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Inhibition of DNA synthesis by phorbol esters through protein kinase C in cultured rabbit aortic smooth muscle cells

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In cultured rabbit aortic smooth muscle cells (SMC), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced DNA synthesis in the presence of plasma-derived serum to a small extent, but inhibited markedly the rabbit whole blood serum (WBS)-, platelet-derived growth factor (PDGF)- and epidermal growth factor-induced DNA synthesis. Phorbol-12,13-dibutyrate (PDBu) mimicked this antiproliferative action of TPA, but 4α-phorbol-12,13-didecanoate was inactive in this capacity. Prolonged treatment of the cells with PDBu caused the partial down-regulation of protein kinase C. In these protein kinase C-reduced cells, WBS still induced DNA synthesis, but TPA did not inhibit the WBS-induced DNA synthesis. We have previously shown that protein kinase C is involved at least partially in the PDGF-induced DNA synthesis in rabbit aortic SMC. The present results together with this earlier observation suggest that protein kinase C has not only a proliferative but also an antiproliferative action in rabbit aortic SMC.

Cell proliferation; Phorbol ester; Protein kinase C; (Vascular smooth muscle)

1. INTRODUCTION

Vascular SMCs play a key role in the development of atherosclerosis. These cells are quiescent normally but damage to the endothelium results in migration of the cells from the medial layer to the intima followed by abnormal cell proliferation (review [1]). Several growth factors have been shown to stimulate proliferation of vascular SMCs: these include PDGF [2], EGF [3], fibroblast growth factor [3] and a growth factor released from endothelial cells [4]. In contrast with these growth factors, several antiproliferative factors have also been identified for this cell type: these include prostaglandin E_1 [5-7], prostaglandin E_2 [5-7], prostaglandin D_2 [6], heparin [8], glucocorticoid [9], adenosine [10], interferon α [11] and type β transforming growth factor [12]. Although it has been suggested that the antiproliferative action of the prostaglandins and adenosine is mediated through the activation of protein kinase A [6,7,10], the modes of action of other growth factors and antiproliferative factors have not been clarified in vascular SMCs.

We have recently proposed that protein kinase C may be involved at least partially in the proliferative action of PDGF in rabbit aortic SMCs

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Abbreviations: SMCs, smooth muscle cells; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; protein kinase A, cyclic AMP-dependent protein kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PDBu, phorbol-12,13-dibutyrate; 4α-PDD, 4α-phorbol-12,13-didecanoate; Bt_2cAMP, dibutyryl cyclic AMP; FCS, fetal calf serum; PDS, plasma-derived serum; WBS, whole blood serum; DMEM, Dulbecco's modified Eagle's medium

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[13]. The present paper describes that protein kinase C has not only a proliferative but also an antiproliferative action in this cell type.

2. MATERIALS AND METHODS

2.1. *Materials*

Japanese White rabbits were purchased from Shizuoka Laboratory Animal Center. [*methyl*-^{3}H]-Thymidine was from Amersham; TPA and PDBu were from CCR Inc; 4α-PDD was from Sigma; Bt_2cAMP from Yamasa Shoyu Co; and FCS from Gibco. Cell-free PDS and WBS were prepared from Japanese White rabbits as described [14]. Human PDGF was kindly supplied by Dr T. Deuel (Washington University School of Medicine, USA). Mouse EGF was purchased from Collaborative Research. Other materials and chemicals were obtained from commercial sources.

2.2. *Cell culture*

Primary cultures of vascular SMCs were obtained from thoracic aortae of male Japanese White rabbits by the explantation method of Ross [15]. Briefly, about 10 pieces of 1 mm × 1 mm intimal-inner medial segments of aortae were placed in 25-cm^2 flasks and grown in DMEM containing 10% FCS. The first outgrowth of cells from the explant was observed after 6–10 days. After 2 weeks, the cells were trypsinized and transferred into 100-mm dishes and maintained in the same medium for 3 days. The cells in these secondary cultures were trypsinized and seeded for each experiment as described below. The cells were always cultured at 37°C in a humidified atmosphere of 5% CO_2:95% air.

