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Distinct functions of down-regulation-sensitive and -resistant types of protein kinase C in rabbit aortic smooth muscle cells

Ken-ichi Kariya and Yoshimi Takai

Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan

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In quiescent cultures of rabbit aortic smooth muscle cells, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced DNA synthesis to some extent in the presence of rabbit plasma-derived serum but inhibited the rabbit whole blood serum (WBS)-induced DNA synthesis and increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Prolonged treatment of the cells with phorbol-12,13-dibutyrate (PDBu) caused the partial down-regulation of protein kinase C to a level of 25–35% of that in control cells. In these PDBu-pretreated cells, TPA neither induced DNA synthesis nor inhibited the WBS-induced DNA synthesis, but still inhibited the WBS-induced increase in $[Ca^{2+}]_i$. These results suggest (i) that there are down-regulation-sensitive and -resistant types of protein kinase C in rabbit aortic smooth muscle cells; (ii) that the down-regulation-sensitive type has the proliferative and antiproliferative actions whereas the down-regulation-resistant type lacks them; and (iii) that the down-regulation-resistant type has the activity to inhibit the WBS-induced increase in $[Ca^{2+}]_i$.

Cell proliferation; Ca^{2+}; Phorbol ester; Protein kinase C; (Vascular smooth muscle)

1. INTRODUCTION

Protein kinase C is activated by diacylglycerol or phorbol esters in the presence of Ca^{2+} and phospholipid (reviews [1,2]). The diacylglycerol is derived from the phospholipase C-mediated hydrolysis of phosphoinositides in a receptor-linked manner (reviews [1–3]). This protein kinase is present in most mammalian tissues and plays important functions in transmembrane signaling.

Recently, three different but highly homologous complementary DNA clones encoding protein kinase C have been identified and sequenced in bovine [4,5], rat [6] and rabbit brains [7]. Subsequently, three different protein molecules of this enzyme have been purified from rat brain [8]. The different types of the purified enzyme show very similar catalytic, kinetic and physical properties but are distinguishable from one another by their respective specific antibodies [8]. These results clearly indicate that there are at least three different isozymic forms of protein kinase C, and have raised the possibility that each form of this enzyme is responsible for the regulation of different cell functions.

We have previously shown that protein kinase C is involved in the proliferative action of platelet-derived growth factor in cultured rabbit aortic

Correspondence address: Y. Takai, Dept of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan

Abbreviations: SMC, smooth muscle cell; WBS, whole blood serum; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; PDBu, phorbol-12,13-dibutyrate; FCS, fetal calf serum; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; 4α-PDD, 4α-phorbol-12,13-didecanoate; PDS, plasma-derived serum; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin

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SMCs [9]. Subsequently, we have clarified that protein kinase C has additionally an antiproliferative action in this cell type, and have proposed that this enzyme may serve as not only a positive but also a negative regulator in the proliferation of rabbit aortic SMCs [10]. During the course of these studies, we have found that protein kinase C inhibits the WBS-induced increase in $[Ca^{2+}]_i$ in rabbit aortic SMCs as described in several cell types [11–15]. Moreover, we have found that protein kinase C is partially down-regulated by prolonged treatment of rabbit aortic SMC with PDBu, and that the partial down-regulation of protein kinase C abolishes completely both the proliferative and antiproliferative actions of this enzyme but does not abolish the inhibitory action of this enzyme in the regulation of $[Ca^{2+}]_i$. These results suggest that there are at least two types of protein kinase C which are involved in the regulation of different functions in rabbit aortic SCMs. This paper provides for the first time evidence concerning the physiological significance of the heterogeneity of protein kinase C.

