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POTENTIATION OF PROSTAGLANDIN E_1-STIMULATED cAMP FORMATION BY 12-*O*-TETRADECANOYLPHORBOL-13-ACETATE IN BALB/C MOUSE 3T3 CELLS

HIROYA UZUMAKI, SATOSHI YAMAMOTO,* HIROMI GOTO and RYUICHI KATO
Department of Pharmacology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan

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Abstract—Prostaglandin E_1 (PGE_1: 0.1–100 μM), forskolin (0.1–100 μM), and cholera toxin (20 ng/ml) stimulated cAMP formation of BALB/c 3T3 cells. The pretreatment of the cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) enhanced PGE_1 (10 μM)-stimulated cAMP formation in a concentration- and a time-dependent manner. If the cells were pretreated with TPA (0.1 μM) for only 1 hr, the above augmentation was not observed. Maximal enhancement was observed by pretreatment of the cells for 5 hr with 0.1 μM TPA. Basal cAMP formation was not affected by TPA pretreatment. Other tumor promoters, such as teleocidin and mezerein, showed a potentiating effect similar to that of TPA on the PGE_1-stimulated cAMP formation. However, phorbol which is not a tumor promoter, failed to potentiate PGE_1 action significantly. These results suggest that the above TPA action may share some common mechanisms with the tumor-promoting action of this agent. On the other hand, the forskolin- and cholera toxin-stimulated cAMP formations were not changed by pretreatment of the cells with TPA. Therefore, our results indicate that the potentiating action of TPA on PGE_1-stimulated cAMP formation in 3T3 cells is not due to the activation of the catalytic unit or the stimulatory guanine nucleotide binding protein (N_s) of adenylate cyclase (AC) system by this agent. It is highly likely that TPA induces some alterations on PGE_1 receptors or on PGE_1 receptor-N_s coupling systems and consequently induces an augmentation of PGE_1-stimulated cellular cAMP response.

It is well known that 12-*O*-tetradecanoylphorbol-13-acetate (TPA)† is a potent promoter of chemical carcinogenesis in mouse skin [1] and shows many biological effects in various types of cells [1–3] including mouse 3T3 cells [4]. DNA synthesis of BALB/c 3T3 cells is stimulated by TPA 28 hr after the addition of this agent to the cells, and as a result TPA stimulates the 3T3 cell division [5].

Cyclic adenosine-3′-5′-monophosphate (cAMP) is reported to be one of the endogeneous regulators of cell growth and proliferation [6]. Recently, Rozengurt *et al.* [4, 7] reported the promoting effect of cAMP on mouse 3T3 cell growth. Although TPA is known to stimulate DNA synthesis [5], the functional relationship between TPA and cAMP formation in 3T3 cells by certain stimuli is not clear at present.

In certain types of cells, it has been demonstrated that the pretreatment of cells with TPA causes the decrease in cellular cAMP response to β adrenergic agonists [8–12]. This might be due to the uncoupling between the β receptor and the adenylate cyclase (AC) system [10, 11]. On the contrary, our preliminary experiments showed that the pretreatment of 3T3 cells with TPA caused a marked enhancement of cellular cAMP response to prostaglandin E_1 (PGE_1). Such an augmentation of cellular cAMP response to PGE_1 and β-adrenergic agonist by pretreating clones of mouse myeloid leukemic cells with TPA has previously been reported [13]. However, the authors [13] did not show which component(s) of the AC system is affected by the TPA pretreatment. In order to investigate the mechanism by which TPA exerts its effect, we examined the effects of TPA on PGE_1-, forskolin- and cholera toxin-stimulated cAMP formation in 3T3 cells.

