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Dual Effect of Protein Kinase C on the Induction of DNA Synthesis by Colcemid in G_0-Arrested Human Diploid Fibroblasts

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ABSTRACT. **G_0-arrested human diploid fibroblasts, TIG-1, was stimulated to induce DNA synthesis by serum, epidermal growth factor (EGF), colchicine, colcemid, or 12-O-tetradecanoylphorbol-13-acetate (TPA). The induction of DNA synthesis was mediated by protein kinase C (PKC) when stimulated with TPA but not when stimulated with other agents. When TPA-stimulated cells were immediately treated with colcemid, induction of DNA synthesis was reduced. This reduction diminished when colcemid was added more than 6 h after TPA treatment. Conversely, when colcemid-stimulated cells were treated with TPA, induction of DNA synthesis was also reduced. This reduction was enhanced when the interval between the addition of two stimulants was extended. PKC-deprivation abolished both stimulatory and inhibitory effects of TPA on DNA synthesis. Staurosporine blocked an induction of DNA synthesis by TPA but appeared to be ineffective on the inhibitory action of TPA on DNA synthesis by colcemid. These results suggest that the inhibitory effect of TPA on the induction of DNA synthesis by colcemid is mediated by down regulation-sensitive and staurosporine-insensitive PKC.**

Growth arrested human diploid fibroblast line, TIG-1, is induced to synthesize DNA following stimulation with various peptide growth factors such as serum, epidermal growth factor (EGF), and DNA synthesis factors (26). This cell line is also stimulated to synthesize DNA by other agents as 12-O-tetradecanoylphorbol-13-acetate (TPA), teleosidine, colchicine, colcemid, vinblastin, vincristin, or TN16 (26). Generally these non-peptide agents individually are very weak inducers of DNA synthesis in various cell lines, but are active as fetal bovine serum in early-passaged TIG-1.

It has been shown that protein kinase C (PKC) is activated by diacylglyceride derived from phosphatidylinositides which are hydrolyzed by phospholipase C through the action of several growth factors including platelet-derived growth factor, fibroblast growth factor, bombesin and vasopressin (4, 15, 25). PKC is a receptor of TPA which is the potent activator of PKC (2, 13, 17, 18). Activation of PKC induces several cell cycle dependent genes such as c-*fos* and c-*myc* and lets cells traverse from the G_0 to the S phase (7, 24). Contrary to the mitogenic action of PKC, it has also been reported that TPA inhibits the induction of DNA synthesis or cell proliferation (1). However, the mode of antiproliferative action of TPA has not been studied extensively except in smooth muscle cells (8, 9).

In the present paper we report that in the human fibroblast, TIG-1, TPA itself has a potent stimulatory effect on the induction of DNA synthesis but it also showed inhibitory effects on the induction of DNA synthesis induced by colcemid. This dual effect suggests that the former is mediated by staurosporine-sensitive PKC and the latter by staurosporine-insensitive PKC.

MATERIALS AND METHODS

Materials. Materials used were: fetal bovine serum (Whittaker Bioproducts Inc., Md.), human epidermal growth factor (a gift from Wakunaga Pharmaceutical Co. Ltd., Hiroshima), colchicine (Wako Pure Chemical Industries Inc., Tokyo), colcemid (Wako Pure Chemical Industries Inc., Tokyo), 12-O-tetradecanoylphorbol-13-acetate (LC Service Corp.), phorbol 12,13-dibutyrate (Sigma Co. Mo.), staurosporine (Boehringer Mannheim), [methyl-^{3}H]thymidine (74 GBq Ci/m mol, Amersham), [^{3}H]phorbol-12,13-dibutyrate (537 GBq/m mol, Amersham).

Cell and cultures. Human diploid fibroblasts, TIG-1, were cultured as described previously (26). They have a proliferative lifespan, about 60 population doublings, and becomes less responsive to various growth factors (26). We used them at the 25–45 population doubling level.

