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Elevation of Intracellular Calcium Ion by Prostaglandin E_1 and Its Inhibition by Protein Kinase C in a Human Megakaryocyte Leukemia Cell Line

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ABSTRACT

In the present study, we examined the effect of prostaglandin E_1 (PGE_1) on Ca^{2+} mobilization in a human megakaryocyte (the progenitor of platelets) leukemia cell line, designated as CMK. PGE_1 caused a rapid and dose-dependent increase in the intracellular free calcium level ($[Ca^{2+}]_i$) associated with the elevation of cyclic AMP. The PGE_1-induced elevation of $[Ca^{2+}]_i$ was decreased by the prior addition of ethylene glycol bis(2-aminoethylether)tetraacetic acid to the medium by approximately 25% of the control. This result indicates that the PGE_1-induced elevation of $[Ca^{2+}]_i$ is due to influx of Ca^{2+} from the external medium and to mobilization of Ca^{2+} from intracellular stores. Pretreatment of CMK cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a stimulus for protein kinase C, further enhanced the PGE_1-induced increase in the cellular cyclic AMP level. Inversely, pretreatment of CMK cells with TPA (10 nM), prior to the addition of PGE_1, inhibited the PGE_1-induced elevation of $[Ca^{2+}]_i$. Dibutyryl cyclic AMP and forskolin did not elevate $[Ca^{2+}]_i$ or affect the PGE_1-induced Ca^{2+} mobilization. The inhibitory action of TPA in the PGE_1-induced elevation of $[Ca^{2+}]_i$ was mimicked by other protein kinase C-activating agents, such as 1-oleoyl-2-acetylglycerol and *N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide, and was selectively restored by protein kinase C inhibitors, such as 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride and *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride. Thus, the inhibitory modulation of TPA on the PGE_1-induced elevation of $[Ca^{2+}]_i$ is mediated through protein kinase C activation. PGE_1 had no inductional effect of megakaryocytic phenotypic changes in CMK cells. The biological role of PGE_1, which increased $[Ca^{2+}]_i$ and cyclic AMP levels in the CMK cells, remains to be determined.

INTRODUCTION

Ca^{2+} plays essential roles in the induction of platelet aggregation and secretion by thrombin, collagen, ADP, platelet-activating factor, stable thromboxane A_2 analogues, and other agonists (1–6). PGE_1[2] inhibits these platelet functions by increasing the intracellular concentration of cAMP (7, 8). cAMP presumably decreases the elevation of $[Ca^{2+}]_i$ and thereby inhibits the platelet functions (9, 10). In contrast to such an inhibitory action of PGE_1, PGE_1 and prostaglandin E_2 have been shown to cause an elevation of $[Ca^{2+}]_i$ in some cell types and stimulate their cellular functions (11–13).

Recently, we have established a megakaryocytic cell line, designated as CMK (14), which releases platelet-like particles that possess α-granule and platelet peroxidase activity, as well as exhibit platelet GPIIb/IIIa antigen on their membranes (15). Since PGE_1 has an inhibitory effect on the agonist-induced elevation of $[Ca^{2+}]_i$ in platelets through cAMP accumulation, it is interesting to examine whether PGE_1 shows a similar effect in CMK cells. In this paper, we describe for the first time that PGE_1 is a potent activator of $[Ca^{2+}]_i$ in CMK cells.

Another line of study has shown that a phorbol ester such as TPA, a potent protein kinase C activator, inhibits the agonist-induced Ca^{2+} mobilization in platelets (16–21). On the basis of this finding, it is hypothesized that protein kinase C activation may have a role in the termination of the agonist-induced signal transduction in platelets. To clarify the relationship between protein kinase C activation and the PGE_1-modulated Ca^{2+} mobilization in CMK cells, we have examined the effects of protein kinase C activators and inhibitors on the PGE_1-induced Ca^{2+} mobilization in the cells. This paper also describes protein kinase C inhibition of the PGE_1-induced Ca^{2+} mobilization in CMK cells.

