Phorbol Ester Inhibits the Phosphorylation of the Retinoblastoma Protein without Suppressing Cyclin D-associated Kinase in Vascular Smooth Muscle Cells*

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To elucidate the role of protein kinase C in vascular smooth muscle cell proliferation, we examined the effects of phorbol 12-myristate 13-acetate (PMA) on G1 events in human arterial cells. About 15 h after G0 cells were stimulated with fetal bovine serum and basic fibroblast growth factor, [3H]thymidine incorporation started. PMA (10 nM) inhibited the incorporation over 90% when added earlier than 3 h after stimulation, but had no effect when added 12 h or later. PMA inhibited the phosphorylation of the retinoblastoma protein (pRb), which normally began at about 9 h. PMA did not inhibit the gene expression of Cdk2, Cdk3, Cdk4, Cdk5, and cyclins G, C, and D, all of which began at 0–3 h. However, PMA reduced the expression of cyclins E and A, which usually began at 3-9 h and about 15 h, respectively. PMA inhibited the histone H1 kinase activity of Cdk2, which increased from about 9 h, whereas PMA did not inhibit the pRb kinase activities of cyclin D-associated kinase(s) and Cdk4, detectable from 0–3 h. These results suggested that the PMA-induced inhibition of pRb phosphorylation is not mediated by suppressing cyclin D-associated kinase(s) including Cdk4, but involves the suppression of Cdk2 activity that results from the reduced expression of cyclins E and A.

Hyperplasia of vascular smooth muscle cells (VSMCs) plays a central role in the formation of atherosclerotic lesions and intimal thickening after angioplasty. It is also one of the pathological changes that occur during the development of hypertension. Therefore, it is essential for understanding the etiology of these disorders to elucidate the mechanism regulating VSMC proliferation (1–3).

VSMC proliferation is promoted by several biological substances, such as growth factors, cytokines, vasoactive peptides, catecholamines, and arachidonate metabolites (1, 2). Most, if not all, of these substances activate protein kinase C (PKC), since upon stimulation of their receptors, phospholipase C is activated, which stimulates phosphoinositide turnover producing 1,2-sn-diacylglycerol (DAG), which then activates PKC (4, 5). PKC activity is sustained by DAG produced from phosphatidylcholine by subsequently activated phospholipases C and D (6). Lyso phosphatidylcholine and fatty acids produced by phospholipase A2 may also contribute to PKC activation (7). Moreover, phosphatidylinositol 3,4-biphosphate and phosphatidylinositol 3,4,5-triphosphate, the products of phosphatidylinositol 3-kinase, reportedly activate some isoforms of the PKC family (8, 9).

The role of the PKC pathway in mitogenesis remains undetermined. PKC activators such as phorbol esters and membrane permeable DAGs mimic competence factors (10, 11) and induce the expression of immediate early genes such as c-fos and c-myc (12). PKC directly phosphorylates and activates Raf-1 (13, 14), which would lead to the activation of MAP kinase. Therefore, PKC may be involved in the early phases of mitogenesis as a positive mediator. Nevertheless, phorbol ester inhibits the transition from G1 to S phase in a variety of cell species including VSMCs (15–18). Since this effect is mimicked by repeated doses of membrane-permeable DAG and is prevented by the down-regulation of α- and ε-isofoms of PKC, we suggested that PKC mediates the G1/S inhibition induced by phorbol ester (18). Moreover, PKC-mediated inhibition seems to operate as a physiological mechanism, since DNA synthesis is accelerated when PKC is down-regulated (18). However, the mechanism by which PKC inhibits the G1/S progression remains to be investigated.

In the present study, we examined the effects of phorbol ester on cellular events during the G1 and S phases, including the phosphorylation of the retinoblastoma protein (pRb) and the activation of cyclin-dependent kinases (Cdks), using VSMCs from human umbilical arteries.

EXPERIMENTAL PROCEDURES

Chemicals—Phorbol 12-myristate 13-acetate (PMA, Sigma) was dissolved in 100% Me2SO and stocked at –20°C until use. The concentration of Me2SO added simultaneously with PMA (vehicle) was 0.1%. Other chemicals were of reagent grade.

