

Possible involvement of protein kinase C in proliferation and differentiation of osteoblast-like cells

Osamu Kozawa, Kensuke Takatsuki, Katsuhiro Kotake, Masahiro Yoneda, Yutaka Oiso and Hidehiko Saito

First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya 466, Japan

Received 21 November 1988

In cloned osteoblast-like cells, MC3T3-E1, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C activating phorbol ester, and 1-oleoyl-2-acetyl glycerol (OAG), a specific activator for protein kinase C, stimulated DNA synthesis in a dose-dependent manner. Both TPA and OAG acted synergistically with insulin-like growth factor I to stimulate DNA synthesis. TPA as well as OAG suppressed the increase in alkaline phosphatase activity of MC3T3-E1 cells induced by parathyroid hormone. These results suggest that protein kinase C is involved in the process which directs osteoblast-like cells toward proliferation.

Protein kinase C; Osteoblast

1. INTRODUCTION

It is now generally accepted that protein kinase C plays important roles in numerous cellular processes including the regulation of cell proliferation, differentiation and secretion [1,2]. In osteoblasts, the actions of certain hormones (e.g. PTH) and prostaglandins are thought to be mediated through the activation of adenylate cyclase [3,4]. However, the involvement of protein kinase C in signal transduction system remains unclear. Therefore, we studied the effects of TPA and OAG, potent activators of protein kinase C [1], on DNA synthesis and ALP activity in osteoblast-like MC3T3-E1 cells cloned from newborn mouse calvaria [5,6]. We also studied the interaction of these protein kinase C activators with other osteoblast modulators, IGF-I [7,8] and PTH [9].

Correspondence address: O. Kozawa, First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya 466, Japan

Abbreviations: PTH, parathyroid hormone; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; OAG, 1-oleoyl-2-acetyl glycerol; ALP, alkaline phosphatase; IGF-I, insulin-like growth factor I; α -MEM, α -minimum essential medium; FCS, fetal calf serum

Our results suggest that protein kinase C is involved in the process which directs osteoblast-like cells toward proliferation.

2. MATERIALS AND METHODS

2.1. Materials

TPA was purchased from Sigma. OAG was from Nakarai Chemical. [*methyl*-³H]Thymidine (22 Ci/mmol) was from Amersham. Synthetic IGF-I was provided by Fujisawa Pharmaceutical Co. (Osaka, Japan). Synthetic PTH(1–34) was provided by Yamasa Shoyu Co. (Chiba, Japan). Other materials and chemicals were from commercial sources.

2.2. Cell culture

Cloned osteoblast-like cells, MC3T3-E1, were generously provided by Dr M. Kumegawa (Meikai University, Sakado, Japan) and maintained at 37°C in a humidified atmosphere of 5% CO₂, 95% air in α -MEM containing 10% FCS. The cells (5×10^4) were seeded into 35-mm diameter dishes in 2 ml of α -MEM containing 10% FCS. After 3 days, the medium was exchanged for 1 ml of α -MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

2.3. Studies on DNA synthesis

The cells cultured as above were incubated in 1 ml of α -MEM containing 0.3% FCS and various doses of TPA or OAG in the absence or presence of IGF-I for 22 h. The cells were then incubated with 2 μ Ci of [*methyl*-³H]thymidine for 6 h. The incubation was terminated by adding 10% trichloroacetic acid, and the radioactivity in the acid-insoluble materials [10] was

determined with Beckman liquid scintillation system, model LS7500.

2.4. Studies on ALP activity

The cells were first exposed to TPA or OAG for 10 min, and then incubated in 1 ml of α -MEM containing 0.3% FCS with or without 1×10^{-7} M PTH for 24 h. At the end of the experiment, the cells were harvested by scraping with a rubber policeman into 0.2% Nonidet P-40 and disrupted by sonication. The resulting homogenate was assayed for ALP activity by the method of Lowry et al. [11].

3. RESULTS

TPA significantly stimulated DNA synthesis of MC3T3-E1 cells at concentrations between 1×10^{-9} M and 1×10^{-7} M (fig.1A), which are known to activate protein kinase C in a variety of eukaryotic cells [1]. OAG also stimulated DNA synthesis in a dose-dependent manner (fig.1B). The pattern of the stimulation by OAG appears to be almost identical to that by TPA, except that the comparable degree of activation was achieved at a molar ratio of approximately 1000:1.