2. MATERIALS AND METHODS

2.1. *Materials*

Japanese White rabbits were purchased from Shizuoka Laboratory Animal Center; FCS from Gibco; and [*methyl*-^{3}H]thymidine and [γ-^{32}P]ATP from Amersham. Aequorin was supplied by Dr J.R. Blinks (Mayo Foundation, Rochester, MN). TPA and PDBu were from CCR Inc. and 4α-PDD was from Sigma. WBS and PDS were prepared from the Japanese White rabbits as described [16]. H1 histone and a phospholipid mixture were prepared from calf thymus and bovine brain, respectively, as described [17]. 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 [18]. Briefly, about 10 pieces of 1 mm × 1 mm intimal-inner medial segments of aortae were placed in a 25-cm^2 flask 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 [19]. 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 deprived of FCS by washing twice with serum-free DMEM and incubating in the same medium for 48 h. After these cells were washed three times with DMEM containing 0.1% BSA, they were incubated in 1 ml of DMEM with various agents for 24 h in the continuous presence of 4.4 μM [*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 Beckman liquid scintillation system LS3801. The cells used for these studies were quiescent before stimulation by various agents as judged by autoradiography of the labeled nuclei.

2.4. *Assay for $[Ca^{2+}]_i$*

Changes in $[Ca^{2+}]_i$ in rabbit aortic SMCs were monitored by use of aequorin, a calcium-sensitive photoprotein. The cells in secondary cultures were seeded into 100-mm dishes at a density of 1.6×10^6 cells/dish in DMEM containing 10% FCS. After the incubation for 3 days, the cells were deprived of FCS by washing twice with serum-free DMEM and incubating in the same medium for 48 h. After these cells were washed three times with DMEM containing 0.1% BSA, they were loaded with aequorin by the scrape-loading method of McNeil and Taylor [20], except that the experiments were performed using suspended cells between 20 and 40 min after the loading with aequorin. Aequorin signal was measured using a Chrono-log platelet ionized calcium aggregometer (Havertown, PA). A suspension of aequorin-loaded cells (2×10^5 cells/ml) was applied into a cuvette and incubated at 37°C under constant stirring. $[Ca^{2+}]_i$ was

estimated by the calibration method of Allen and Blinks [21] assuming an intracellular magnesium concentration of 1 mM.

2.5. *Down-regulation of protein kinase C by PDBu-treatment*

Down-regulation of protein kinase C was performed by incubating rabbit aortic SMCs with 100 ng/ml of PDBu for 48 h during the period of the FCS deprivation as described [9,10].

3. RESULTS

Incubation of quiescent cultures of rabbit aortic SMCs with WBS induced DNA synthesis (table 1). TPA also induced DNA synthesis in the presence of PDS to a level of about 30% of that induced by WBS. Either TPA or PDS alone was almost inactive. In contrast to this proliferative action of TPA, this phorbol ester reduced the WBS-induced DNA synthesis to the same level as that induced by TPA in the presence of PDS. Prolonged treatment of the cells with PDBu caused the partial down-regulation of protein kinase C to a level of 25–35% of that in the control cells [9]. In these PDBu-pretreated cells, WBS induced DNA synthesis to the same level as that in the control cells, but TPA neither induced DNA synthesis in the presence of PDS nor inhibited the WBS-induced DNA synthesis. PDBu showed the actions similar to those of TPA but 4α-PDD was inactive in these capacities (not shown). These results are consistent with the earlier observations [9,10].

Addition of WBS to quiescent cultures of rabbit aortic SMCs caused a rapid increase in $[Ca^{2+}]_i$ (fig.1A). TPA inhibited this WBS-induced increase in $[Ca^{2+}]_i$ (fig.1B). TPA did not affect the basal level of $[Ca^{2+}]_i$. Although the active components of WBS involved in the increase in $[Ca^{2+}]_i$ have not been identified, these results are consistent with the earlier observations that angiotensin II raises $[Ca^{2+}]_i$ and TPA inhibits this increase in $[Ca^{2+}]_i$ in rat aortic SMCs [15]. In the PDBu-pretreated cells, WBS increased $[Ca^{2+}]_i$ to the same level as that observed in the control cells (fig.1C). In these cells, TPA still inhibited the WBS-induced increase in $[Ca^{2+}]_i$ (fig.1D), in contrast to the ineffectiveness of TPA on DNA synthesis and the WBS-induced DNA synthesis. PDBu similarly inhibited the

Table 1

Effect of TPA on DNA synthesis

Additions	DNA synthesis (cpm × 10^{-4})	
	Control cells	PDBu-pretreated cells
None	0.1	0.1
WBS (10%)	10.1	10.0
TPA (100 ng/ml)	0.1	0.2
PDS (10%)	0.6	0.6
TPA + PDS	2.9	0.6
WBS + TPA	3.0	9.8

