Vol. 157, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1988 Pages 337-345

INDEPENDENT INHIBITION OF DNA SYNTHESIS BY PROTEIN KINASE C, CYCLIC AMP AND INTERFERON α/β IN RABBIT AORTIC SMOOTH MUSCLE CELLS[1]

Yasuo Fukumoto, Yasuhiro Kawahara*, Ken-ichi Kariya*, Shun-ichi Araki*, Hisashi Fukuzaki*, and Yoshimi Takai[2]

Departments of Biochemistry and *Internal Medicine (1st Division), Kobe University School of Medicine, Kobe 650, Japan

Received October 10, 1988

SUMMARY: In quiescent cultures of rabbit aortic smooth muscle cells, whole blood serum-induced DNA synthesis was inhibited markedly by protein kinase C-activating 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and phorbol-12,13-dibutyrate (PDBu), cyclic AMP-derivatives, such as dibutyryl cyclic AMP (Bt_2cAMP) and 8-bromo-cyclic AMP, and interferon α/β. Neither TPA nor interferon α/β elevated the cellular cyclic AMP level. Neither Bt_2cAMP nor interferon α/β induced the phospholipase C-mediated hydrolysis of phosphoinositides. The down-regulation of protein kinase C by prolonged treatment with PDBu abolished the antiproliferative action of TPA but did not affect that of Bt_2cAMP or interferon α/β. TPA and Bt_2cAMP inhibited the serum-induced DNA synthesis when added within 12 h after the addition of the serum, while interferon α/β was active only when added within 6 h. These results suggest that there are at least three independent signaling systems, protein kinase C- and cyclic AMP-mediated systems and an unidentified system for interferon α/β, which are involved in the antiproliferative mechanisms in rabbit aortic smooth muscle cells.

Many growth factors are known to induce DNA synthesis in vascular SMC : these include platelet-derived growth factor (1), fibroblast growth factor (2), epidermal growth factor (2) and

1. This investigation was supported by Grants-in-Aid for Scientific Research and Cancer Research from the Ministry of Education, Science and Culture, Japan (1987,1988), Grants-in-Aid for Abnormalities in Hormone Receptor Mechanisms (1987,1988), Cardiovascular Diseases (1987,1988) and for Cancer Research (1988) from the Ministry of Health and Welfare, Japan, and by grants from the Yamanouchi Foundation for Research on Metabolic Disease (1987) and the Research Program on Cell Calcium Signal in the Cardiovascular System (1987,1988).

2. To whom all correspondence should be addressed.

The abbreviations used are: SMC, smooth muscle cells; TPA, 12-O-tetradecanoylphorbol-13-acetate; PDBu, phorbol-12,13-dibutyrate; 4αPDD, 4α-phorbol-12,13-didecanoate; Bt_2cAMP, dibutyryl cyclic AMP; PGE_1, prostaglandin E_1; WBS, whole blood serum; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; IP_1, inositol 1-monophosphate; IP_2, inositol 1,4-bis-phosphate; IP_3, inositol 1,4,5-trisphosphate; IBMX, 3-isobutyl-1-methylxanthine.

0006-291X/88 $1.50

endothelial cell-derived growth factor (3). In contrast to these growth factors, many agents have been identified to serve as antiproliferative factors in this cell type: these include prostaglandins E_1, E_2 and D_2 (4-6), adenosine (7), type β transforming growth factor (8), interferon α (9) and heparin (10,11). Among these antiproliferative factors, prostaglandins and adenosine have been suggested to exert their antiproliferative action through cyclic AMP (5-7), but the modes of action of other factors have not yet been clarified.

We have shown in preceding reports that, in addition to these antiproliferative factors, TPA also serves as an antiproliferative factor in rabbit aortic SMC (12-14). The antiproliferative action of TPA is mimicked by another protein kinase C-activating PDBu but not by 4αPDD known to be inactive for protein kinase C (12-14). The down-regulation of protein kinase C induced by prolonged treatment of the cells with PDBu abolishes the antiproliferative action of TPA (12-14). On the basis of these observations, we have proposed that the antiproliferative action of TPA is mediated through the activation of protein kinase C. The studies on the mode of antiproliferative action of protein kinase C have revealed that this enzyme inhibits the progression from the G_1 into S phase of the cell cycle and thereby suppresses DNA synthesis in this cell type (14). The similar antiproliferative action of TPA has recently been confirmed in rat vascular SMC (15).