2.3. *Assay for DNA synthesis*

DNA synthesis was assayed by measuring the incorporation of [*methyl*-^{3}H]thymidine into acid-insoluble materials as described [16]. The cells in secondary cultures were seeded into 24-well cluster plates at a density of 4×10^4 cells/well in DMEM containing 10% FCS. After the incubation for 3 days, the cells were washed twice with serum-free DMEM, incubated in the same medium for 48 h and washed three times with DMEM containing 0.1% bovine serum albumin. These cells were quiescent as judged by autoradiography of the labeled nuclei. The cells were then incubated in 1 ml of DMEM with various agents for 24 h in the continuous presence of 4.4 μM of [*methyl*-^{3}H]-thymidine (2 μCi/ml). The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid, and the radioactivity in the acid-insoluble materials was determined with a Packard Tri-Carb (model 3330) liquid scintillation spectrometer.

3. RESULTS

Incubation of quiescent cultures of rabbit aortic SMCs with various doses of rabbit WBS caused DNA synthesis in a dose-dependent manner (fig.1). The addition of TPA caused a marked decrease in the WBS-induced DNA synthesis. This effect of TPA was dose-dependent (fig.2). The maximal effect was observed with 25–50 ng/ml of TPA, and the IC_{50} value was about 3 ng/ml. TPA alone at any dose did not stimulate DNA synthesis in the absence of PDS. TPA stimulates this reaction slightly in the presence of PDS. PDS alone was inactive. Similar results were obtained when DNA synthesis was assayed by autoradiography of the labeled nuclei (not shown).

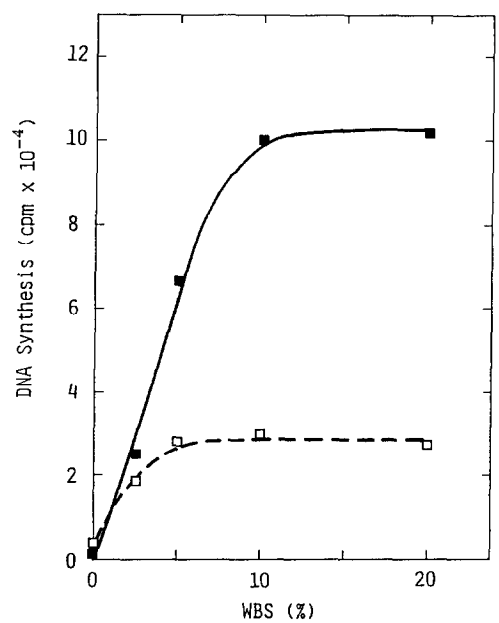

Fig.1. Inhibition by TPA of the WBS-induced DNA synthesis. Quiescent cultures of rabbit aortic SMCs were incubated with various doses of WBS in the absence (■—■) or presence (□--□) of 100 ng/ml of TPA. Each value is the mean of triplicate determinations.