Cell Culture and Cell Cycle Synchronization—VSMCs, obtained from the media of human umbilical arteries by explant culture, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% (v/v) fetal bovine serum (HyClone) and 10 ng/ml human recombinant basic fibroblast growth factor (Amersham Corp.) (growth medium), and used at the third passage. Cultured cells were identified as VSMCs as described (19). Cell cycle synchronization in the quiescent state (G0) was achieved by incubating the cells in serum-free DMEM containing 0.1% bovine serum albumin (BSA, Sigma) for 48 h. Thereafter, the synchronized cells were stimulated with growth medium to re-enter the
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RESULTS

G₁ Inhibition by PMA—We used human umbilical artery smooth muscle cells, because the cell cycle was the easiest to synchronize in this cell type among several VSMC species screened in a pilot study. Flow cytometry showed that the content of DNA was 2C in almost all the cells after serum starvation for 48 h, indicating that the cells were synchronized in the G₁ phase (Fig. 1). In control experiments, cells with an S phase DNA content began to increase about 15 h after supplementation with fetal bovine serum and basic fibroblast growth factor, with the maximal accumulation being obtained at 24 h. Cells with a G₂/M phase DNA content (4C) appeared from about 30 h, peaking at 36 h. When PMA (10 nM) was added at time 0, cells with an S or G₂/M phase DNA content were nearly completely absent, indicating that the cell cycle was arrested before the entry into S phase.

However, as shown in Fig. 1, some of the control cells did not enter S phase even after mitogenic stimulation. Since the cell viability was over 95% according to trypan blue exclusion, it was unlikely that these non-responding cells were injured or dead. Therefore, we considered that these cells were those remaining in G₀ phase due to contact inhibition, which cannot be avoided when using normal diploid cells. Nevertheless, we used these cells, because we felt that contamination with G₀ cells would not interfere with the interpretation of our experimental results.

The incorporation of [³H]Thymidine started to increase about 15 h after mitogenic stimulation and reached a plateau at about 33 h (Fig. 2a), which corresponded well with the results of the flow cytometry (Fig. 1). There was little incorporation when cells were notstimulated, suggesting that entry into S phase was strictly dependent on mitogenic stimulation (Fig. 2a). To determine when PMA inhibits the cell cycle, PMA (10 nM) was applied at several time points during the G₁ and S phases (Fig. 2b). PMA added earlier than 3 h after stimulation inhibited [³H]Thymidine incorporation over 90%. However, the effect was attenuated when PMA was added 6 h or later, and there was no effect when added 12 h or later, suggesting that PMA inhibited the G₁, but not S phase progression. The inhibition was maximal at a PMA concentration of 10 nM and partially reversed above this concentration (Fig. 2c).

The Effect of PMA on the Phosphorylation of PRB—To elucidate the mechanism for the G₁ arrest by PMA, we examined the effect of PMA on the phosphorylation of pRB, since this phenomenon is a crucial milestone for the cell to advance into the S phase. Hyperphosphorylated pRB moves more slowly in SDS-polyacrylamide gels than the hypophosphorylated form. In quiescent cells, a 110-kDa protein, which seemed to be the hypophosphorylated form, was predominantly detected by immunoblotting (Fig. 3a). After mitogenic stimulation, a shift of the apparent molecular mass from 110 to 115 kDa on SDS-
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PMA (10 nM) failed to reverse the shift from 115 to 110 kDa (Fig. 3b). This effect peaked at 10 nM and was partially reversed at higher concentrations. Once PMA was phosphorylated, however, PMA could not reverse the phosphorylation, because when added 18 h after mitogenic stimulation, when the mobility of pRb had shifted, PMA failed to reverse the shift from 115 to 110 kDa (Fig. 3a, 100% inhibition).

The Effect of PMA on the Expression of Cdk5—To understand how PMA inhibits pRb phosphorylation, we examined the effects of PMA on the gene expression of Cdk5 by Northern blotting, since pRb may be the physiological substrate for Cdk5, such as Cdk2, Cdk2, Cdk4, and Cdk6 (22-25). As shown in Fig. 4, Cdc2 expression was not clear until 24 h, suggesting that it begins to be expressed after the entry into S phase. However, the expression of all of the other Cdk5s we tested, namely Cdk2, Cdk3, Cdk4, and Cdk5, started from the G₀ or early G₁ phase. The level of Cdk2 expression, which was low in quiescent cells, was elevated from early G₁ and further increased after the advance into S phase. The expression of other Cdk5s was markedly expressed even in quiescent cells, but the level was not dramatically altered throughout the G₁.