MATERIALS AND METHODS

Chemicals. Thrombin was a product of Mochida Pharmaceutical Co. (Tokyo, Japan). PGE_1 was donated by Ono Pharmaceutical Co. (Tokyo, Japan). TPA, dibutyryl cAMP, and forskolin were purchased from Sigma Chemical Co. (St. Louis, MO). OAG was from Nakarai Chemicals (Kyoto, Japan). H7, H8, HA1004, and SC9 were from Seikagaku Kogyo Co. (Tokyo, Japan). Fura 2-AM was from Dojindo Laboratories (Kumamoto, Japan). The cAMP radioimmunoassay kit was from Amersham Japan Co. (Tokyo, Japan).

Cell and Cell Cultures. The human megakaryocytic leukemia cell line (CMK) (14, 15) was maintained in polystyrene tissue culture flasks (Corning Glass Works, Corning, NY) that contained RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd, Tokyo) supplemented with 10% (v/v) fetal bovine serum (M. A. Bioproducts, Walkersville, MD). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every 2–4 days to maintain a density of 2×10^5 to 8×10^5 cells/ml. Stock CMK cells were stored in liquid nitrogen in a solution of 10% dimethyl sulfoxide in otherwise standard growth medium. Fresh cells were thawed every month during the course of the study to maintain the biological stability in the culture.

Assay for $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured by using a fluorescent Ca^{2+} indicator, fura 2-AM. Cells (5×10^6 cells/ml) were suspended in RPMI 1640 medium that contained 20 mM HEPES and were loaded by incubation with 2 μM fura 2-AM, final concentration. After incubation for 20 min at 37°C, the cells were washed twice with medium that contained 10% fetal bovine serum and were kept in the medium at room temperature until use. Just before use, aliquots of the cells (3×10^6 cells) were centrifuged in a microcentrifuge (Hitachi Koki Co., Tokyo), resuspended in 0.5 ml of a HEPES-Tyrode solution (10 mM HEPES at pH 7.4, 129 mM NaCl, 8.9 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$, and 5.6 mM dextrose), and transferred to the cuvet. Before experimental manipulations, the external Ca^{2+} was adjusted by adding 1 mM Ca^{2+} or 3 mM EGTA as required. The fura 2 fluorescence of these cells, stirring at 1000 rpm at 37°C, was recorded using a dual excitation wavelength spectrofluorometer (CAF100; Nihon Bunko Co., Tokyo, Japan), with excitation at 340 and 380 nm and emission at 500 nm. Values of $[Ca^{2+}]_i$ were calculated from fluorescence signals, as described by Tsien *et al.* (22).

Assay for cAMP. Cells (3×10^6 cells/ml) in suspension were washed

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[2] The abbreviations used are: PGE_1, prostaglandin E_1; cAMP, cyclic adenosine-3′,5′-monophosphate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)tetraacetic acid; $[Ca^{2+}]_i$, intracellular free calcium level; GP, platelet glycoprotein; OAG, 1-oleoyl-2-acetylglycerol; SC9, *N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; H8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; HA1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride; fura 2-AM, fura-2 acetoxymethyl ester.

twice with a HEPES-Tyrode solution, at 37°C, and were incubated with PGE_1 in 500 μl of the same solution. After the incubation, the medium was rapidly removed and cAMP was extracted with 500 μl of 12% perchloric acid, neutralized with $KHCO_3$, and kept at −80°C until measured. The cAMP concentrations produced by agonists were measured using the radioimmunoassay kit.

Flow Cytometric Analysis. To examine quantitatively the phenotypic differentiation of CMK cells induced by PGE_1 or TPA, cell surface antigenic changes toward greater maturation were measured using a flow cytometer (Epics; Coulter Electronics Inc., Hialeah, FL), with monoclonal antibodies in indirect immunofluorescent assays, as described in the previous paper (23). The monoclonal antibodies 224B, 227A, and Thr-4 (Serotec; Blackthorn, Bicester, United Kingdom) recognize GPIIb/IIIa, GPIb, and GPIIIa, respectively (23, 24). PLT1 (Coulter Immunology, Hialeah, FL) also reacts with platelets.

