analysis using monoclonal antibody for the monocytic surface antigen CD14 was determined. The CD14 antigen is expressed exclusively when cells are differentiated into monocytes (Wright et al., 1990). As shown in Fig. 4, HL-60 cells treated with a mixture of capsaicin and 1,25-$(OH)_2D_3$ reacted very

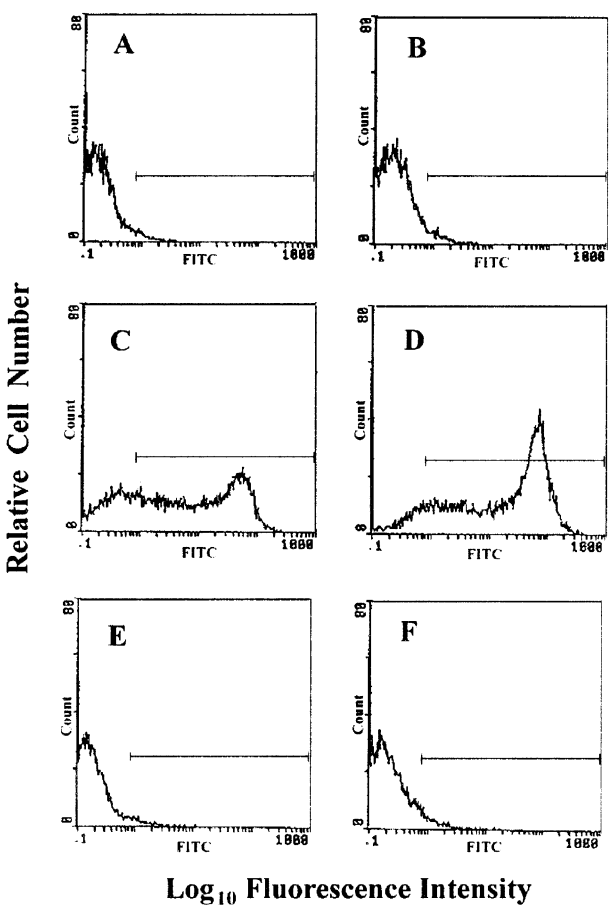

Fig. 4. Fluorocytometric analysis of HL-60 cell differentiation using monoclonal antibody for monocytic cell surface marker CD14. HL-60 cells were treated for 72 h with medium alone (A), 25 μg/ml capsaicin (B), 5 nM 1,25-$(OH)_2D_3$ (C), 25 μg/ml capsaicin in combination with 5 nM 1,25-$(OH)_2D_3$ (D), 50 nM all-*trans* retinoic acid (E), or 25 μg/ml capsaicin in combination with 50 nM all-*trans* retinoic acid (F). The cells were incubated with FITC-conjugated anti-CD14 monoclonal antibody. CD14 expression was assessed by fluorocytometric analysis.

strongly with anti-CD14 monoclonal antibody. Cells treated with 1,25-$(OH)_2D_3$ alone also reacted with anti-CD14 monoclonal antibody, but to a lesser extent than did the cells treated with a mixture of capsaicin and 1,25-$(OH)_2D_3$. These results indicate that capsaicin stimulated 1,25-$(OH)_2D_3$-induced HL-60 cell differentiation along the monocytic pathway. In contrast, HL-60 cells treated with a mixture of capsaicin and all-*trans* retinoic acid showed little staining with anti-CD14 monoclonal antibody, although synergistic induction of cell differentiation was observed as shown by the nitroblue tetrazolium reduction assay. In addition, HL-60 cells treated with a mixture of capsaicin and all-*trans* retinoic acid stained strongly with a monoclonal antibody against HL-60 cell differentiation marker CD11b (Fig. 3), indicating that capsaicin stimulated all-*trans* retinoic acid-induced HL-60 cell differentiation along the granulocytic pathway.

3.4. Effect of inhibitors for protein kinase C or extracellular signal-regulated kinase on HL-60 cell differentiation induced by capsaicin in combination with 1,25-$(OH)_2D_3$ or all-trans retinoic acid

Previous studies have provided evidence that activation of protein kinase C has been shown to be necessary for differentiation of HL-60 cells (Martell et al., 1988; Wu et al., 1989; Pan et al., 1997). To explore the possibility of a relationship between the effect of capsaicin on cellular differentiation and protein kinase C activation, HL-60 cells were treated with capsaicin alone or in combination with either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid, and protein kinase C activity in the treated cells was determined. As shown in Fig. 5A, capsaicin increased protein kinase C activity in HL-60 cells and the increased level of protein kinase C activity was near-maximal at 2 h after the treatment. Furthermore, capsaicin enhanced protein kinase C activity in both 1,25-$(OH)_2D_3$- and all-*trans* retinoic acid-treated HL-60 cells (Fig. 5B), and the levels were sustained during the entire 72-h observation period.