The relationships among the inhibitory actions of cyclic AMP, TPA and other antiproliferative factors, however, have not been clarified. In the present study, we have compared the modes of antiproliferative action of cyclic AMP, TPA and interferon α/β. We describe here that cyclic AMP, TPA and interferon α/β independently inhibit the progression from the G_1 into S phase of the cell cycle in rabbit aortic SMC.

EXPERIMENTAL PROCEDURES

Materials and Chemicals——Japanese White rabbits were purchased from Shizuoka Animal Center. Rabbit interferon α/β was purchased from LEE Biomolecular Research Inc.. TPA and PDBu were from CCR Inc.. 4αPDD was from Sigma. Bt_2cAMP, 8-bromo-cyclic AMP and the cyclic AMP assay kit were purchased from Yamasa Shoyu Co.. PGE_1 was from Funakoshi Pharmaceutical Co.. WBS was prepared from Japanese White rabbits as described previously (16). FCS was from GIBCO. [methyl-^{3}H]Thymidine and myo-[^{3}H]inositol were from Amersham. Other materials and chemicals were obtained from commercial sources.

Cell Culture——Primary cultures of rabbit aortic SMC were obtained from thoracic aortae of male Japanese White rabbits by the explantation method of Ross (17) as described previously (12). The cells in secondary cultures maintained in DMEM containing 10% FCS were trypsinized and seeded for each experiment. The cells were always cultured at 37°C in a humidified atmosphere of 5% CO_2 : 95% air.

Assays——DNA synthesis was assayed by autoradiography of labeled nuclei or by measuring the incorporation of [^{3}H]thymidine into acid-insoluble materials as described previously (14). The cellular cyclic AMP level was assayed by radioimmunoassay (18). Rabbit aortic SMC in secondary cultures were seeded into 35-mm dishes at a density of 2 x 10^5 cells/dish in DMEM containing 10% FCS. After 3 days, the cells were washed twice with DMEM and incubated in serum-free DMEM for 48 h. The cells were then incubated with various agents in 1 ml of balanced salt solution containing 20 mM Hepes at pH 7.4, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.5 mM $CaCl_2$ at 37°C for 30 min. After the incubation, the medium was rapidly removed and cyclic AMP was extracted with 400 µl of 0.1 N HCl. Appropriate dilutions of the extracts were succinylated, and the cyclic AMP concentrations were determined by radioimmunoassay (18). The phospholipase C-mediated hydrolysis of phosphoinositides was assayed by measuring the formation of radioactive IP_1, IP_2 and IP_3 in rabbit aortic SMC prelabeled with [^{3}H]inositol as described previously (14).

Determinations——The radioactivities of ^{3}H- and ^{125}I-labeled samples were determined with a Beckman liquid scintillation system LS3801 and Aloka gamma scintillation system ARC-251, respectively.

RESULTS

Incubation of quiescent cultures of rabbit aortic SMC with WBS induced DNA synthesis as estimated by measuring the incorporation of [^{3}H]thymidine into acid-insoluble materials. This serum-induced DNA synthesis was inhibited by TPA in a dose-dependent manner as described previously (12-14). The dose of TPA necessary for the half maximal inhibition was about 3 ng/ml. The serum-induced DNA synthesis was also inhibited by Bt_2cAMP and interferon α/β in a dose-dependent manner as described (6,9). The similar inhibitory action of TPA, Bt_2cAMP and interferon α/β in the serum-induced DNA synthesis was confirmed when DNA synthesis was assayed by autoradiography (data not shown).

PDBu, known to be another protein kinase C-activating phorbol ester (19), also inhibited the serum-induced DNA synthesis as described (12-14). 4αPDD, known to be inactive for protein kinase C (19), was ineffective in this capacity. These results suggest that the antiproliferative action of phorbol esters is mediated through the activation of protein kinase C as described previously (12-14).