RESULTS

Fig. 1 shows the effect of 1 μM PGE_1 on $[Ca^{2+}]_i$ of fura 2-loaded CMK cells in the presence or absence of extracellular Ca^{2+} in the medium. In the presence of extracellular Ca^{2+}, PGE_1 provoked an acute rise in $[Ca^{2+}]_i$ (depending on the concentration used), which was followed by a rapid decrease. PGE_1 at 1 μM caused an elevation of $[Ca^{2+}]_i$ from a resting value of 143 ± 11 to 1128 ± 48 nM (means ± SD; three experiments). A second addition of PGE_1, 4 min after the first one, triggered a small elevation in $[Ca^{2+}]_i$. The PGE_1-induced spike phases were reduced by the prior addition of 3 mM EGTA but still induced a marked rise in $[Ca^{2+}]_i$, as shown in Fig. 1*B*. These results imply that the increase in $[Ca^{2+}]_i$ after the addition of PGE_1 was mainly dependent on the extracellular Ca^{2+} but

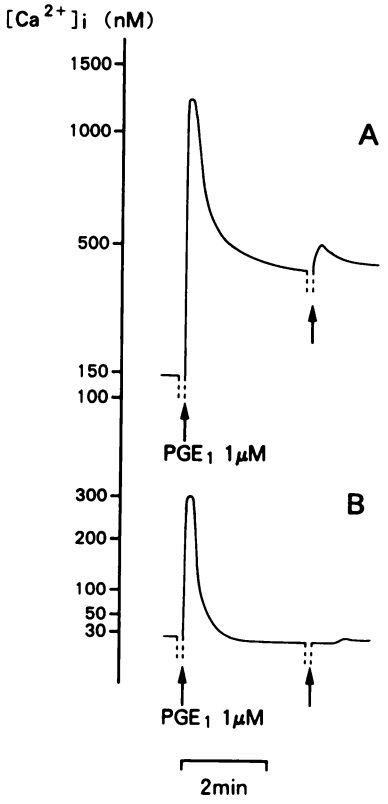

Fig. 1. PGE_1-induced elevation of $[Ca^{2+}]_i$ and its dependency on extracellular Ca^{2+} in medium. The cells loaded with fura 2-AM were washed, resuspended in either HEPES-Tyrode solution that contained 1 mM Ca^{2+} or Ca^{2+}-free medium that contained 3 mM EGTA, and then stimulated with 1 μM PGE_1 twice. Other details are described under "Materials and Methods." *A*, with PGE_1 in HEPES-Tyrode solution containing 1 mM Ca^{2+}; *B*, with PGE_1 in the Ca^{2+}-free medium. *Arrows*, two points of addition of PGE_1. The *panel* shows a typical pattern of fluorescent monitoring from three different experiments.

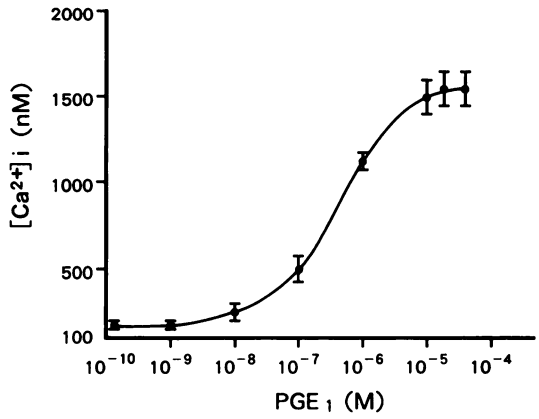

Fig. 2. Dose-dependent elevation of $[Ca^{2+}]_i$ by PGE_1. The cells loaded with fura 2-AM were washed, resuspended in HEPES-Tyrode solution that contained 1 mM Ca^{2+}, and then stimulated with various doses of PGE_1. Other details are described under "Materials and Methods." *Points* and *vertical bars*, mean ± SD of three separate experiments.

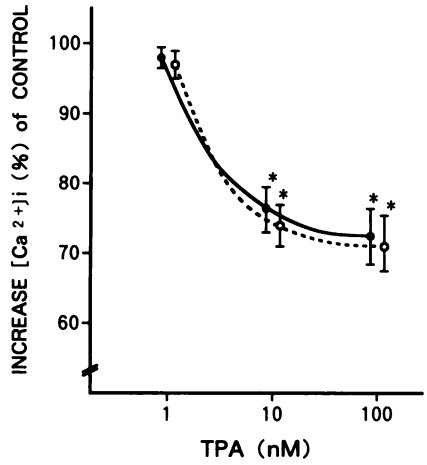

Fig. 3. Effect of TPA on the PGE_1- or thrombin-induced elevation of $[Ca^{2+}]_i$. The cells loaded with fura 2-AM were washed, resuspended in HEPES-Tyrode solution that contained 1 mM Ca^{2+}, exposed to the indicated concentration of TPA for 5 min, and then stimulated with 1 μM PGE_1 (——) or 0.5 units/ml thrombin (– – –). The increase in $[Ca^{2+}]_i$ over basal level without TPA addition was taken as 100% control. Other details are described under "Materials and Methods." Results are expressed as means ± SD of three independent experiments. *, values significantly different ($P < 0.01$) compared to the corresponding values without TPA, as measured by Student's *t* test.