Assay of DNA synthesis. DNA synthesis was assayed by measuring the incorporation of [^{3}H]thymidine into acid insoluble materials (20) or by autoradiography (6). The cells were plated on 13-mm round cover slips in 24-multiwell, and were cultured until they became subconfluent. Then they were

growth-arrested in G_0 phase by maintaining subconfluent cultures in serum-deprived (0.5%) medium for 2–3 days as described (26). Growth-stimulation was done by adding serum or other growth factors at desired concentrations. The cells were continuously labeled with [methyl-^{3}H]thymidine (74 GBq/m mol, 74 KBq/ml) for desired periods of time as indicated in each experiment. After labeling, the cells were washed with phosphate-buffered saline, fixed with 10% formalin and washed extensively with water, rinsed with ethanol and air-dried. The radioactivity was determined with a liquid scintillation spectrometer. After determination of radioactivity, coverslips were rinsed with ethanol, air-dried and processed for autoradiography (6).

Assay of PKC. The cells were washed with phosphate buffered saline and scraped. The cell pellet was homogenized in homogenizing buffer (20 mM Tris-HCl, 50 mM 2-mercaptoethanol, 5 mM EGTA, 2 mM EDTA, 2 μM leupeptin, 100 μM phenylmethylsulfonyl fluoride, pH 7.5) by Dounce A type homogenizer. The homogenate was centrifuged for 10 min at 1,500 × g and the resultant supernatant (postnuclear fraction) was recentrifuged for 30 min at 10,000 × g. The supernatant was saved as cytosol fraction. The pellet was suspended in homogenizing buffer containing 2% NP-40 and centrifuged for 30 min at 10,000 × g. The final supernatant was saved as membrane fraction. PKC in each fraction was partially purified through DE52 columun (12). Binding of [^{3}H]phorbol-12,13-dibutyrate (16) and assay of enzyme activity using a synthetic octapeptide as a substrate (4, 5) were done as previously described.

Northern blot analysis. Isolation of RNA and analysis by Northern blot were done as reported previously on TIG-1 cells (10).

RESULTS

Induction of DNA synthesis in G_0-arrested TIG-1. TIG-1 cells were growth-arrested in G_0 phase by culturing subconfluent cells in serum-deprived (0.5%) medium for 2–3 days. They entered the S phase following stimulation with serum, EGF, colcemid, or TPA (Table I) as previously reported (26). Entrance into the S phase began at about 14–16 h after growth stimulation (data not shown), though the extent of induction of DNA synthesis varied among experiments. Stimulatory effect on DNA synthesis by TPA and colcemid was highly dependent upon population doubling level (PDL) of TIG-1 as previously reported (26), and was sometimes higher than that by serum before 30 PDL but generally lower after 35 PDL. Frequencies in cells entering the S phase monitored by autoradiography (Table I) were always correlated with the amount of incorporated [^{3}H]thymidine, and therefore incorporation of [^{3}H]thymidine into DNA is refered to as "induction of DNA synthesis" or "entrance into the S phase" in the following experiments.

Table I. INDUCTION OF DNA SYNTHESIS IN G_0-ARRESTED TIG-1.

Stimulants	DNA synthesis (percent of labeled nuclei)
unstimulated	1.7
serum (10%)	60.6
EGF (10 ng/ml)	21.1
TPA (10 ng/ml)	47.6
colcemid (10^{-5} M)	35.8
serum + EGF	85.1
serum + colcemid	55.6
EGF + TPA	9.1
serum + TPA	40.0
colcemid + TPA	8.6

G_0-arrested TIG-1 was stimulated with various agents and labeled with [^{3}H] thymidine (37 KBq/ml) for 36 h.

When two stimulants were added simultaneously, no additive or synergistic effect for induction of DNA synthesis was observed. Induction of DNA synthesis by combination of two stimulants was generally not less than that observed after stimulation with one stimulant which showed higher response than the other (Table I). However, when TPA was added with EGF or colcemid, induction of DNA synthesis was much lower than expected. We focused the following experiments on the action of colcemid and TPA. In repeated experiments, colchicine and colcemid gave quantitatively similar effects at concentrations between 10^{-6}–10^{-5} M and therefore, the results from only one of these conditions were presented in the following studies.