DNA synthesis was assayed in control and PDBu-pretreated rabbit aortic SMCs in the presence of various combinations of WBS, TPA and PDS as indicated. Each value is the mean of triplicate determinations

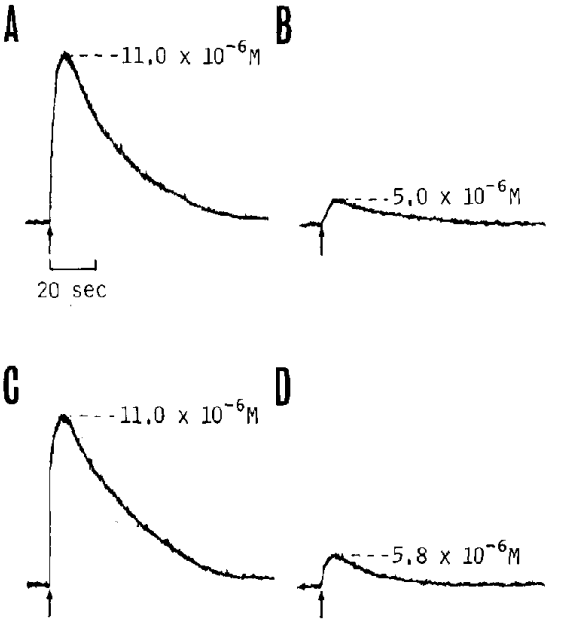

Fig.1. Effect of TPA on the WBS-induced increase in $[Ca^{2+}]_i$. After the control and PDBu-pretreated rabbit aortic SMCs loaded with aequorin were preincubated in the absence or presence of 100 ng/ml of TPA for 10 min, the cells were incubated with 10% WBS and changes in $[Ca^{2+}]_i$ were assayed. (A,B) Control cells; (C,D) PDBu-pretreated cells. (A,C) In the absence of TPA; (B,D) in the presence of TPA. The arrows indicate the point at which WBS was added. The numbers indicate the maximum value of $[Ca^{2+}]_i$. Each panel shows a typical trace from three different experiments.

WBS-induced increase in $[Ca^{2+}]_i$ but 4α-PDD was inactive in this capacity in both the control and PDBu-pretreated cells (not shown). The PDBu-treatment did not change the basal level of $[Ca^{2+}]_i$.

Fig.2 shows the dose-dependent effects of TPA on DNA synthesis and the WBS-induced DNA synthesis and increase in $[Ca^{2+}]_i$ in the control and PDBu-pretreated cells. In the control cells, TPA induced DNA synthesis in the presence of PDS in a dose-dependent manner (fig.2A). This phorbol ester also inhibited the WBS-induced DNA synthesis and increase in $[Ca^{2+}]_i$ in a dose-dependent manner. The doses of TPA necessary for these three actions were nearly the same. In the PDBu-pretreated cells, TPA, at any dose, neither induced DNA synthesis nor inhibited the WBS-induced DNA synthesis, but it inhibited the WBS-induced increase in $[Ca^{2+}]_i$ in a dose-dependent manner (fig.2B). The doses of TPA necessary for inhibiting the WBS-induced increase in $[Ca^{2+}]_i$ in the control and PDBu-pretreated cells were nearly the same.