was partly attributed to mobilization from intracellular Ca^{2+} storage sites.

Fig. 2 shows the dose-response curve for the PGE_1-induced increase in $[Ca^{2+}]_i$. The effect of 1 μM PGE_1, which increased $[Ca^{2+}]_i$ over the resting level by 1128 ± 48 nM, was taken as 100% control. The maximal and half-maximal increases in $[Ca^{2+}]_i$ were found at about 20 and 0.5 μM PGE_1, respectively. The concentrations of PGE_1 necessary for the increase in $[Ca^{2+}]_i$ were similar to those necessary for the inhibition of the agonist-induced elevation of $[Ca^{2+}]_i$ in platelets (9, 10).

As shown in Fig. 3, incubation of CMK cells with 10 nM TPA, a stimulus for protein kinase C, prior to the addition of PGE_1 impaired the PGE_1-induced increase in $[Ca^{2+}]_i$ by about 30%. TPA did not alter the resting level of $[Ca^{2+}]_i$ (data not shown). This inhibitory effect of TPA on the PGE_1-induced rise was dose dependent (1–100 nM). Thrombin (0.5 units/ml), which is a potent Ca^{2+}-mobilizing agonist in platelets, caused an elevation of $[Ca^{2+}]_i$ from a resting value to 1114 ± 82 nM (three experiments).[3] TPA caused the same effect on thrombin-

[3] R. Ryo, M. Adachi, A. Yoshida, W. Sugano, N. Yamaguchi, and T. Sato. Cytosolic Ca^{2+} mobilization and thromboxane synthesis in a human megakaryocytic leukemia cell, submitted for publication.

induced Ca^{2+} mobilization. The doses of TPA necessary for these inhibitions were the same as those necessary for the activation of protein kinase C (25). The inhibitory effect of TPA was mimicked by OAG or SC9, other protein kinase C activators, as shown in Table 1. Preincubation of CMK cells for 5 min with protein kinase C inhibitors, H7 and H9, abolished the inhibitory effect of TPA on the PGE_1-induced elevation in $[Ca^{2+}]_i$ in a dose-dependent manner (1–100 nM), as shown in Fig. 4. These inhibitors did not alter the resting $[Ca^{2+}]_i$ (data not shown). With H8 at a concentration of 100 nM, the PGE_1-induced increase in $[Ca^{2+}]_i$ of the TPA-treated CMK cells was restored to the level of the cells treated with H8 alone. In contrast, the same doses of HA1004, which had little inhibitory action for protein kinase C, failed to restore the TPA-induced inhibition of $[Ca^{2+}]_i$.

As illustrated in Fig. 5, TPA alone had little measurable effect on the basal cellular cAMP level, while TPA enhanced the PGE_1-induced cAMP accumulation in a time-dependent manner. The cAMP levels in the control samples in these experiments (i.e., without TPA or PGE_1), or in the samples containing TPA alone, were less than 1.0 pmol/10^5 cells. TPA caused the remarkable increase in the cAMP accumulation in response to PGE_1. The TPA-induced augmentation was demonstrated at 30 s after the addition of PGE_1.

Table 1 *Effect of protein kinase C activators on the PGE_1-induced elevation in $[Ca^{2+}]_i$*

The cells, loaded with fura 2-AM, were washed, resuspended in HEPES-Tyrode solution containint 1 mM Ca^{2+}; preincubated with TPA (10 nM), OAG (300 μM), or SC9 (10 mM) at 37°C for 5 min; and then stimulated with 1 μM PGE_1. The effect of 1 μM PGE_1, which increased $[Ca^{2+}]_i$ over the resting level, was taken as 100% control for the inhibition of PGE_1-induced elevation of $[Ca^{2+}]_i$ by protein kinase C activators. Other details are described under "Materials and Methods." Results are expressed as means ± SD of three independent experiments.