MATERIALS AND METHODS

BALB/c mouse 3T3 cells were cultured in a minimum essential medium (Gibco, Grand Island, NY) supplemented with 2.2 g/l $NaHCO_3$ and 10% fetal calf serum (Gibco, Grand Island, NY) in 100 mm plastic tissue culture plates (Nunc, Roskilde, Denmark) at 37° in a CO_2 incubator with a humidified atmosphere of 95% air–5% CO_2. 3T3 cells were subcultured every 3 days. Before experimental use, subcultures in 16 mm plastic tissue culture plates (Nunc, Roskilde, Denmark) were initiated with 5×10^4 cells in 0.5 ml culture medium. The cells were grown to confluence within 4–5 days. One or two days after reaching confluence, the cells were used for experiments. The number of cells used was about 2×10^5/well. The cells were preincubated with or without the indicated concentrations of TPA, teleocidin, mezerein, or phorbol at 37° for the indicated time periods in the CO_2 incubator. At the end of this incubation, the culture medium was aspirated and the cells were washed twice with Krebs–Ringer bicarbonate solution containing 120 mM NaCl, 4.8 mM KCl, 25.5 mM $NaHCO_3$, 1.2 mM KH_2PO_4,

* To whom all requests should be addressed.

† Abbreviations used: PGE_1, prostaglandin E_1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; IBMX, 1-methyl-3-isobutylxanthine; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; N_s, stimulatory nucleotide binding protein; AC, adenylate cyclase; cAMP, cyclic adenosine-3′,5′-monophosphate.

1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1 mg/ml glucose, 1 mg/ml bovine serum albumin, buffered with 10 mM Hepes pH 7.4. The cells were then incubated in 500 μl of the above Krebs–Ringer bicarbonate solution at 37° with the indicated concentrations of various drugs in the presence of 0.5 mM 1-methyl-3-isobutylxanthine (IBMX). After incubation, 500 μl of 10% trichloroacetic acid was added to the incubation medium and the cells were sonicated for 1 min on the ice. The sonicated mixture was transferred into the test tubes and centrifuged at 800 g, for 10 min. The supernatant was washed twice with water-saturated diethylether. One hundred microliters of aqueous portion were used for measuring cAMP. cAMP was assayed by a commercially available radioimmunoassay kit. ^{3}H-Thymidine incorporation into confluent 3T3 cells was determined as previously described [14].

The ^{3}H-thymidine and radioimmunoassay kit for cAMP were purchased from New England Nuclear (Boston, MA) and Yamasa Shoyu Co., Ltd. (Choshi, Japan), respectively. BALB/c 3T3 cells were kindly supplied by Dr. M. Umeda, Kihara Institute for Biological Research, Yokohama City University, Yokohama, Japan. TPA, cholera toxin and IBMX were purchased from Sigma Chemical Co. (St. Louis, MO). Mezerein was obtained from Chemicals for Cancer Res. Inc. (Prairie, MN). Phorbol was purchased from LC Service Co. (Woburn, MA). Forskolin was a gift from Nippon Kayaku Co., Ltd. (Tokyo, Japan). PGE_1 was kindly supplied by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Teleocidin was a generous gift from Dr. H. Fujiki, National Cancer Research Institute, Tokyo, Japan.

TPA, teleocidin, mezerein, phorbol, PGE_1 and forskolin were dissolved in dimethyl sulfoxide (DMSO). DMSO (final concentration 0.3%) itself affected neither the basal, PGE_1-, nor forskolin-stimulated cAMP formation.

Statistical significance was calculated using Student's t-test, when the two groups were being compared.

RESULTS

Figure 1 shows the time course of PGE_1- and forskolin-stimulated cAMP formation in 3T3 cells. PGE_1 caused a time-dependent and almost linear increase in cAMP formation up to 5 min after the addition of 10 μM PGE_1; then the cAMP formation leveled off and even decreased 15 min after the PGE_1 addition. Forskolin also stimulated cAMP formation markedly. The forskolin-stimulated cAMP formation increased linearly up to 10 min, then leveled off. cAMP level became nearly maximal at 10 min for both PGE_1 and forskolin. Therefore the incubation time was set for 10 min in the following studies. The concentration–response curves for PGE_1 and forskolin on cAMP formation were shown in Fig. 2. Significant increase in cAMP formation was observed at 0.1 μM with either PGE_1 or forskolin. The maximal effects were observed at 10 μM with PGE_1 and 30 to 100 μM with forskolin. Thus, the concentrations of PGE_1 and forskolin were selected at 10 μM and 30 μM, respectively.