Activation of PKC following growth stimulation. Before studying the action of TPA and colcemid, the possible participation of PKC on the induction of DNA synthesis after addition of various growth stimulants was examined. When the cells were stimulated with TPA, induction of DNA synthesis was likely to be mediated by PKC activation, because 1) translocation of PKC from cytosol to membrane fraction occurred after 15 min of TPA treatment as revealed by assay of enzyme activity and by [^{3}H]phorbol-dibutyrate binding (data not shown); 2) PKC was down regulated after TPA treatment (Fig. 1); 3) staurosporine, an inhibitor of PKC (23), blocked induction of DNA synthesis by TPA (Table II); and 4) PKC deprivation resulted in the inability of DNA induction by TPA (Table III).

On the other hand, when the G_0-arrested cells were stimulated with serum, EGF, colcemid or colchicine, the induction of DNA synthesis appeared to be bypassed, or was not mediated, by PKC, because 1) down regulation of PKC did not occur (Fig. 1); 2) staurosporin did not block induction of DNA synthesis (Table II); and 3) PKC deprivation did not block but rather enhanced induction of DNA synthesis (Table III).

Effect of colcemid and TPA on induction of DNA

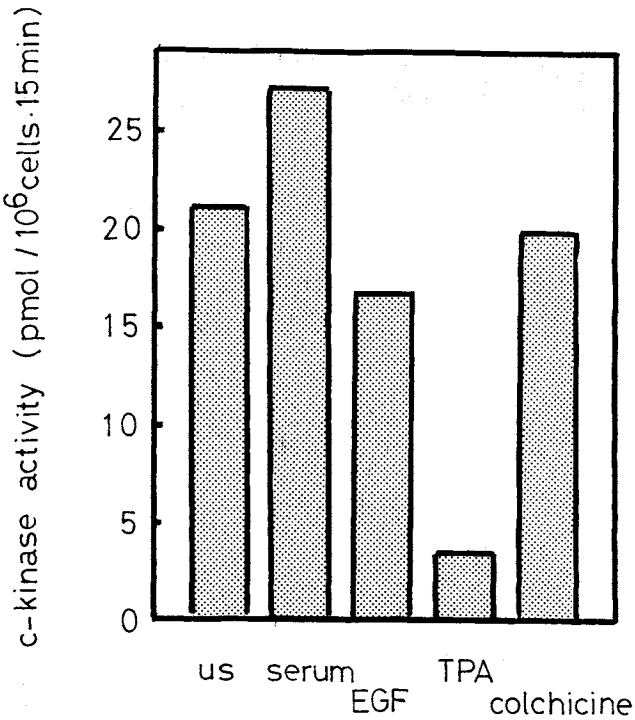

Fig. 1. Down regulation of PKC after the stimulation with various agents.
G_0-arrested TIG-1 was stimulated with serum (10%), EGF (10 ng/ml), TPA (10 ng/ml), or colchicine (10^{-6} M), and after 24 h the PKC activity in postnuclear fraction was assayed.

synthesis. When colcemid was added immediately (within a few minutes) after the addition of TPA, induction of DNA synthesis was reduced: compare closed square (TPA alone) and closed circle (colcemid after TPA) at 0 time in Fig. 2. However, the longer the interval between addition of TPA and colcemid, the similler the reduction of DNA synthesis (Fig. 2, closed circles). When colcemid was added more than 3 h later, DNA synthesis began to recover to the level of that induced by TPA alone. The reason why DNA synthesis was re-

Table II. Effects of staurosporine on the induction of DNA synthesis.

stimulants	[^{3}H]thymidine incorporation (cpm)	
	staurosporine (0 nM)	staurosporine (1 nM)
serum (10%)	14,321 (100)	12,267 (86)
EGF (10 ng/ml)	14,979 (100)	14,870 (99)
TPA (10 ng/ml)	14,033 (100)	3,954 (28)
colchicine (10^{-5} M)	8,434 (100)	6,859 (81)

G_0-arrested TIG-1 in 24-multiwell dishes was stimulated with various agents to induce DNA synthesis in the presence or absence of staurosporine. Incorporation of [^{3}H]thymidine (37 KBq/ml) during 36 h was assayed. Parenthesis; percentage of incorporation in the presence of staurosporine to that in its absence.