To further investigate the involvement of protein kinase C in the HL-60 cell differentiation enhanced by capsaicin, HL-60 cells were treated with specific protein kinase C inhibitors, bisindolylmaleimide (GF-109203X), chelerythrine (CE) or 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), in the presence of capsaicin alone or as combinations of either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid. Afterward, cell differentiation was assessed by the nitroblue tetrazolium reduction assay. As shown in Fig. 6, all three protein kinase C inhibitors significantly inhibited HL-60 cell differentiation whether treated with capsaicin alone or in combination with either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid. Especially, the

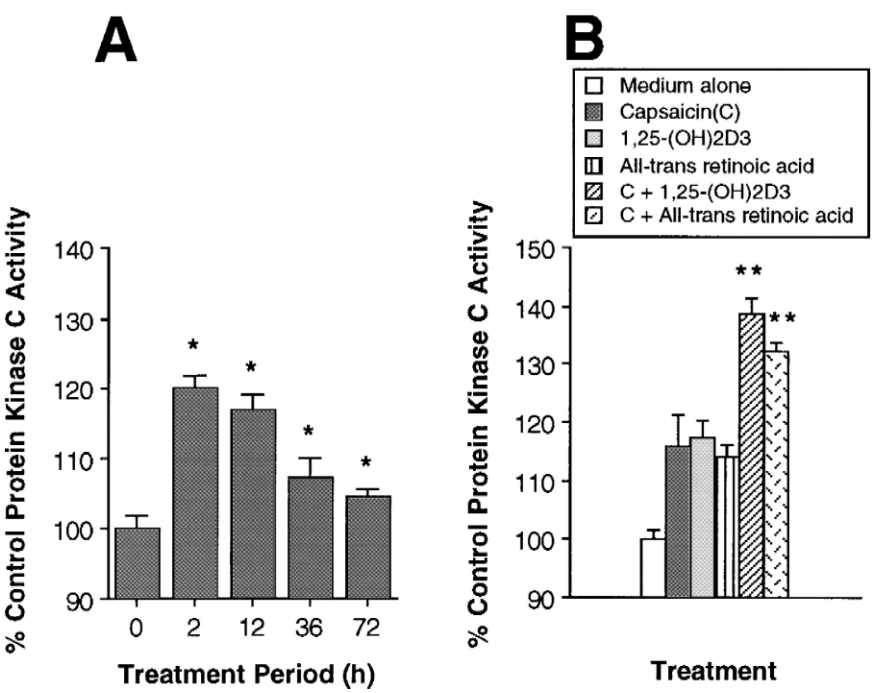

Fig. 5. Effect of capsaicin on protein kinase C activity in 1,25-$(OH)_2D_3$- and all-*trans* retinoic acid-treated HL-60 cell. HL-60 cells were treated with 25 μg/ml capsaicin for 2, 12, 36 or 72 h and total protein kinase C activity in the treated cells was determined as described in Section 2 (A). The cells were treated with medium alone, 25 μg/ml capsaicin, 5 nM 1,25-$(OH)_2D_3$, 50 nM all-*trans* retinoic acid, or 25 μg/ml capsaicin in combination with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM all-*trans* retinoic acid for 2 h (B). The % control protein kinase C activity represents the percentage of protein kinase C activity of each treated group relative to the untreated control group. The values represent the means ± S.D. of triplicate determinations. The experiment was repeated twice with similar results. $^{*}P < 0.05$, compared with an untreated group. $^{**}P < 0.01$, compared with any other groups.