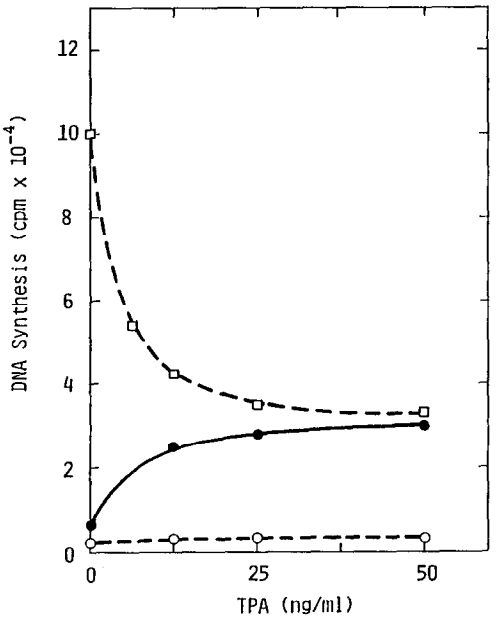

Fig.2. Dose-dependent inhibition by TPA of the WBS-induced DNA synthesis. Quiescent cultures of rabbit aortic SMCs were incubated with 10% WBS (□--□) or 10% PDS (●—●) in the presence of various doses of TPA. (○--○) TPA alone. Each value is the mean of triplicate determinations.

PDGF and EGF also induced DNA synthesis in the presence of PDS, although the maximal levels obtained by these growth factors were 45–65% of those obtained by WBS (table 1). TPA inhibited the PDGF- and EGF-induced DNA synthesis. Neither PDGF nor EGF alone in the absence of PDS induced DNA synthesis (not shown).

PDBu mimicked the inhibitory effect of TPA on the WBS-induced DNA synthesis, but 4α-PDD was ineffective in this capacity (not shown). Since it has been shown that TPA and PDBu activate protein kinase C whereas 4α-PDD is inactive for this enzyme (review [17]), these results suggest that the antiproliferative action of TPA and PDBu is mediated through the activation of protein kinase C. This suggestion was further confirmed by the following experiments using protein kinase C-reduced cells.

It has been described that preincubation of rabbit aortic SMCs with 100 ng/ml of PDBu for 48 h causes the down-regulation of protein kinase C to a level of 25–35% of that in control cells [13]. In

Table 1

Effect of TPA on the PDGF- and EGF-induced DNA synthesis

Additions	DNA synthesis (cpm × 10^{-4})	
	Without TPA	With TPA
None	0.1	0.4
WBS	10.1	3.0
PDGF + PDS	4.6	3.0
EGF + PDS	6.5	3.1

Quiescent cultures of rabbit aortic SMC were incubated with 10% WBS or with 25 ng/ml of PDGF or 10 ng/ml of EGF in the presence of 10% PDS. TPA was added as indicated at a final concentration of 100 ng/ml. Each value is the mean of triplicate determinations

cells treated in this way, WBS still induced DNA synthesis to the same extent as that in control cells (fig.3). However, in these protein kinase C-reduced cells the inhibitory effect of TPA on the

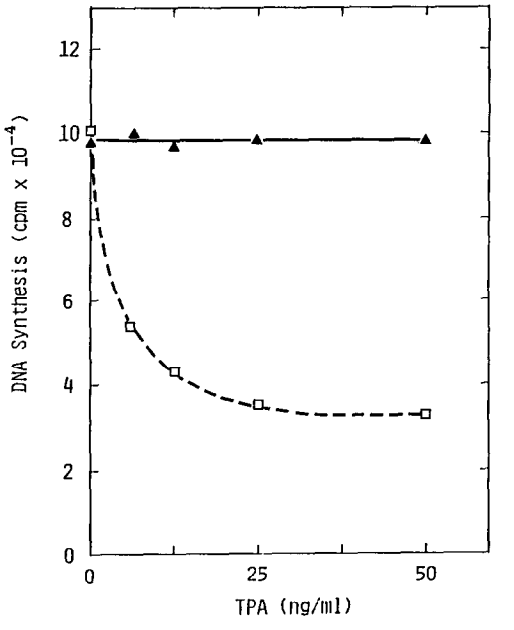

Fig.3. Effect of the down-regulation of protein kinase C on the inhibition by TPA of the WBS-induced DNA synthesis. Quiescent cultures of control rabbit aortic SMCs (□--□) or cells pretreated with 100 ng/ml of PDBu (▲—▲) for 48 h were incubated with 10% WBS in the presence of various doses of TPA. Each value is the mean of triplicate determinations.