The effects of PMA on the gene expression of Cdk5s were incubated in growth medium in the presence of PMA (10 nM) or vehicle (0.1% Me₂SO) for various times as indicated below, after which they were collected and lysed. The proteins precipitated by the anti-pRb antibody (XZ104) were separated by 7.5% SDS-PAGE and immunoblotted against another anti-pRb antibody (PMG3-245). Lanes 1 and 2, not stimulated with growth medium; lanes 3-18, incubated in growth medium for 3 h (lanes 3 and 4), 6 h (lanes 5 and 6), 9 h (lanes 7 and 8), 12 h (lanes 9 and 10), 15 h (lanes 11 and 12), 18 h (lanes 13 and 14), 21 h (lanes 15 and 16), and 24 h (lanes 17 and 18), respectively. Odd-numbered lanes, vehicle; even-numbered lanes, PMA. b, G₀ cells were incubated in DMEM containing 0.1% BSA (no stimulation) or in growth medium containing various concentrations of PMA as indicated. *, 0.1% Me₂SO (vehicle) was added. Cells were lysed at 24 h and immunoblotted. c, after G₀ cells were stimulated with growth medium, PMA (10 nM) or vehicle (0.1% Me₂SO) was added at 18 h. Cells were lysed at the indicated times and immunoblotted. To indicate the position of hypophosphorylated pRb, the immunoprecipitate from the G₀ cell lysate was also blotted (shown as time 0).
and S phases. The expression of Cdk5 was also marked from G₀ until the late G₁ phase, but this was attenuated after 12 h. PMA (10 nM) suppressed the expression of Cdk2, which seemed to result from the inhibition of the advance into S phase. On the other hand, the expression of Cdk2, Cdk3, Cdk4, and Cdk5 was not inhibited for the first 12 h after growth stimulation, indicating that PMA did not inhibit their expression during the G₁ phase. Further elevation of Cdk2 after the entry into S phase was lost in the presence of PMA, probably due to the inability of cells to go into this phase. In contrast, the amount of Cdk5 mRNA after 12 h did not decrease in the presence of PMA.

We then examined the effects of PMA on the gene expression of cyclins, since association with a cyclin is required to activate Cdkks. Cyclins G, C, D₁, D₂, and D₃ were all induced from early G₁ (Fig. 5a). Cyclin G was expressed even in quiescent cells without significant changes in its mRNA levels throughout the G₁ and S phases. Cyclin C was also expressed in quiescent cells, but this increased after mitogenic stimulation. The expression of cyclins D₁, D₂, and D₃ was low in quiescent cells, but all were induced from the early G₁ phase. PMA (10 nM) did not reduce the expression of any of these cyclins at least for the first 12 h, but rather tended to enhance that of cyclins G, C, D₁, and D₂ in the early G₁ phase. PMA attenuated the expression of cyclin D₂ after 12 h.

Fig. 5b demonstrates the mRNA expression of cyclins induced later than those shown in Fig. 5a. It was difficult to clearly determine when the expression of cyclin E began, since small amounts of the mRNA were detected 3–9 h after stimulation and before the expression was markedly enhanced. Cyclin A was expressed from about 15 h, which corresponded to around the G₂/S border, and cyclin B was expressed a few hours later than cyclin A, probably after entry into S phase. In the presence of PMA (10 nM), however, the expression of all these mRNAs was suppressed.

The effects of PMA on the activities of Cdkks—We considered that the suppressed expression of cyclins E and A would reduce Cdk2 activity, since this kinase is activated by associating with these cyclins (26–29). The activity of Cdk2, determined by the in vitro phosphorylation of histone H₁, started to increase from about 9 h, and continued to increase until 24 h (Fig. 6a). PMA (10 nM) markedly inhibited this activation. The inhibitory effect was maximal when PMA was added at 0–6 h (Fig. 6b). PMA added at 15–21 h, which corresponded to the early S phase, also significantly inhibited Cdk2 activity. The inhibition was maximal at PMA concentration of 10 nM and partially reversed above this concentration, similarly to the effect on the [³H]Tdr incorporation (Fig. 6c).

Since Cdk2 phosphorylates pRb in vitro (23), the PMA-induced inhibition of Cdk2 activity seemed to explain the inhibition of pRb phosphorylation. However, it remained to be determined whether PMA inhibits the cellular events that arise before Cdk2 activation. Cyclins of the D class and their major partner Cdk4 may play crucial roles in the G₁/S transition by phosphorylating pRb (30, 31). As shown in Figs. 4 and 5, D-type cyclins and Cdk4 were expressed from the early G₁ phase, probably preceding the expression of cyclins D and A. We therefore tested whether PMA influences the activity of cyclin D-associated kinases, including Cdk4.

To confirm that PMA does not inhibit the expression of Cdk4 and D-type cyclins during the G₁ phase, the protein levels of Cdk4 and cyclin D were analyzed by Western blotting (Fig. 7). Consistent with the result of Northern blotting, PMA did not reduce their protein levels. Moreover, PMA did not reduce the amount of Cdk4 that co-purified with cyclin D, indicating that PMA did not inhibit the association of cyclin D with Cdk4.