	Increase in $[Ca^{2+}]_i$ (% of control)
TPA (10 nM)	76.6 ± 2.8[a]
OAG (300 μM)	75.1 ± 3.9[a]
SC9 (10 mM)	75.7 ± 3.6[a]

[a] The values are significantly different ($P < 0.01$) compared to the corresponding values without protein kinase C activators, as measured by Student's *t* test.

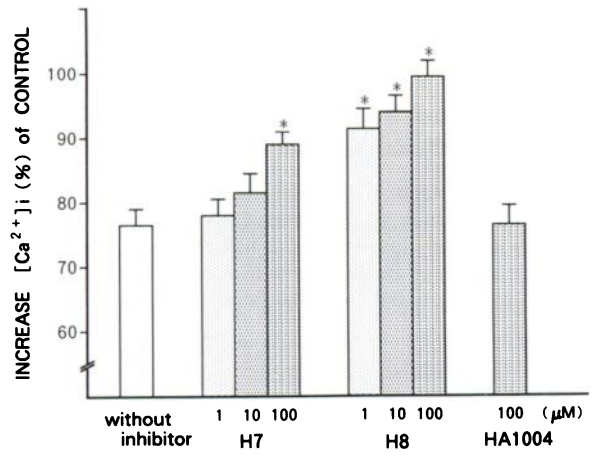

Fig. 4. Effect of protein kinase C inhibitors on the inhibitory effect of TPA in the PGE_1-induced elevation of $[Ca^{2+}]_i$. The cells loaded with fura 2-AM were washed, resuspended in HEPES-Tyrode solution that contained 1 mM Ca^{2+}, exposed to H7, H8, or HA1004 for 5 min at 37°C, treated with TPA (10 nM) for 5 min, and then stimulated with 1 μM PGE_1. The effect of 1 μM PGE_1, which increased $[Ca^{2+}]_i$ over the resting level without TPA and inhibitors, was taken as 100% control. Other details are described under "Materials and Methods." Results are expressed as means ± SD of three independent experiments. *, values are significantly different ($P < 0.01$) compared to the corresponding values without inducers, as measured by Student's *t* test.

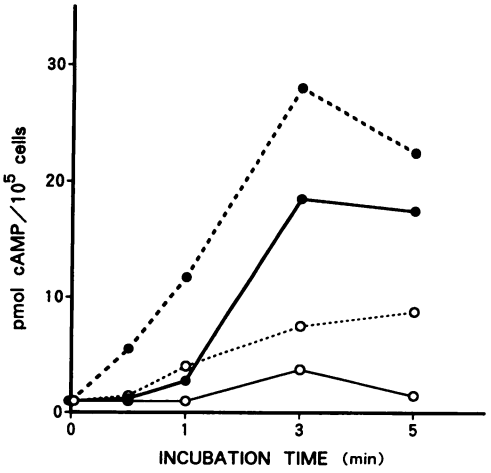

Fig. 5. Kinetics of modulation by TPA (10 nM) of the PGE_1-stimulated cAMP accumulation in CMK cells. CMK cells were treated with (– – –) or without (——) 10 nM TPA for 5 min and then stimulated with 1 μM (○) or 10 μM (●) PGE_1 for various periods of time. Other details are described under "Materials and Methods." No change in cAMP was observed in the absence of PGE_1, and the basal level of cAMP was less than 1.0 pmol/10^5 cells in the unstimulated CMK cells. This figure represents the mean of duplicates for independent experiments and data points were within 10% of the mean.

Table 2 *Phenotypic differentiation of CMK cells in the absence and presence of TPA and PGE_1*

CMK cells were treated with TPA for 48 h. The cell surface antigenic changes toward more mature megakaryocytes were analyzed with monoclonal antibodies (224B, 227A, Thr-4, and PLT1). Other details are described under "Materials and Methods." Results are expressed as means ± SD of three independent experiments.