Table III. Effect of PKC-deprivation on the induction of DNA synthesis.

stimulants	[^{3}H]thymidine incorporation	
	control	PKC-deprived
–	1,125 (100)	2,380 (212)
serum (10%)	9,491 (100)	15,298 (161)
EGF (10 ng/ml)	11,646 (100)	15,303 (131)
TPA (10 ng/ml)	19,625 (100)	4,194 (21)
colcemid (10^{-5} M)	8,606 (100)	14,634 (170)

TIG-1 was PKC-deprived and arrested in G_0 phase. It was stimulated with various agents and DNA synthesis was assayed as described in legends to Table II.

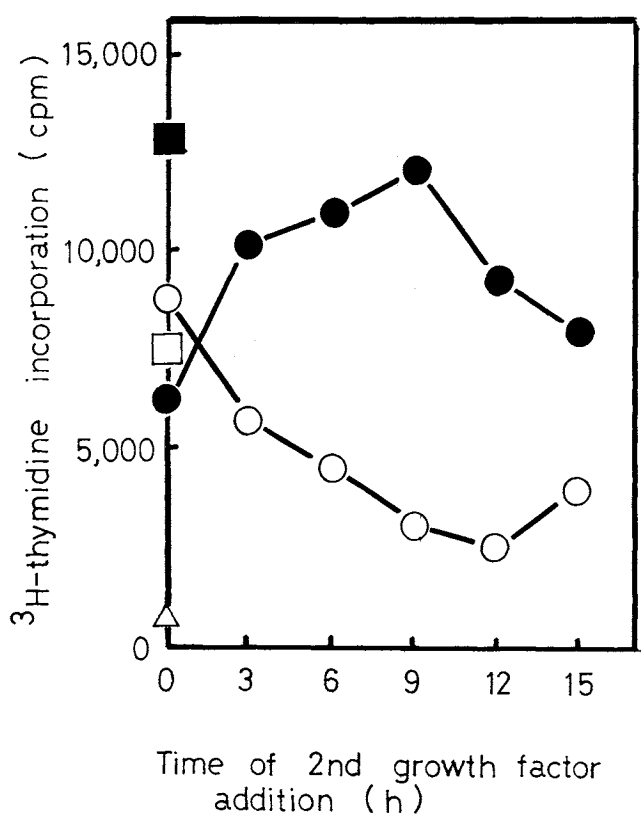

Fig. 2. Effects of colcemid and TPA on the induction of DNA synthesis.
G_0-arrested TIG-1 was stimulated with TPA (10 ng/ml) alone (■) or also with colcemid (10^{-6} M) after various periods of time (indicated along abscissa) of TPA addition (●). Closed circle at 0 time represents point at which colcemid was added within a few minutes after TPA addition. G_0-arrested TIG-1 was stimulated with colcemid alone (□) or also with TPA after various periods of time (indicated along abscissa) of colcemid addition (○). Open circle at 0 time represented that TPA was added within a few minutes after colcemid addition. Triangle at 0 time represented unstimulated control. The cells were labeled with [^{3}H]thymidine from 15 through 39 h.

duced when colcemid was added after 12 h is unclear. Conversely, when TPA was added immediately (within a few minutes) after colcemid addition, induction of DNA synthesis was somewhat affected. However, the longer the interval between the addition of colcemid and TPA, the greater the reduction of DNA synthesis (Fig. 2, open circles).

It should be emphasized that colcemid or TPA was individually an inducer of DNA synthesis and that during the majority of the culture period up to 39 h the cells were incubated in medium containing both colcemid and TPA, but that the extent of the induction of DNA synthesis was influenced by the order of addition of the two agents. An inhibitory effect of TPA on DNA synthesis was also observed when TPA was added after treatment of G_0-arrested TIG-1 cells with serum or EGF, but it was very weak (data not shown). When phorbol was used instead of TPA, it showed neither stimulatory effect on DNA synthesis by itself nor inhibitory effect on induction of DNA synthesis by colcemid (data not shown).