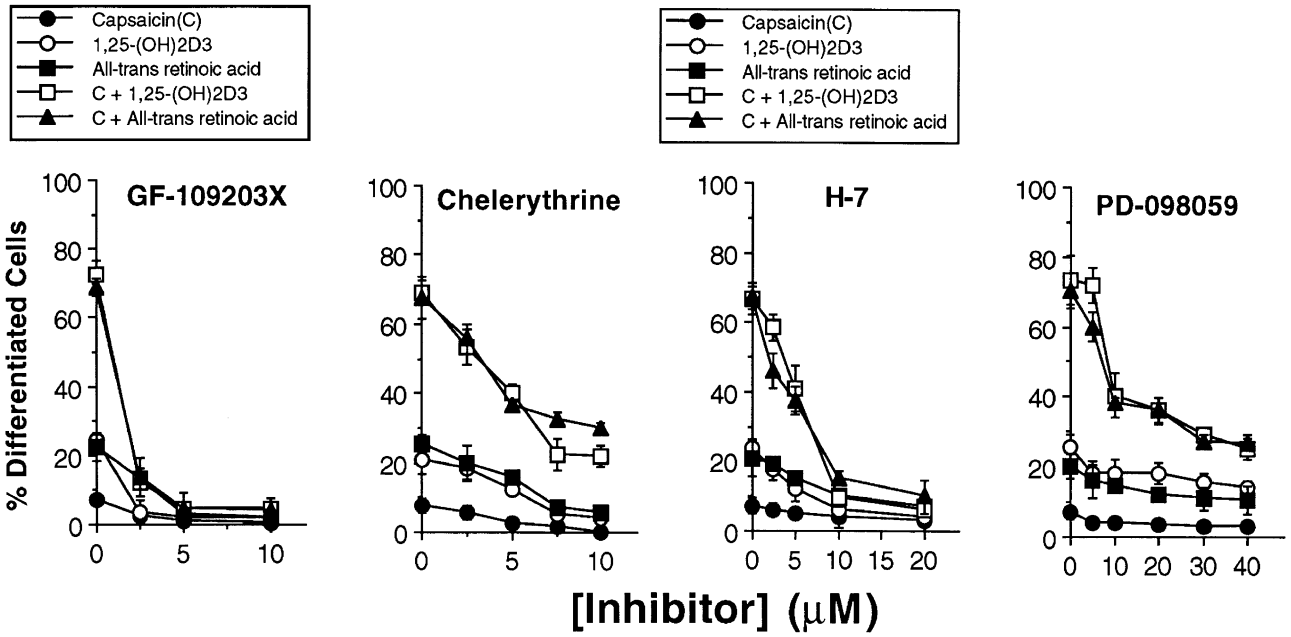

Fig. 6. Effect of protein kinase C and ERK kinase inhibitors on HL-60 cell differentiation induced by capsaicin alone or in combination with either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid. Cells were treated for 40 min with varying concentrations of protein kinase C inhibitors (GF-109203X, chelerythrine or H-7) or an ERK kinase inhibitor PD-098059, followed by incubation with 25 μg/ml capsaicin, 5 nM 1,25-$(OH)_2D_3$, 50 nM all-*trans* retinoic acid, or 25 μg/ml capsaicin in combination with either 5 nM 1,25-$(OH)_2D_3$ or 50 nM all-*trans* retinoic acid for 72 h. Cellular differentiation was assessed by the NBT reduction assay. Data are presented as percentages of differentiated cells with the means ± S.D. of triplicate determinations. The experiment was repeated twice with similar results.

treatment with GF-109203X completely inhibited both the 1,25-$(OH)_2D_3$- and the all-*trans* retinoic acid-induced cell differentiation enhanced by capsaicin. Protein kinase C inhibitors also inhibited 1,25-$(OH)_2D_3$- and all-*trans* retinoic acid-induced cell differentiation in the absence of capsaicin, however, to a lesser extent than that induced by combinations of capsaicin and either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid.

Extracellular signal-regulated kinase (ERK) is one of the mitogen-activated protein kinases (MAPK) and is a downstream element in the protein kinase C signaling pathway of HL-60 cells (Marcinkowska et al., 1997). To determine the involvement of ERK in 1,25-$(OH)_2D_3$- and all-*trans* retinoic acid-induced cell differentiation enhanced by capsaicin, HL-60 cells were treated with 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD-098059), a specific ERK kinase inhibitor, in the presence of capsaicin alone or in combinations of either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid. The synthetic compound, PD-098059, inhibits the ERK pathway by preventing the activation of ERK kinase by c-Raf (Alessi et al., 1995). As shown in Fig. 6, PD-098059 significantly inhibited HL-60 cell differentiation after treatment with capsaicin in combination with either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid. Therefore, capsaicin may potentiate 1,25-$(OH)_2D_3$- or all-*trans* retinoic acid-induced HL-60 cell differentiation, and both protein kinase C and ERK may be involved in the cell differentiation synergistically enhanced by capsaicin.