	% of positive cells			
Inducers	224B (GPIIb/IIIa)	227A (GPIb)	Thr-4 (GPIIIa)	PLT1
Control	76.1 ± 1.3	19.9 ± 5.2	74.2 ± 3.8	10.8 ± 2.4
TPA (10 nM)	91.0 ± 7.5	60.5 ± 4.1	90.2 ± 8.3	64.8 ± 2.7
PGE_1 (1 μM)	78.2 ± 1.3	17.7 ± 6.1	72.3 ± 5.0	9.7 ± 1.1
PGE_1 + TPA	97.1 ± 1.1	64.3 ± 4.1	97.0 ± 1.3	64.6 ± 4.7

As shown in Table 2, treatment of CMK cells with 10 nM TPA enhanced the reactivity with antiplatelet antibodies (anti-GPIIb/IIIa, -GPIb, and -GPIIIa and PLT1). In contrast, 1 μM PGE_1 had no inductional effect on the maturation of CMK cells. PGE_1 plus TPA also enhanced the expression of megakaryocytic markers, which was similar to augmentation with TPA alone.

DISCUSSION

We have previously observed the thrombin-induced thromboxane A_2 synthesis and calcium mobilization in CMK (26), a megakaryocytic leukemia cell line, as well as the release of platelet-like particles from these cells (15). These results indicate that CMK cells have the same cellular transduction systems for thrombin as do platelets. Our present study, however, has shown for the first time that PGE_1 has a stimulatory effect on calcium mobilization in megakaryocytic cells, although PGE_1 inhibits the agonist-induced elevation of $[Ca^{2+}]_i$ in platelets (7, 8). Prostaglandin E_2 and prostaglandin I_2 similarly cause the elevation of $[Ca^{2+}]_i$ in CMK cells, while these prostaglandins are known to inhibit platelet activation (7–9) (data not shown).

PGE_1 is known to possess many biological actions through cAMP accumulation (27). Since PGE_1 increases the cAMP level in CMK cells, as in platelets, the elevation of $[Ca^{2+}]_i$ may be postulated to be related to the cAMP accumulation. However, the increase in $[Ca^{2+}]_i$ is not mimicked by the addition of dibutyryl cAMP (1–5 μM) and forskolin (10–100 μM). More-

over, TPA alone fails to stimulate the cAMP formation in CMK cells, but TPA causes about a 2-fold increase in the PGE_1-induced cAMP synthesis. Nevertheless, TPA has an inhibitory effect on the PGE_1-induced elevation of $[Ca^{2+}]_i$. These results suggest that cAMP is not involved in the PGE_1-induced Ca^{2+} mobilization in CMK cells. Similar findings have been shown in Swiss 3T3 fibroblasts (28). In contrast to CMK cells, cAMP plays an inhibitory role in controlling Ca^{2+} mobilization in platelets (9, 10). Thus, it seems likely that the transmembrane signaling mechanism for PGE_1 might be different in cells with platelet-megakaryocyte lineage. Since CMK cells are induced into more differentiated cells by TPA, CMK cells and platelets may be good models for studying the possible mechanisms of PGE_1 and cAMP on the Ca^{2+} mobilization in cells with platelet-megakaryocyte lineage.

Our study has also revealed that TPA produces about a 30% inhibition of the PGE_1- and thrombin-induced Ca^{2+} signal. Several works have demonstrated that the inhibitory effects of phorbol esters on the agonist-induced cellular processes are mediated through protein kinase C activation (16–21). We have examined the role of protein kinase C in the inhibition by TPA of the PGE_1-induced elevation of $[Ca^{2+}]_i$. In our study, the TPA-induced inhibition is mimicked by OAG (29) and SC9 (30, 31), known to be other protein kinase C activators. Moreover, H7 and H8, known to be protein kinase C inhibitors (32), restore the inhibition by TPA of the PGE_1-induced elevation of $[Ca^{2+}]_i$. These results indicate that protein kinase C is related to the regulation of the PGE_1-induced Ca^{2+} mobilization and support the notion that protein kinase C plays an important role in negative feedback regulation in various cells (33).

PGE_1 is known to be one of the growth factors present in some types of cells. The elevation of $[Ca^{2+}]_i$ and the accumulation of cAMP induced by PGE_1 have been reported to be independently involved in the expression of c-*myc* and c-*fos* genes in mouse 3T3 fibroblasts (11). We have studied the effects of PGE_1 on the phenotypic differentiation of CMK cells. However, PGE_1 shows no inductional effect on CMK cells. The biological role of PGE_1 remains to be determined in this cell line.

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