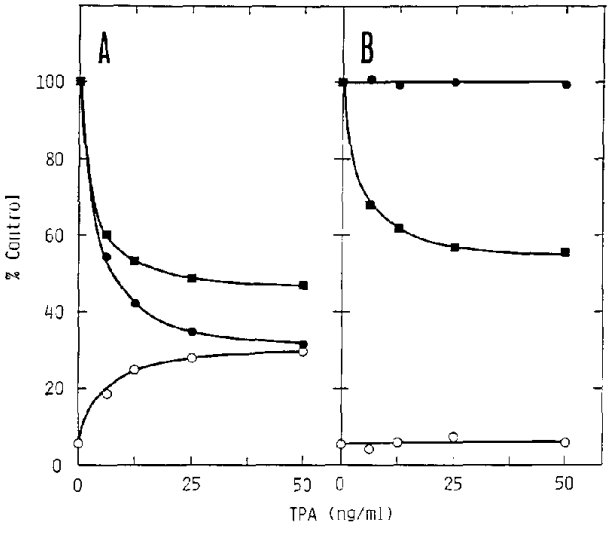

Fig.2. Dose-dependent effects of TPA on DNA synthesis and the WBS-induced DNA synthesis and increase in $[Ca^{2+}]_i$. DNA synthesis and changes in $[Ca^{2+}]_i$ were assayed in control and PDBu-pretreated rabbit aortic SMCs in the presence of various doses of TPA, 10% WBS and 10% PDS as indicated. The values are presented as percentages of those obtained by 10% WBS in the absence of TPA. (A) Control cells; (B) PDBu-pretreated cells. (○—○) DNA synthesis in the presence of PDS; (●—●) DNA synthesis in the presence of WBS; (■—■) increase in $[Ca^{2+}]_i$ in the presence of WBS.

Table 2

Down-regulation of protein kinase C

	Protein kinase C activity (cpm)
Control cells	1180
PDBu-pretreated cells	330

Rabbit aortic SMC in secondary cultures were further seeded into 100-mm dishes at a density of 1.6×10^6 cells/dish in DMEM containing 10% FCS. After the incubation for 3 days, the cells were deprived of FCS by washing twice with serum-free DMEM and incubating in the same medium for 48 h. During the period of this serum-deprivation, the down-regulation of protein kinase C was performed by adding 100 ng/ml of PDBu to the cells. After these cells were washed three times with DMEM containing 0.1% BSA, the Triton X-100 extract was prepared as described [22]. The protein kinase C activity in the Triton X-100 extract (equivalent to 4×10^4 cells) prepared from the control and PDBu-pretreated cells was assayed by measuring the incorporation of ^{32}P into H1 histone from [γ-^{32}P]ATP as specified earlier [17]. The protein kinase C activity was determined by subtracting the enzymatic activity measured in the absence of Ca^{2+}, TPA and a phospholipid mixture from that in the presence of Ca^{2+}, TPA and a phospholipid mixture. Each value is the mean of triplicate determinations

In our preceding report [9], the down-regulation of protein kinase C was estimated by assaying the [^{3}H]PDBu-binding to the intact rabbit aortic SMC after pretreatment with PDBu. The down-regulation of this enzyme was again confirmed here by assaying the protein kinase activity with H1 histone as a substrate in the Triton X-100 extract prepared from the control and PDBu-pretreated cells. As shown in table 2, prior incubation of the cells with PDBu reduced the enzymatic activity of protein kinase C to the level of about 25% of that in the control cells.

4. DISCUSSION

In rabbit aortic SMCs, prior treatment of the cells with PDBu causes the partial down-regulation of protein kinase C to a level of 25–35% of that in the control cells. This result suggests that there are two types of protein kinase C: one is the down-

regulation-sensitive type and the other is the down-regulation-resistant type. The mechanism underlying the selective down-regulation of one type of protein kinase C is unknown, but it can be speculated that the intracellular compartment of protein kinase C may be different between the down-regulation-sensitive and -resistant types.

Since the actions of TPA in inducing DNA synthesis and inhibiting the WBS-induced DNA synthesis and increase in $[Ca^{2+}]_i$ are mimicked by PDBu but not by 4α-PDD, it is most likely that these actions of TPA are mediated through the activation of protein kinase C. The proliferative and antiproliferative actions of protein kinase C are completely abolished by the down-regulation of the enzyme. Therefore, it is conceivable that the enzyme responsible for these actions may be the down-regulation-sensitive type and the down-regulation-resistant type may lack these actions. In contrast, the inhibitory action of protein kinase C in the WBS-induced increase in $[Ca^{2+}]_i$ is not abolished by the down-regulation of the enzyme. Protein kinase C responsible for this action may be the down-regulation-resistant type. However, it cannot be concluded from these results whether the down-regulation-sensitive type also has the activity to inhibit the WBS-induced increase in $[Ca^{2+}]_i$.