4. Discussion

In the present study we have demonstrated that capsaicin potentiates 1,25-$(OH)_2D_3$-and all-*trans* retinoic acid-induced differentiation in HL-60 promyelocytic leukemia cells that are widely used as a model system for differentiation studies. HL-60 cells were synergistically differentiated into monocytes or granulocytes when treated with capsaicin in combination with either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid. Many previous studies have shown some chemical combinations which exerted an additive or synergistic effect on HL-60 cell differentiation. These combinations include butyrate and all-*trans* retinoic acid or hexafluoro-vitamin D_3 (Breitman and He, 1990; Yoshida et al., 1992), vitamin D_3 and interferon-γ (Weinberg et al., 1986), vitamin D_3 and tumor necrosis factor-α (Wang et al., 1991), all-*trans* retinoic acid and α-tocopherol (Makishima et al., 1996), and vitamin D_3 and vitamin E succinate (Sokoloski et al., 1997).

The mechanism by which capsaicin potentiates 1,25-$(OH)_2D_3$- or all-*trans* retinoic acid-induced HL-60 cell differentiation is not clear. 1,25-$(OH)_2D_3$ and all-*trans* retinoic acid are believed to mediate biological responses

including cell differentiation as a consequence of their interaction with nuclear receptors to regulator gene transcription (Haussler et al., 1998) and with a putative cell membrane receptor to generate rapid non-genomic effects (Norman et al., 1997), including the opening of voltage-gated calcium and chloride channels (Zanello and Norman, 1997), and activation of protein kinase C and mitogen-activated protein kinase (Pan et al., 1997; Song et al., 1998). In our study, inhibitors for protein kinase C or ERK kinase significantly inhibited the HL-60 cell differentiation induced with capsaicin in combinations with either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid, suggesting that potentiation of cell differentiation by capsaicin may be, at least in part, via protein kinase C and ERK-mediated signaling pathway. Capsaicin itself is known to activate protein kinase C (Harvey et al., 1995). Furthermore, inhibitors for protein kinase C and ERK kinase inhibited both 1,25-$(OH)_2D_3$- and all-*trans* retinoic acid-induced HL-60 cell differentiation potentiated by capsaicin, suggesting that both protein kinase C and ERK may be common signaling components involved in the capsaicin-enhanced HL-60 cell differentiation into granulocytic and monocytic lineage. Previous studies have demonstrated that protein kinase C and ERK might be involved in the signaling pathway of both lineages (Solomon et al., 1991; Koike et al., 1992; Marcinkowska et al., 1997; Yen et al., 1999).

The increased levels of protein kinase C activity were approximately the same in HL-60 cells treated with 1,25-$(OH)_2D_3$, all-*trans* retinoic acid or with capsaicin (Fig. 5), although treatment with 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid induced much greater cell differentiation than did capsaicin (23.4 ± 3.2, 20.3 ± 2.7 and 7.2 ± 1.4, respectively). This may have been due to different time courses of enhancement of protein kinase C activity between capsaicin and either 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid. Capsaicin increased protein kinase C activity much earlier than did 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid, which is consistent with effect of other calcium ionophores such as ionomycin (Pan et al., 1997). The levels of protein kinase C activity in capsaicin-treated cells were approximately maximal at 2 h after the treatment (Fig. 5A). In contrast, 1,25-$(OH)_2D_3$ was known to increase protein kinase C activity much later and required 24 h to significantly enhance protein kinase C activity (Solomon et al., 1991).

All-*trans* retinoic acid has been used for the treatment of leukemia patients (Warrell et al., 1991) and its analogues have been used for the treatment of psoriasis (Orfanos et al., 1987). Vitamin D_3 and some of its analogues are also used for the treatment of psoriasis (Kragballe, 1992). The results presented here suggest that treatment of patients with combinations of capsaicin and 1,25-$(OH)_2D_3$, or capsaicin and all-*trans* retinoic acid may produce a greater therapeutic response than 1,25-$(OH)_2D_3$ or all-*trans* retinoic acid alone, possibly with less toxicity. Clinical studies are needed to evaluate this possibility, especially at concentrations of capsaicin that do not induce known side-effects. It is possible that many dietary chemicals such as curcuminoids, tocopherols, carotenoids, and other edible plants can prevent human cancer, in part by synergizing with endogenously produced stimulators of differentiation such as retinoic acids and vitamin D_3. Epideminology studies suggest that people who eat large amounts of fruit and some vegetables have a lower risk of many kinds of cancer (Negri et al., 1991).

In conclusion, capsaicin potentiates 1,25-$(OH)_2D_3$- and all-*trans* retinoic acid-induced HL-60 cell differentiation via the protein kinase C signaling pathway. These results may explain some known activities of capsaicin, including its anti-carcinogenic effects and suggest a possible use of capsaicin in the treatment of neoplastic diseases.

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