The complex results shown in Fig. 2 could be explained tentatively by the following: 1) the inhibitory effect on DNA synthesis is expressed when the signal transduction process by colcemid is operating and, at the same time, activation of PKC occurs; and 2) some of the molecular species of PKC are responsible for the inhibitory action of TPA by influencing the processes operating after colcemid treatment. In the experimental protocol of "TPA first and colcemid second", when colcemid was added within 3 h of TPA addition, there was enough active PKC is available to bring out an inhibitory effect that is expressed through pathways driven by colcemid. When colcemid is added 6 h after TPA addition, PKC is no longer available, and so the inhibitory effect of TPA can not be expressed. Contrarily, in the experimental protocol of "colcemid first and TPA second", colcemid does not down-regulate PKC at all, and so when TPA treatment is applied anytime after colcemid addition, PKC is activated and functions on pathways driven by colcemid to express an inhibition of DNA synthesis. The result that the inhibitory effect of DNA synthesis was more extensive when the interval between addition of colcemid and TPA was extended, suggests that the the inhibitory action of PKC is expressed in the progression of cells from late G_1 to the S phase as reported in smooth muscle cells (8, 9).

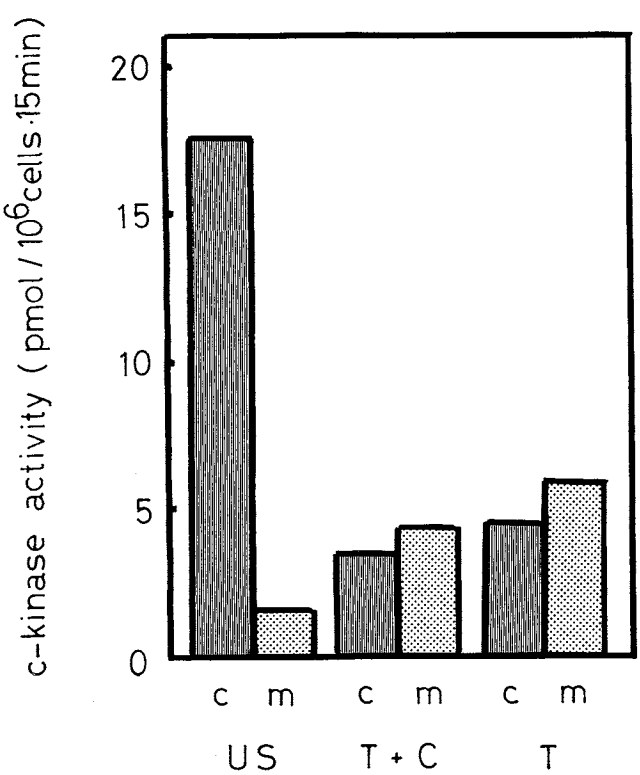

Fig. 3. Activation of PKC after 15-min treatment with TPA in cells incubated with colchicine for 6 h.
PKC activity in membrane (m) and cytosolic (c) fractions was assayed. us; unstimulated control, T; G_0-arrested cells were treated with TPA (10 ng/ml) for 15 min, and C+T; G_0-arrested TIG-1 was treated with colchicine for 6 h and then treated with TPA for 15 min.