Table 2

Effect of the down-regulation of protein kinase C on the inhibition by Bt_2cAMP of the WBS-induced DNA synthesis

Additions	DNA synthesis (cpm × 10^{-4})	
	Without PDBu treatment	With PDBu treatment
WBS	10.1	10.0
WBS + TPA	3.0	9.8
WBS + Bt_2cAMP	3.3	3.2

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 absence or presence of 100 ng/ml of TPA or 5 mM Bt_2cAMP. Each value is the mean of triplicate determinations

WBS-induced DNA synthesis was completely abolished.

It has been described that Bt_2cAMP inhibits the serum-induced DNA synthesis in human aortic SMCs [7]. In confirmation of this observation, Bt_2cAMP inhibited the WBS-induced DNA synthesis (table 2). This inhibition was not affected by the down-regulation of protein kinase C. These results suggest that the two intracellular messenger systems, protein kinases C and A, serve as negative regulators for cell proliferation in rabbit aortic SMCs.

4. DISCUSSION

We have previously shown that protein kinase C is involved at least partially in the PDGF-induced DNA synthesis in rabbit aortic SMCs [13]. In contrast, evidence has been presented in this paper that protein kinase C has additionally an antiproliferative action in this cell type, indicating that this enzyme has a dual action in the regulation of proliferation of SMCs. A similar dual action of protein kinase C has recently been observed in the regulation of Na^+/H^+ exchange in human epidermoid carcinoma A431 cells [18], smooth muscle contraction [19] and exocytosis in platelets [20,21] and neutrophils [22,23]. For instance, in platelets protein kinase C is activated by the action of thrombin and is involved in the exocytosis induced by this agonist [20]. Nevertheless, the preactivation of this enzyme by prior treatment of platelets with PDBu inhibits this reaction [21]. It has been suggested that protein kinase C serves as not only a positive but also a negative regulator. Similarly, protein kinase C may serve as both positive and negative regulators also in the proliferation of rabbit aortic SMCs.

The mode of antiproliferative action of protein kinase C in rabbit aortic SMCs remains to be clarified. It has been described in other cell types that protein kinase C phosphorylates the EGF receptors resulting in their desensitization to EGF [24–26]. However, since TPA inhibits not only the EGF-induced but also the PDGF-induced DNA synthesis, it is likely that protein kinase C may have another mode(s) of antiproliferative action in addition to the desensitization of the EGF receptors in rabbit aortic SMCs.

In the present experiments, protein kinase C is artificially activated by use of TPA. It is possible that some naturally occurring agonists which inhibit DNA synthesis through the activation of this enzyme may be present. It has been shown that heparin [8], interferon α [11] and type β transforming growth factor [12] inhibit DNA synthesis in vascular SMCs. Therefore, it is tempting to speculate that some of these factors may inhibit DNA synthesis through the activation of protein kinase C.

We have shown here that in the rabbit aortic SMCs whose protein kinase C is down-regulated by prior treatment with PDBu, WBS still induces DNA synthesis to the same extent as that in control cells. This result is apparently inconsistent with the observation that protein kinase C is involved in the PDGF-induced DNA synthesis in this cell type. However, it is conceivable that protein kinase C is involved in but is not essential for the WBS-induced DNA synthesis since WBS contains various growth factors. Presumably, there are other intracellular messenger systems which can substitute for protein kinase C and induce DNA synthesis in this cell type, since it has been shown in Swiss 3T3 cells that protein kinase C and Ca^{2+} are independently involved in PDGF- and fibroblast growth factor-induced DNA synthesis [27,28], that cyclic AMP and Ca^{2+} are involved in prostaglandin E_1-induced DNA synthesis [29–31], and that neither protein kinase C, Ca^{2+} nor cyclic AMP is involved in EGF-induced DNA synthesis

[32]. Further investigation is necessary to identify other intracellular messenger systems responsible for the mitogenic action of WBS than protein kinase C in rabbit aortic SMCs.

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