If all the actions of TPA are mediated through the activation of protein kinase C, the present results clearly indicate that there are at least two functionally different types of this enzyme in cultured rabbit aortic SMCs. It has been shown that there are at least α, β and γ types of protein kinase C, and that α and β types are expressed in most tissues [6,7] (the nomenclature for the isozymic forms of protein kinase C here has followed that of Coussens et al. [5]). Although it is unknown which types of protein kinase C are present in rabbit aortic SMCs, α and β types may also be present in this cell type and either of them may correspond to the down-regulation-sensitive or -resistant type. It is also conceivable that the down-regulation-sensitive type of protein kinase C is further divided into subtypes and each subtype has the proliferative or antiproliferative action. Further studies are necessary to clarify which types of protein kinase C have the activities to induce DNA synthesis and to inhibit the WBS-induced DNA synthesis and increase in $[Ca^{2+}]_i$ in rabbit aortic SMCs.

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REFERENCES

[1] Takai, U., Kikkawa, U., Kaibuchi, K. and Nishizuka, Y. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res. 18, 119–158.
[2] Nishizuka, Y. (1984) Nature 308, 693–697.
[3] Majerus, P.W., Connolly, T.M., Deckmyn, H., Ross, T.S., Bross, T.E., Ishii, H., Bansal, V.S. and Wilson, D.B. (1986) Science 234, 1519–1526.
[4] Parker, P.J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M.D. and Ullrich, A. (1986) Science 233, 853–859.
[5] Coussens, L., Parker, P.J., Rhee, L., Yang-Feng, T.L., Chen, E., Waterfield, M.D., Francke, U. and Ullrich, A. (1986) Science 233, 859–866.
[6] Knopf, J.L., Lee, M.-H., Sultzman, L.A., Kriz, R.W., Loomis, C.R., Hewick, R.M. and Bell, R.M. (1986) Cell 46, 491–502.
[7] Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T. and Hidaka, H. (1987) Nature 325, 161–166.
[8] Huang, K.-P., Nakabayashi, H. and Huang, F.L. (1986) Proc. Natl. Acad. Sci. USA 83, 8535–8539.
[9] Kariya, K., Kawahara, Y., Tsuda, T., Fukuzaki, H. and Takai, Y. (1987) Atherosclerosis 63, 251–255.
[10] Kariya, K., Fukumoto, Y., Tsuda, T., Kawahara, Y., Fukuzaki, H., Yamamoto, T. and Takai, Y. (1987) FEBS Lett. 217, 69–73.
[11] Naccache, P.H., Molski, T.F.P., Borgeat, P., White, J.R. and Sha'afi, R.I. (1985) J. Biol. Chem. 260, 2125–2131.
[12] Vicentini, L.M., Di Virgilio, F., Ambrosini, A., Pozzan, T. and Meldolesi, J. (1985) Biochem. Biophys. Res. Commun. 127, 310–317.
[13] Zavoico, G.B., Halenda, S.P., Sha'afi, R.I. and Feinstein, M.B. (1985) Proc. Natl. Acad. Sci. USA 82, 3859–3862.

[14] Misbahuddin, M., Isosaki, M., Houchi, H. and Oka, M. (1985) FEBS Lett. 190, 25–28.
[15] Brock, T.A., Rittenhouse, S.E., Powers, C.W., Ekstein, L.S., Gimbrone, M.A. jr and Alexander, R.W. (1985) J. Biol. Chem. 260, 14158–14162.
[16] Vogel, A., Raines, E., Kariya, B., Rivest, M.-J. and Ross, R. (1978) Proc. Natl. Acad. Sci. USA 75, 2810–2814.
[17] Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692–3695.
[18] Ross, R. (1971) J. Cell Biol. 50, 172–186.
[19] Dicker, P. and Rozengurt, E. (1980) Nature 287, 607–612.
[20] McNeil, P.L. and Taylor, D.L. (1985) Cell Calcium 6, 83–93.
[21] Allen, D.G. and Blinks, J.R. (1979) in: Detection and Measurement of Free Ca^{2+} in Cells (Ashley, C.C. et al. eds) pp.159–174, Elsevier-North Holland, Amsterdam.
[22] Kaibuchi, K., Tsuda, T., Kikuchi, A., Tanimoto, T., Yamashita, T. and Takai, Y. (1986) J. Biol. Chem. 261, 1187–1192.