In order to examine the effects of TPA on the

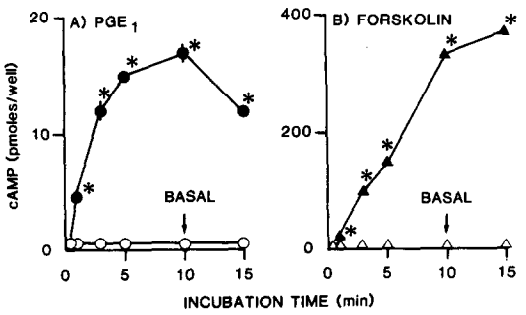

Fig. 1. Time course of PGE_1(A)- and forskolin(B)-stimulated cAMP formation in 3T3 cells. The number of cells per well was approximately 2×10^5. 3T3 cells were incubated with 10 μM PGE_1 or 30 μM forskolin in a Krebs–Ringer bicarbonate solution at 37° for the indicated time periods. Each value represents the mean ± S.E. of 3 independent experiments in triplicate determinations. *$P < 0.01$ when compared with the corresponding basal value at the indicated time.

cAMP-generating system of 3T3 cells, the cells were pretreated with TPA (0.1 μM) for various time periods. Then the cellular cAMP responses to PGE_1 and forskolin were determined (Fig. 3). Basal cAMP level was not affected by TPA pretreatment. Although PGE_1-stimulated cAMP formation was not changed by treating the cells with TPA (0.1 μM) for 1 hr, it was markedly augmented by treating the cells with TPA for 3 hr or more. The maximal potentiating effect of TPA was observed by treating the cells with TPA for 5–10 hr. TPA, however, failed to change the cellular cAMP response to forskolin (Fig. 3).

Figure 4 shows the effect of the changes in TPA concentrations on the cellular cAMP response to PGE_1 and forskolin. Cells were treated with or without various concentrations of TPA for 5 hr; cellular cAMP responses to PGE_1 and forskolin were then determined. Significant augmentation of cellular cAMP response to PGE_1 was observed with 1 nM TPA. The effect of TPA was concentration-dependent and the maximal effect was observed with 0.1 μM TPA. PGE_1-stimulated cAMP formation was rather decreased by pretreating the cells with higher

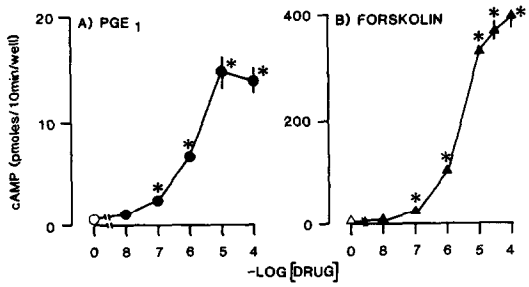

Fig. 2. Concentration-dependent effects of PGE_1(A) and forskolin(B) on cAMP formation in 3T3 cells. 3T3 cells were incubated at 37° for 10 min in the presence or absence of the various concentrations of the above agents. Each value represents the mean ± S.E. of 3 independent experiments in triplicate determinations. *$P < 0.01$ when compared with the basal value.

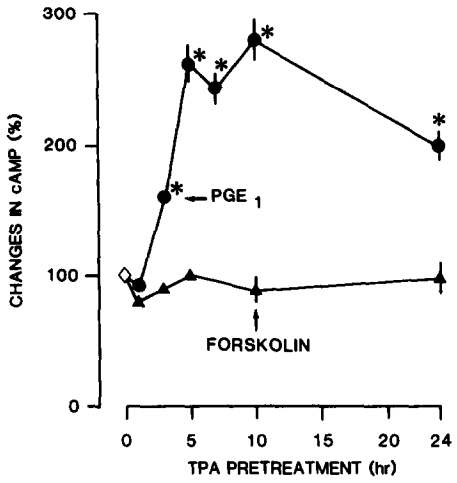

Fig. 3. Effects of TPA pretreatment on cAMP formation stimulated by PGE_1(●) and forskolin(▲). 3T3 cells were preincubated with TPA (0.1 μM) or vehicle for the indicated time periods, then the cells were washed and incubated again for 10 min in the presence of 10 μM PGE_1 or 30 μM forskolin to measure cAMP formation. Values are expressed as percentile change in cAMP formation. The cAMP formation stimulated by 10 μM PGE_1 or 30 μM forskolin with vehicle treatment at indicated time periods was set at 100%. Each value represents the mean ± S.E. of 3 independent experiments in triplicate determinations. *P < 0.01 when compared with the PGE_1-stimulated cAMP response with vehicle treatment at the indicated times.