The activities of cyclin D-associated kinase and Cdk4 were measured by the in vitro phosphorylation of GST-Rb, since pRb may be the physiological substrate for these enzymes and the histone H₁ kinase activity of Cdk4 was very low (not shown). The activity that co-purified with cyclin D, of which the level was low in quiescent cells, increased during the G₁ phase to peak at 7.5 h (Fig. 8a). After it dropped once at 9 h, the activity again increased from 12 until 24 h, when the activity was maximal during the observation period. In the presence of PMA (10 nM), the elevation in the G₁ phase was enhanced and lasted for a much longer period, although there was no second increase. The activity of Cdk4 was relatively high even in
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**Fig. 7.** The effect of PMA on the association between cyclin D and Cdk4. a, G0 cells were incubated in growth medium in the presence of PMA (10 nM) or vehicle and lysed 7.5 h after the release from G0. Lysates were immunoprecipitated (IP) with antibodies to Cdk4 or cyclin D as indicated. Precipitates were separated by 10% SDS-PAGE, and immunoblotted with antibodies to Cdk4 or cyclin D as indicated. The position of the IgG heavy chain (about 50 kDa) is also shown.

**Fig. 8.** The effects of PMA on the activities of cyclin D-associated kinase and Cdk4. a, G0 cells were incubated in growth medium in the presence of PMA (10 nM) or vehicle for the indicated periods. Cell lysates were then prepared and precipitated with anti-cyclin D antibody. After the kinase reaction, assay mixtures were resolved by 10% SDS-PAGE. Proteins were transferred to membranes and autoradiographed. The arrows indicate the positions of GST-Rb (46 kDa). b, the procedure was the same as described in a, except that anti-Cdk4 antibody was used for immunoprecipitation.

**DISCUSSION**

The cellular event mediating the cell cycle arrest induced by PMA should take place during G1, but not S phase, since PMA inhibited pRb phosphorylation (31, 35). PMA also inhibited pRb phosphorylation in vivo in melanoma and U937 cells, which showed the inability of phosphorylation. On the other hand, PMA did not inhibit the activity of Cdk4, either. Consistently, PMA did not inhibit the activity of Cdk4, either. Consistently, PMA did not inhibit the expression of cyclins D1, D2, and D3, when pRb had already undergone phosphorylation. Therefore, PMA seemed to arrest cells at the G1 phase by inhibiting an event that occurs before pRb phosphorylation.

Cdk2 phosphorylates pRb in vitro and it may be a mediator of G1/S transition (23, 38). Cdk2 activity increased from about 9 h after the release from G0. Therefore, the onset of the activation appeared to correspond to that of pRb phosphorylation. The mobility of pRb appeared to have shifted by 15–18 h, whereas Cdk2 activity increased until 24 h. The progressive elevation of Cdk2 activity after completion of the mobility shift could contribute to the maintenance of phosphorylation, or induce the phosphorylation of other sites on pRb that is undetectable by the mobility shift. PMA inhibited Cdk2 activity in a dose-dependent manner similar to that for the inhibition of DNA replication. Therefore, suppressed Cdk2 activity may explain the PMA-induced inhibition of pRb phosphorylation.

In vascular endothelial cells, cyclin A appeared to be expressed from the late G1; therefore, we suggested that PMA caused G1/S arrest by reducing cyclin A expression (39). In VSMCs, however, there was little expression of cyclin A until the cells entered the S phase. Therefore, we suggested that the endothelial cells assumed to be synchronized in the G0 phase contained a substantial number of G1 cells, which expressed cyclin A earlier than truly G0-synchronized cells.

The onset of Cdk2 activation appeared to be simultaneous with that of pRb phosphorylation, implying that these two events were closely linked. However, Cdk2 activation did not clearly precede pRb phosphorylation. It was conceivable, therefore, that the initiation of pRb phosphorylation is mediated by other kinases, and that PMA inhibits their activities. Cdk2 is associated with D-type cyclins, such as Cdk4 and Cdk6, also phosphorylate pRb in vitro (21, 24, 25). They may be the kinases most prominently involved in the phosphorylation of pRb (31). In contrast to Cdk2, the cyclin D-associated kinase activity increased in the early to middle G1 phase forming a peak 7.5 h after the release from G0, thus clearly preceding the initiation of pRb phosphorylation. PMA did not suppress this activity, but rather enhanced it in the early to middle G1 phase. Therefore, it was unlikely that the PMA-induced inhibition of pRb phosphorylation resulted from the inhibition of cyclin D-dependent kinases. It remains to be investigated why Cdk4 activity was relatively high even in quiescent cells and its phosphorylation.
High p16 expression restrains pRb phosphorylation, even in the presence of active cyclin D-Cdk complexes.