Table 1. Effect of cyclic AMP derivatives and PGE_1 on the WBS-induced DNA synthesis

Additions	DNA synthesis (cpm x 10^{-4})
None	0.1 ± 0.1
WBS (10%)	10.4 ± 0.4
WBS + Bt_2cAMP (5 mM)	2.0 ± 0.3
WBS + 8-bromo-cyclic AMP (5 mM)	2.3 ± 0.3
WBS + PGE_1 (20 μM)	9.5 ± 0.2
WBS + IBMX (50 μM)	9.2 ± 0.2
WBS + PGE_1 + IBMX	4.9 ± 0.3

Quiescent cultures of rabbit aortic SMC were incubated with WBS in the presence of indicated agents. After the incubation for 24 h, DNA synthesis was assayed by measuring the incorporation of [^{3}H]thymidine into acid-insoluble materials. Other details are described under EXPERIMENTAL PROCEDURES. Results are the means ± SE of three independent experiments.

8-Bromo-cyclic AMP as well as Bt_2cAMP inhibited the serum-induced DNA synthesis as shown in Table 1. PGE_1, known to elevate the cellular cyclic AMP level (20), showed the similar inhibitory effect in the presence of IBMX, an inhibitor for phosphodiesterase (21). Either PGE_1 or IBMX alone showed little effect. PGE_1 elevated markedly the cellular cyclic AMP level in the presence of IBMX, but elevated it to a small extent in the absence of IBMX (see Table 2).

It has been shown that TPA increases agonist-stimulated as well as basal, guanine nucleotide- and fluoride ion-stimulated adenylate cyclase activities in frog erythrocytes (22). In the next set of experiments, we examined whether TPA and interferon α/β inhibit the serum-induced DNA synthesis through the formation of cyclic AMP. As shown in Table 2, neither TPA nor interferon α/β elevated this cyclic AMP level even in the presence of IBMX. Either PGE_1 or IBMX alone elevated slightly the cellular cyclic AMP level, but the simultaneous addition of both agents elevated it markedly.

In order to know whether Bt_2cAMP and interferon α/β inhibit the serum-induced DNA synthesis through the activation of protein kinase C which could be induced by the phospholipase C-mediated hydrolysis of phosphoinositides (19), the effects of Bt_2cAMP and interferon α/β on the formation of IP_1, IP_2 and IP_3 were examined.

Table 2. Effect of PGE_1, TPA and interferon α/β on the cellular cyclic AMP level

Additions	cyclic AMP (pmol/10^6 cells)
None	4.0 ± 0.5
PGE_1 (20 μM)	9.5 ± 1.1
TPA (100 ng/ml)	3.8 ± 0.8
Interferon α/β (500 U/ml)	4.0 ± 0.7
IBMX (100 μM)	6.2 ± 0.7
IBMX + PGE_1	28.0 ± 2.1
IBMX + TPA	6.4 ± 0.8
IBMX + Interferon α/β	6.7 ± 1.0

Quiescent cultures of rabbit aortic SMC were incubated with PGE_1, TPA or interferon α/β in the presence or absence of IBMX. After the incubation for 30 min, cyclic AMP was extracted and assayed as described under EXPERIMENTAL PROCEDURES. Results are the means ± SE of three independent experiments.

As shown in Fig. 1, WBS induced the formation of these inositol phosphates. Under the same conditions, neither Bt_2cAMP nor interferon α/β induced the formation of IP_1, IP_2 and IP_3.

We have previously shown that prolonged treatment of SMC with PDBu causes the down-regulation of protein kinase C (12-14,23). This down-regulation of protein kinase C abolished the antiproliferative action of TPA as described previously (12-14) but did not affect the antiproliferative action of Bt_2cAMP or interferon α/β as shown in Fig. 2.

In the last set of experiments, the effect of time of the addition of TPA, Bt_2cAMP and interferon α/β on the inhibition of the serum-induced DNA synthesis was examined. As shown in Fig. 3A, DNA synthesis started at about 18 h after the addition of the serum. TPA and Bt_2cAMP inhibited the serum-induced DNA synthesis when added within 12 h after the addition of the serum as shown in Fig. 3B. However, interferon α/β inhibited the serum-induced DNA synthesis only when it was added within 6 h after the addition of the serum.