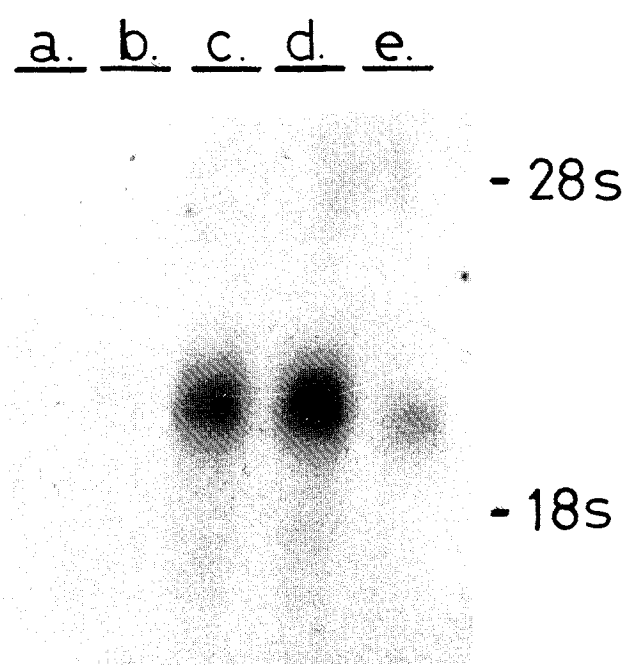

Fig. 4. Induction of c-*myc* after 2 h-treatment with TPA in cells incubated with colcemid for 6 h.
G_0-arrested TIG-1 was treated with colcemid (10^{-6} M) for 6 h and treated with TPA (10 ng/ml) for 30 min. Amount of c-*myc* mRNA was assayed by Northern blot analysis. a; unstimulated control, b; colcemid-treated for 2 h, c; TPA-treated for 2 h, d; colcemid-treated for 6 h and then TPA-treated for 2 h, and e; colcemid-treated for 6 h.

Activation and down regulation of PKC by TPA. To see whether the recovery from inhibition of DNA synthesis observed in "TPA first and colcemid second experiments" is due to the down regulation of PKC, the time course of down regulation of PKC was monitored after TPA addition. PKC Activity in postnuclear fraction was regulated down to about 50% and 20% of control level at 3 h and 6 h after TPA addition, respectively. After 9 h, PKC activity was less than 10% of control level. This time course of PKC down regulation was in good agreement with that of recovery from inhibition of DNA synthesis shown in Fig. 2 (closed circles).

Next, to see whether PKC was activated by TPA in "colcemid first and TPA second experiments", TPA was added at 6 h after colcemid addition and PKC activation was monitored by two different methods. First, translocation of PKC to membrane was observed at 15 min after TPA addition (Fig. 3). Second, c-*myc* mRNA accumulated 2 h after TPA addition in both G_0-arrested and colcemid-treated cells (Fig. 4). Accumulation of c-*fos* mRNA also occurred 30 min after TPA addition in both G_0-arrested and colcemid-treated cells (data not shown). Treatment with colcemid alone did not bring about the accumulation of these mRNAs. These results indicated that TPA could activate PKC even at 6 h after colcemid addition.

Effect of staurosporine on the induction of DNA synthesis. If an inhibitory effect, as well as stimulatory effect, on DNA synthesis by TPA was mediated by PKC, it could be abolished when PKC was inhibited by metabolic inhibitors. To test this hypothesis, similar experiments as shown in Fig. 2 were carried out in the presence or absence of staurosporine. Fig. 5a shows the "TPA first and colcemid second" experiment. Since an induction of DNA synthesis by TPA alone was inhibited in the presence of staurosporine but that by colcemid was not, the remaining DNA synthesis observed in the presence of staurosporine being attributable to the effect of colcemid alone. The induction of DNA synthesis was reduced in the presence of staurosporine (Fig. 5a). However, the inhibitory effect on DNA synthesis by TPA was observed when TPA was added soon after colcemid addition, and was not abolished even in the presence of staurosporine (Fig. 5a). Fig. 5b showed that DNA synthesis was reduced in the presence of staurosporine as compared with that in the absence of the in-