Alternatively, PMA could activate pRb phosphatase. Although studies on the mechanism of pRb dephosphorylation are so far limited, evidence suggests that one of the pRb phosphatases is a type 1 protein phosphatase (PP1) (41). The pRb directly binds to the catalytic subunit of PP1, and the complex is detected in G1/G0, mid-G1, and G phase cells, suggesting the importance of PP1 for the maintenance of hypophosphorylated pRb from late M to mid-G1 phase (42). Therefore, if PMA can stimulate pRb phosphatase, our apparently contradictory results would be coincident, since pRb may be able to interact with pRb phosphatase in vivo, but not in our in vitro pRb kinase assay system, which lacks pRb phosphatase.

Negative growth signals generated by TGF-β, cAMP, and cell-cell contact may be mediated by inhibitor proteins of Cdkks (43–45). Several Cdk inhibitors have been found in mammalian cells, including p21Cip1/WAF1/Sdi1, p27Kip1, p16INK4a, and p15INK4b (46). Although there are only a few reports on their mechanisms of action, p27 may inhibit Cdk4 and Cdk2 by preventing the phosphorylation with Cdk-activating kinase (44, 47). p16 interrupts the association of cyclin D1 with either Cdk4 or Cdk6 (48, 49). However, PMA inhibited neither the activities of cyclin D-associated kinase and Cdk4 nor the binding of cyclin D to Cdk4, suggesting that the effect of PMA is not mediated by these inhibitors.

Alternative mechanisms have been suggested for TGF-β and cAMP. TGF-β reduces the levels of Cdk2 and Cdk4 in human keratinocytes (50) and of Cdk4 in mink lung epithelial cells (51), and cAMP inhibits the expression of cyclin D in murine macrophages (52). However, in contrast, PMA did not inhibit, but sometimes enhanced the cellular events arising in the G0 to early G1 phase, that included the expression of cyclins C, D, Cdk2, Cdk3, Cdk4, and Cdk5. This corresponded to other reports that phorbol ester stimulates the G0/G1 transition by mimicking competence factors (10). Significantly, these contrasts between PMA and other growth inhibitory signals indicate that PMA is a unique inhibitory agent that suppresses pRb phosphorylation in a different manner from others. PMA inhibits the proliferation of a lymphoma cell line by inducing TGF-β (53). However, this may not be so in our cells due to the above reason, and moreover, because neutralizing antibodies to TGF-β did not attenuate the PMA-induced inhibition of G1/S transition. 2

We showed that PCK mediates the phorbol ester-induced inhibition of G1/S transition, since the inhibition was mimicked by repeated doses of membrane-permeable DAG and abolished by down-regulation of PCK by a preincubation with phorbol ester (18, 39). The PMA dose-effect relationship also suggested the involvement of PKC, because the maximal inhibitory effects on DNA synthesis, pRb phosphorylation, and Cdk2 activity were always obtained around 10 nM. This was in agreement with the reported concentrations for PKC activation. This inhibition was always partially reversed at concentrations over 100 nM, which we assumed to result from the down-regulation of PKC.

It seems that the PKC pathway inhibits the cell cycle of VSMCs at the mid-to-late G1, but not the early G1 phase. Cells remain competent for proliferation while PKC is being activated. We thus speculated that PKC is a physiological regulator of the G1, phase, hindering DNA synthesis just before the R point by preventing pRb phosphorylation until the circumference are favorable for advance into the remainder of the cell cycle. Alternatively, PKC could contribute to cell differentiation, since several lines of evidence have suggested that the prevention of pRb phosphorylation and the resulting cell cycle arrest are necessary for subsequent cell differentiation (31). To test these possibilities, it is essential to understand how PKC activity is controlled during the cell cycle and cell differentiation.

In addition, we found that vascular endothelial cells are arrested in the G1 phase, as well as the G1 phase by PMA and DAG (54). If this is also a phenomenon universal among various cell species, it would suggest that PKC operates as a regulator of the cell cycle clock at two points, namely in the G1 and G2 phases. There are multiple phosphorylation sites on pRb (55), and phosphorylation progresses by multiple steps, not only in the G1, but also in the G2 phase (56). Hence, the G1 inhibition could also involve the inhibition of pRb phosphorylation. It is essential to characterize each phosphorylation site in terms of the regulation of pRb function.

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