DISCUSSION

We have confirmed here the earlier observations that TPA, cyclic AMP and interferon α/β show an antiproliferative action in

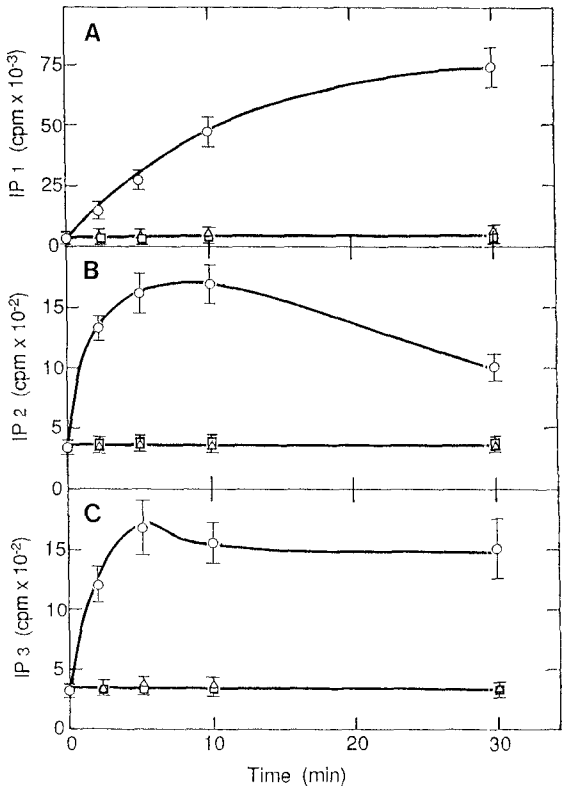

Fig. 1. Failure of the formation of inositol phosphates by Bt_2cAMP and interferon α/β. Quiescent cultures of rabbit aortic SMC prelabeled with [^{3}H]inositol were incubated with 10% WBS, 5 mM Bt_2cAMP or 500 U/ml of interferon α/β for various periods of time. Other details are described under EXPERIMENTAL PROCEDURES. (A), IP_1; (B), IP_2; (C), IP_3. (○), with WBS; (△), with Bt_2cAMP; (□), with interferon α/β. Results are the means ± SE of three independent experiments.

vascular SMC (5,6,9,12-15). Extending these observations, we have examined here the relationships among the modes of action of these antiproliferative agents in this cell type. Since TPA does not elevate the cellular cyclic AMP level, the antiproliferative action of TPA is not mediated through cyclic AMP. Inversely, since Bt_2cAMP does not induce the phospholipase C-mediated hydrolysis of phosphoinositides, and the down-regulation of protein kinase C does not affect the antiproliferative action of Bt_2cAMP under the condition where it completely abolishes the antiproliferative action of TPA, the antiproliferative action of Bt_2cAMP is not mediated through protein kinase C. Moreover, since interferon α/β does not elevate the cellular cyclic AMP level nor induces the phospholipase C-mediated hydrolysis of phospho-inositides and the down-regulation of protein kinase C does not affect the antiproliferative action of interferon α/β, the

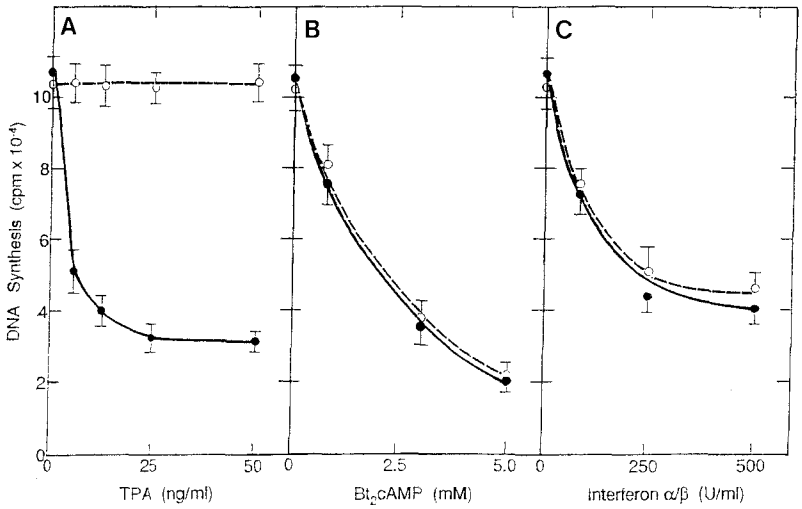

Fig. 2. Effect of the down-regulation of protein kinase C on the inhibition of the WBS-induced DNA synthesis by TPA, Bt_2cAMP and interferon α/β. Quiescent cultures of the control rabbit aortic SMC or the cells pretreated with 100 ng/ml of PDBu for 48 h were incubated with 10% WBS in the presence of various doses of TPA, Bt_2cAMP or interferon α/β. After the incubation for 24 h, DNA synthesis was assayed by measuring the incorporation of [^{3}H]thymidine into acid-insoluble materials. Other details are described under EXPERIMENTAL PROCEDURES. (A), in the presence of TPA; (B), in the presence of Bt_2cAMP; (C), in the presence of interferon α/β. (●), control cells; (○), the cells treated with PDBu. Results are the means ± SE of three independent experiments.

antiproliferative action of interferon α/β is not mediated through cyclic AMP or protein kinase C. The intracellular messenger system of interferon α/β remains to be clarified.