IGF-I, a known activator of the proliferation of calvaria osteoblasts [7,8], doubled the DNA synthesis in MC3T3-E1 cells at a concentration of 1×10^{-7} M. When OAG was added to the culture at concentrations between 1×10^{-7} M and $1 \times$

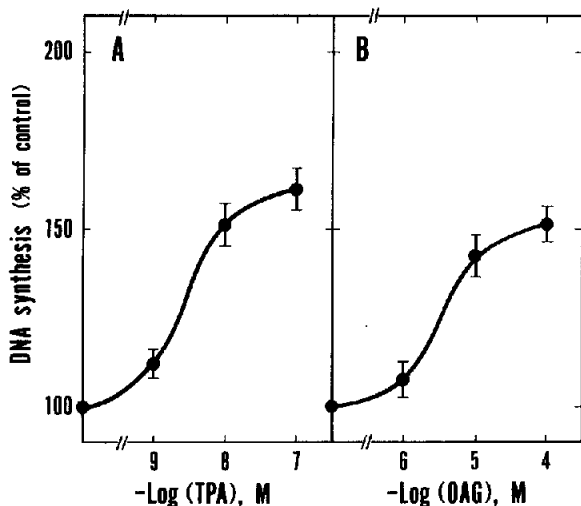


Fig.1. Effect of TPA or OAG on DNA synthesis of MC3T3-E1 cells. The [3 H]thymidine incorporation into the acid-insoluble materials of the cells incubated without TPA or OAG is expressed as 100% (control). (A) Cells incubated with TPA; (B) cells incubated with OAG. Each point represents the mean \pm SD of three separate experiments.

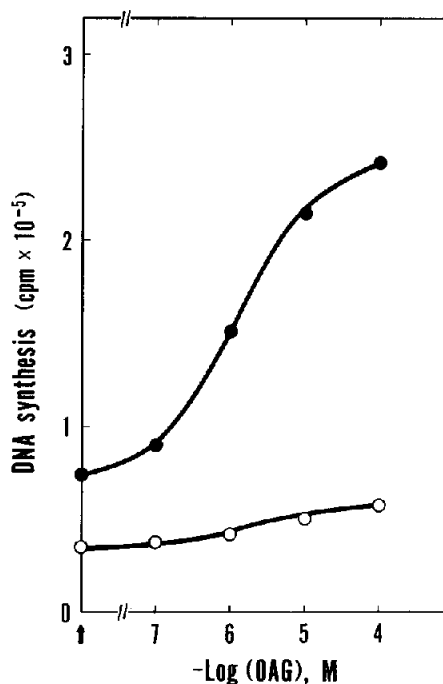


Fig.2. Synergistic effect of OAG and IGF-I on DNA synthesis of MC3T3-E1 cells. The arrow indicates the DNA synthesis of the cells incubated in the absence of OAG. (\bullet — \bullet) 1×10^{-7} M IGF-I added; (\circ — \circ) no IGF-I added. Each point represents the mean of duplicate determinations.

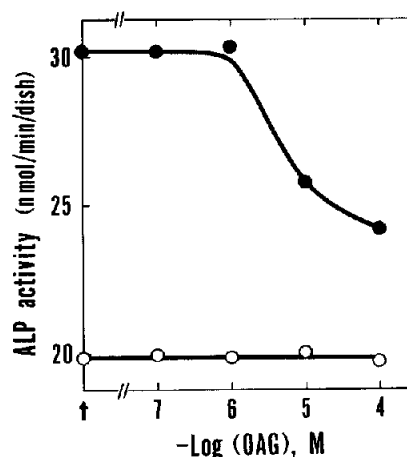


Fig.3. Effect of OAG on PTH-induced increase in ALP activity of MC3T3-E1 cells. The cells were first exposed to OAG or vehicle (arrow) for 10 min, and then incubated with (\bullet — \bullet) or without (\circ — \circ) 1×10^{-7} M PTH for 24 h. Each point represents the mean of duplicate determinations.

10^{-4} M, it acted synergistically with IGF-I to stimulate DNA synthesis in a dose-dependent manner (fig.2). TPA had a similar synergistic effect with IGF-I upon DNA synthesis (not shown).

We next examined the effect of these protein kinase C activators upon ALP activity, a marker of mature osteoblast phenotype [12]. OAG, which by itself had no effect on ALP activity, suppressed the increase in ALP activity induced by PTH (1×10^{-7} M) in a dose-dependent manner in a range between 1×10^{-6} M and 1×10^{-4} M (fig.3). Similarly, TPA at a concentration of 1×10^{-7} M also resulted in a 34% suppression of the increase in ALP activity induced by 1×10^{-7} M PTH.