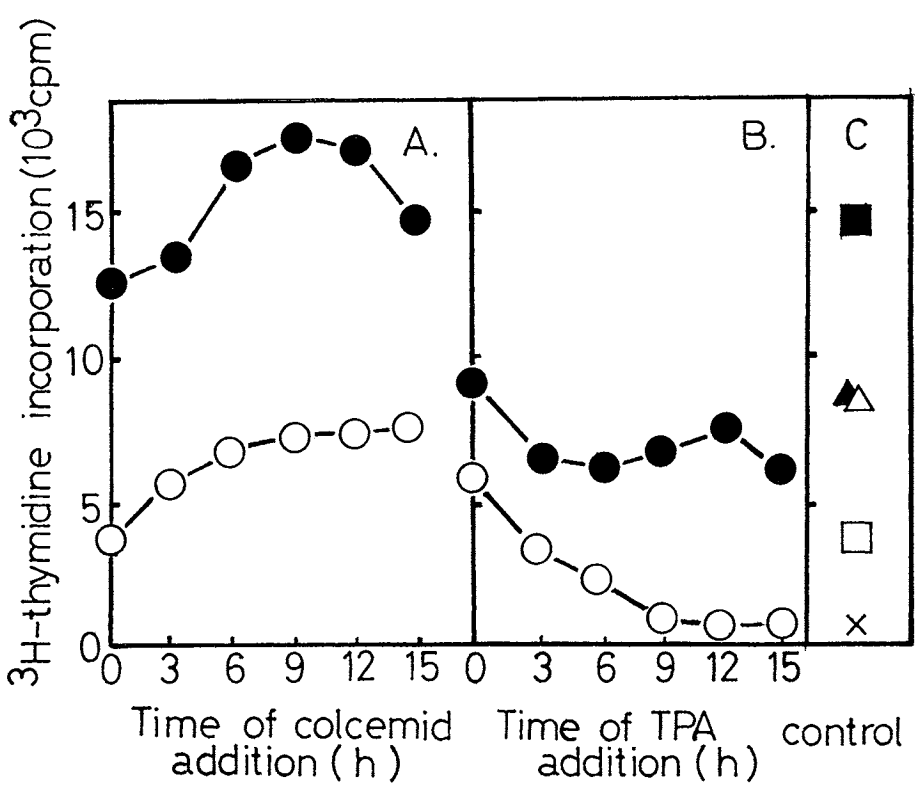

Fig. 5. Effect of staurosporine on the induction of DNA synthesis by TPA and colcemid.
Experimental protocol was essentially the same as shown in Fig. 3 except for the addition of staurosporine (1 nM). Open symbols; with staurosporine, closed symbols; without staurosporine. (A) G_0-arrested TIG-1 was stimulated with TPA first and then with colchicine after various periods of time (indicated along abscissa) of TPA addition. (B) G_0-arrested TIG-1 was stimulated with colcemid first and then with TPA after various periods of time (indicated along abscissa) of colcemid addition. (C) TPA alone (□, ■), colcemid alone (△, ▲), and unstimulated control (×).

hibitor. The inhibitory effect on DNA synthesis by TPA was not abolished but appeared rather to be enhanced (Fig. 5b). These observations suggest that PKC which mediates a stimulatory signal by TPA for DNA synthesis is inhibited by staurosporine but that PKC which mediates an inhibitory signal by TPA for DNA synthesis is resistant to staurosporine.

Induction of DNA synthesis in PKC deprived cells. Since the presence of staurosporine-resistant PKC has not been reported in the course of the signal transduction process, other means should be used to confirm whether PKC is concerned with the inhibitory process. We then carried out similar experiments as shown in Fig. 2 using PKC deprived cells. Subconfluent culture of TIG-1 was incubated in serum-deprived (0.5%) medium containing 100 ng/ml of TPA for 2 days. It was confirmed that 24-h incubation with TPA was enough to down regulate PKC to a negligible level of activity. As expected, these cells were stimulated to synthesize DNA following addition of serum, EGF, or colcemid, but not by TPA (Table III). When these G_0-arrested and PKC-deprived cells were stimulated with both TPA and colchicine, the extent of DNA induction was largely constant, regardless of the order of addition of the two stimulants (Fig. 6). In these experiments, induction of DNA synthesis appeared to be stimulated simply by colcemid alone. When colcemid was added at 12 h, induction of DNA synthesis apparently declined because of 12-h delay of induction of DNA synthesis. These results strongly suggested that the inhibitory effect on DNA synthesis by TPA is mediated by PKC that is down regulation-sensitive and staurosporine-insensitive. The results shown in Table III, the DNA synthesis is induced by serum, EGF, or colcemid enhanced in PKC-deprived cells but not in a staurosporine-treated cells, might also indicate some degree of participation of down regulation-sensitive and staurosporine-insensitive PKC on the repression of DNA synthesis.