The chronological analysis of the antiproliferative action of TPA, Bt_2cAMP and interferon α/β has revealed that these three factors suppress the serum-induced DNA synthesis even when they are added long after the completion of the transition from the G_0 to G_1 phase, suggesting that they inhibit the progression from the G_1 into S phase. Since time courses of the antiproliferative actions of TPA and Bt_2cAMP are similar, it is possible that these two agents exert their actions through a common pathway distal to the activation of each specific protein kinase, for example, the phosphorylation of common substrate(s) for protein kinase C and cyclic AMP-dependent protein kinase. Interferon α/β does not show a potent inhibitory effect on DNA synthesis when it is added more than 6 h after the addition of the serum, while TPA and Bt_2cAMP still show a potent antiproliferative action even when they are added at 12 h after the addition of the serum. These results suggest that the mechanism of antiproliferative action of interferon α/β is different from those of TPA and Bt_2cAMP.

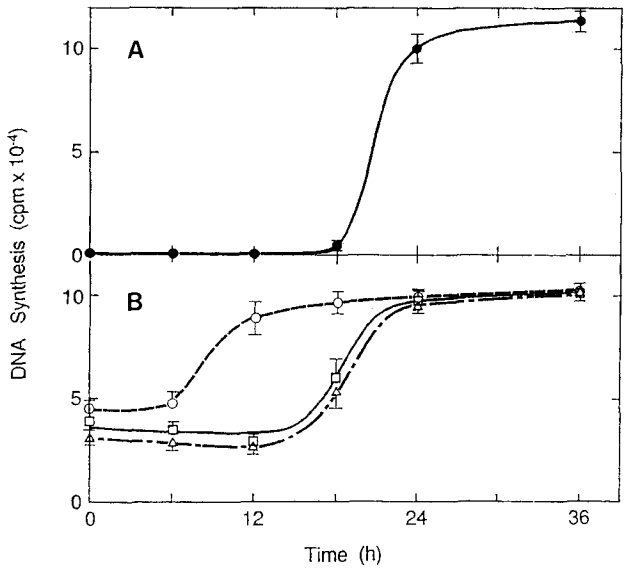

Fig. 3. Chronological analysis of the effect of TPA, Bt_2cAMP and interferon α/β on the WBS-induced DNA synthesis. (A), Time course of the WBS-induced DNA synthesis. Quiescent cultures of rabbit aortic SMC were incubated with 10% WBS. After the incubation for various periods of time, DNA synthesis was assayed by measuring the incorporation of [^{3}H]thymidine into acid-insoluble materials. Other details are described under EXPERIMENTAL PROCEDURES. (B), Time-dependent effect of addition of TPA, Bt_2cAMP and interferon α/β on the WBS-induced DNA synthesis. Quiescent cultures of rabbit aortic SMC were incubated with 10% WBS. DNA synthesis was assayed 40 h after the addition of WBS by measuring the incorporation of [^{3}H]thymidine into acid-insoluble materials. During this 40 h-incubation, TPA, Bt_2cAMP or interferon α/β was added at the indicated time at the final concentration of 100 ng/ml, 5 mM and 500 U/ml, respectively. Other details are described under EXPERIMENTAL PROCEDURES. (□), in the presence of TPA; (△), in the presence of Bt_2cAMP; (○), in the presence of interferon α/β. Results are the means ± SE of three independent experiments.

Although the molecular mechanisms of antiproliferative action of these three factors remain to be clarified, our present results indicate that there are at least three independent signaling systems which inhibit the progression from the G_1 into S phase of the cell cycle in rabbit aortic SMC.

ACKNOWLEDGEMENT

The authors are grateful to Junko Yamaguchi for her skillful secretarial assistance.

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