4. DISCUSSION

The present results show that TPA stimulates DNA synthesis in the cloned osteoblast-like cells as in many other cell types such as fibroblasts [13,14] and smooth muscle cells [15]. It is well established that the phorbol ester receptor is protein kinase C complexed with phosphatidylserine and Ca^{2+} , and that the diverse actions of the phorbol esters are mediated through the activation of this enzyme [1,2]. In our experiments, DNA synthesis was enhanced also by OAG, a specific activator of protein kinase C [1]. Therefore, it is most likely that the action of TPA to stimulate DNA synthesis in the osteoblast-like cells is mediated through the activation of protein kinase C.

There is ample evidence indicating that two sets of growth factors, namely 'competence factors' and 'progression factors', successively and synergistically promote the proliferation of cells [16]. In the case of fibroblasts, platelet-derived growth factor and fibroblast growth factor that process cells from G_0 to G_1 phase, belong to the competence factors, whereas insulin, epidermal growth factor and IGF-I that induce progression of cells from G_1 to S phase, belong to the progression factors [17]. Evidence is accumulating that, in fibroblasts [17] and smooth muscle cells [15], the action of the competence factors is mediated through activation of protein kinase C and mobilization of calcium ion. The results of our experiments that TPA (and OAG) stimulated DNA synthesis synergistically with IGF-I (a progression factor in fibroblasts), might allow the speculation that, in MC3T3-E1 cells, protein kinase C mediates

the signal from a putative competence factor, which is to be identified.

MC3T3-E1 cells retain many osteoblast-like features such as high ALP activity, type I collagen synthesis and mineralization in vitro [5,6]. The ALP of this cell is known to be activated by PTH [9]. From our observation that TPA and OAG suppressed the increase in ALP activity induced by PTH, it seems likely that protein kinase C plays an antagonistic role to the action of PTH as far as ALP activity is concerned. Since ALP activity is considered to be a mature osteoblast phenotype [12], our results as a whole may suggest that protein kinase C is involved in the process which suppresses the differentiation of osteoblast-like cells and directs them toward proliferation.

Acknowledgements: We are grateful to Masumi Hiramatsu and Yasuyo Jo for their skillful secretarial assistance.

REFERENCES

- [1] Takai, Y., Kikkawa, U., Kaibuchi, K. and Nishizuka, Y. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* 18, 119-158.
- [2] Nishizuka, Y. (1986) *Science* 233, 305-312.
- [3] Majeska, R.J., Rodan, S.B. and Rodan, G.A. (1980) *Endocrinol.* 107, 1494-1503.
- [4] Partridge, N.C., Alcorn, D., Michelangeli, V.P., Kemp, B.E., Ryan, G.B. and Martin, T.J. (1981) *Endocrinology* 108, 213-219.
- [5] Kodama, H., Amagai, Y., Sudo, H., Kasai, S. and Yamamoto, S. (1981) *Jpn. J. Oral Biol.* 23, 899-901.
- [6] Sudo, H., Kodama, H., Amagai, Y., Yamamoto, S. and Kasai, S. (1983) *J. Cell. Biol.* 96, 191-198.
- [7] Canalis, E. (1980) *J. Clin. Invest.* 66, 709-719.
- [8] Schmid, C., Steiner, T. and Froesch, E.R. (1983) *Calcif. Tissue Int.* 35, 578-585.
- [9] Nakatani, Y., Tsunoi, M., Hakeda, Y., Kurihara, N., Fujita, K. and Kumegawa, M. (1984) *Biochem. Biophys. Res. Commun.* 123, 894-898.
- [10] Dicker, P. and Rozengurt, E. (1980) *Nature* 287, 607-612.
- [11] Lowry, O.H., Roberts, N.R., Wu, M.L., Hixon, W.S. and Crawford, E.J. (1954) *J. Biol. Chem.* 207, 19-37.
- [12] Robinson, R.J., Doty, S.B. and Cooper, R.R. (1973) in: *Biological Mineralization* (Zipkin, I. ed.) pp.257-296, Academic Press, New York.
- [13] Tsuda, T., Kaibuchi, K., Kawahara, Y., Fukuzaki, H. and Takai, Y. (1985) *FEBS Lett.* 191, 205-210.
- [14] Rozengurt, E. (1986) *Science* 234, 161-166.
- [15] Kariya, K., Kawahara, Y., Tsuda, T., Fukuzaki, H. and Takai, Y. (1987) *Atherosclerosis* 63, 251-255.
- [16] Stiles, C.D., Capone, G.T., Scher, C.D., Antoniadis, H.N., Van Wyk, J.J. and Pledger, W.J. (1979) *Proc. Natl. Acad. Sci. USA* 76, 1279-1283.
- [17] Callahan, M., Cochran, B.H. and Stiles, C.D. (1985) *Ciba Foundation Symposium* 116, 87-96.