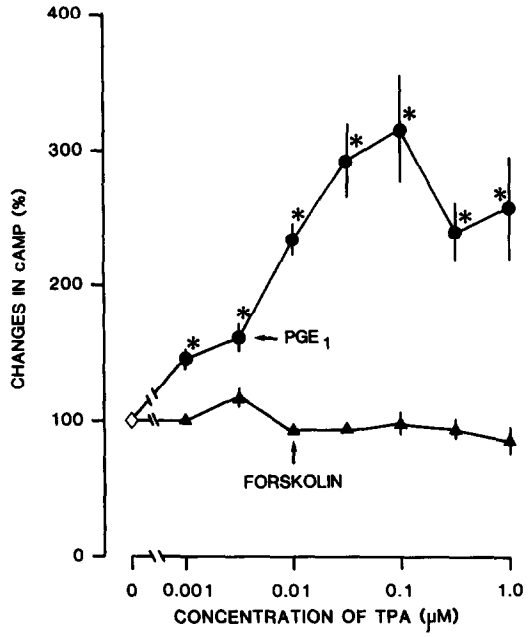

Fig. 4. Concentration-dependent effects of TPA-pretreatment on cAMP formation stimulated by PGE_1(●) and forskolin(▲). 3T3 cells were pretreated with the indicated concentrations of TPA or vehicle for 5 hr at 37°. The cells were then washed and incubated again for 10 min in the presence of 10 μM PGE_1 or 30 μM forskolin. cAMP formation stimulated by 10 μM PGE_1 or 30 μM forskolin with vehicle treatment was set at 100%. Each value represents the mean ± S.E. of 3 independent experiments in triplicate determinations. *P < 0.01 when compared with the value with vehicle treatment.

concentrations of TPA (Fig. 4). On the contrary, forskolin-stimulated cAMP formation was not affected by TPA pretreatment at any concentrations which we tested.

The effect of TPA pretreatment on cholera toxin-caused cAMP formation is shown in Fig. 5. The cells were treated with or without 0.1 μM TPA for 5 hr, and then cellular cAMP response to cholera toxin was determined. Cholera toxin started to stimulate cAMP formation after a lag time of 30 min. As clearly shown in Fig. 5, TPA failed to affect the cellular cAMP response to cholera toxin.

The effects of other tumor promoters and analogue on cellular cAMP response to PGE_1 were also examined. Pretreatment of cells for 5 hr either with 0.1 μM teleocidin, an indole alkaloid tumor promoter [15], or 0.1 μM mezerein, a second stage tumor promoter [16], also caused marked increases in cellular cAMP response to PGE_1. Cellular cAMP responses to PGE_1 were 128% for teleocidin and 84% for mezerein, when the TPA (0.1 μM)-caused potentiation of cellular cAMP response to PGE_1 was set at 100%. A non-tumor promoter, phorbol (0.1 μM), failed to potentiate the cellular cAMP response to PGE_1.

The effect of TPA on ^{3}H-thymidine incorporation into the confluent 3T3 cells was also examined. Basal ^{3}H-thymidine incorporation into the cells was 5301 ± 121 cpm/plate (N = 3). TPA did not affect the ^{3}H-thymidine incorporation at least up to 10 hr incubation (data not shown). The result is consistent with the previous data [5].