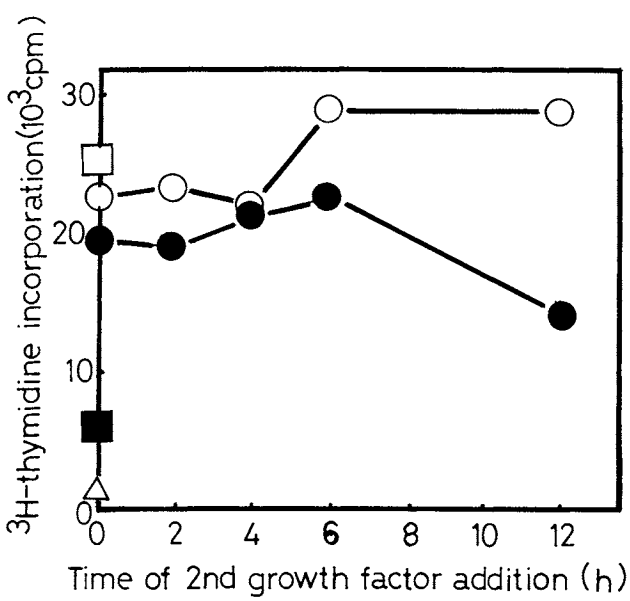

Fig. 6. Effect of PKC deprivation on the induction of DNA synthesis by TPA and colcemid.
Experimental protocol was essentially the same as shown in Fig. 3 except for the use of PKC-deprived cells. G_0-arrested TIG-1 was stimulated with TPA alone (■) or also with colcemid after various periods of time (indicated along abscissa) of TPA addition (●). G_0-arrested TIG-1 was stimulated with colcemid alone (□) or also with TPA after various periods of time (indicated along abscissa) of colcemid addition (○), (△); Unstimulated control.

DISCUSSION

Kariya *et al.* (8) and Kawahara *et al.* (9) reported that an induction of DNA synthesis by serum was reduced by TPA in rabbit aortic smooth muscle cells. Although both of their and our studies were on the inhibitory effect of TPA on an the induction of DNA synthesis following stimulation with various mitogens, these two systems showed several differences: 1) In smooth muscle cells TPA alone did not stimulate DNA synthesis, whereas in TIG-1 cells TPA alone did stimulate DNA synthesis; 2) in smooth muscle cells TPA extensively reduced DNA synthesis after stimulation of cells with serum, whereas in TIG-1 cells it was only slightly reduced after stimulation with serum but was extensively reduced with colcemid; and 3) in smooth muscle cells there exists two forms of PKCs, those which are down regulation-sensitive and those which are -insensitive, and the former is concerned with both growth stimulation and growth repression, whereas in TIG-1 cells it is suggested that there exists staurosporine-sensitive and -insensitive PKCs and both of these are subject to down regulation. In both systems, the inhibitory effect of TPA on induction of DNA synthesis was mediated by PKC, and PKC appeared to exert its inhibitory action at the G_1 phase to block the progression of cells from the late G_1 to the S phase.

Recently, four different but highly homologous PKCs, i.e. PKC-α, PKC-β1, PKC-β2, and PKC-γ, have been identified as cDNAs (14, 21) and as purified enzymes (3, 11, 22). Additionally, three other types of nPKCs, although less homologous to the conventional PKCs, were also reported (19). However, the functional discrimination of these PKC molecules in the transduction processes of growth signal has yet to be clarified. Our results suggest, as one of several possible interpretations, that there are two forms of PKC; one of which is staurosporine-sensitive and acts as a mediator of growth induction, and an other which is staurosporine-insensitive and acts as a mediator of growth repression, and may shed new light to differentiate each function of

the PKC molecule family. However, *in vitro* assay of PKC activity using a synthetic peptide as a substrate revealed the absence of staurosporine-resistant PKC (data not shown). A simple interpretation of this finding, although only one of possible interpretations, would be that some form of PKC is staurosporine-resistant toward physiological substrate(s) critical for growth repression. Studies on the possible presence of phosphorylation substrate(s) *in vivo* which are down regulation-sensitive and staurosporine-resistant is under investigation. Since TPA activated both forms of PKC and other mitogens such as serum, EGF, and colcemid appeared to bypass PKC activation, it is of interest to distinguish the growth stimulatory or inhibitory factors that separately activate different forms of PKC leading to growth induction or growth repression. Studies on the action mechanisms of TGF-β which express an inhibitory effect on the induction of DNA synthesis by serum in TIG-1 are currently in progress.

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