DISCUSSION

Our present results demonstrate the potentiating effect of tumor promoter TPA on the cellular cAMP response to PGE_1 without affecting the cAMP formation stimulated by forskolin or cholera toxin in BALB/c 3T3 cells. The potentiating effect of TPA was observed by pretreating the cells with TPA for 3–24 hr. Maximal effect was observed by treating the cells for 5 hr with this agent. The potentiating effect

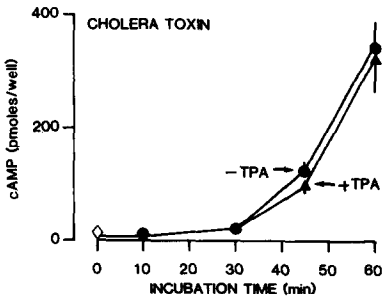

Fig. 5. Effect of TPA pretreatment on cAMP formation stimulated by cholera toxin. 3T3 cells were pretreated with 0.1 μM TPA or vehicle for 5 hr at 37°. The cells were then washed and incubated with 20 ng/ml cholera toxin for the indicated time periods. Each value represents the mean ± S.E. of 3 independent experiments in triplicate determinations.

of TPA on cellular cAMP response to PGE_1 has also been observed in clones of mouse leukemia cells [13]. The enhanced response of the leukemia cells to PGE_1 is, however, observed by treating the cells with TPA for 60 min. Therefore, it seems possible that TPA-induced enhancement of cAMP responses observed in leukemia cells and 3T3 cells is exerted through two different mechanisms.

The effect of TPA was not due to the stimulation of 3T3 cell division, since the ^{3}H-thymidine incorporation into the cells was not increased, at least up to 10 hr after the addition of TPA.

Other promoters, such as teleocidin and mezerein, also showed a potentiating action similar to that of TPA, but phorbol, which has no tumor-promoting activity, failed to potentiate the PGE_1-stimulated cAMP formation. Therefore, it seems likely that this potentiating action of TPA shares some mechanisms in common with the tumor-promoting action of this agent.

It has been reported that TPA decreased the β-adrenergic responsiveness of certain types of cells [8–12] by inducing the uncoupling between β receptor and AC systems [10, 11]. It has been also reported that glucagon-stimulated adenylate cyclase activity is markedly reduced by TPA in rat hepatocyte [17]. The authors suggest that C-kinase activation is involved in the above TPA action [17]. It seems possible that C-kinase activation is also involved in the TPA-induced enhancement of cellular cAMP response to PGE_1 in 3T3 cells.

It is unknown at present whether this potentiating effect of TPA on PGE_1-stimulated cAMP formation is specific for PGE_1-stimulated AC system in BALB/c 3T3 cells. In order to investigate the above possibility, we tried to examine the β-adrenergic responsiveness of 3T3 cells. However, in our cell system, cAMP formation was not stimulated by the β adrenergic agonist (data not shown). We also examined the cellular cAMP response to dopamine, histamine, adenosine and serotonin, but none of the above agents stimulated cAMP formation (data not shown). Therefore we could not investigate whether or not the potentiating effect of TPA is specific for the PGE_1-stimulated adenylate cyclase system.

The effect of TPA on PGE_1-stimulated cAMP formation was observed only after 1 hr of lag time, and the maximal effect was observed by pretreating the cells with TPA for 5 hr. Therefore, it is highly unlikely that TPA directly interacts with PGE_1-stimulated AC systems. In fact, TPA failed to stimulate cAMP formation by itself or to potentiate the PGE_1-stimulated cAMP formation when TPA and PGE_1 were incubated together for 10 min (data not shown). Therefore, it is evident that TPA does not interact with the AC system directly, but alters cellular cAMP response to PGE_1 by modulating some cellular functions.

Cholera toxin stimulated cAMP formation in 3T3 cells after a lag time of 30 min. The result confirmed the previous data [18]. It has been reported that cholera toxin stimulates cAMP formation via the activation of the stimulatory guanine nucleotide binding protein (N_s) of AC systems [19]. On the contrary, forskolin, a diterpen compound, directly activates the catalytic unit of AC [20]. Guanine nucleotide binding protein may or may not be involved in the forskolin action [21–25]. The stimulatory actions of both cholera toxin and forskolin on cAMP formation were not influenced by pretreating the cells with TPA. Therefore, it is highly unlikely that TPA acts to induce a change in the N_s–AC complex or catalytic unit of AC, but rather induces an alteration of PGE_1 receptor function or PGE_1 receptor–N_s coupling. At present, it is also possible that TPA Modifies the function of inhibitory nucleotide binding protein (N_i) and consequently enhances cellular cAMP response to PGE_1.

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