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Stimulation of ribosomal protein S6 kinase activity by pp60$^{v\text{-}src}$ or by serum: Dissociation from phorbol ester-stimulated activity

(Rous sarcoma virus/growth factors/protein kinase C/cycloheximide/vanadate)

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ABSTRACT **Ribosomal protein S6 kinase activity was measured in lysates prepared from serum-deprived chicken embryo fibroblasts (CEF) treated for various times with phorbol 12-myristate 13-acetate (PMA). Maximal activity was observed within 15 min, and it declined to the initial level by 4 hr. Incubation of these cells with PMA 4–60 hr after the initial treatment did not result in an additional increase in S6 protein kinase activity. These results are consistent with down-regulation of the PMA receptor, protein kinase C, and the dependence of PMA-stimulated S6 kinase activity on this enzyme. Long-term pretreatment of CEF with PMA only partially attenuated the stimulation of the S6 protein kinase activity by serum or by expression of the Rous sarcoma virus transforming gene product, pp60$^{v\text{-}src}$. A similar protein kinase activity also was stimulated in cells treated with cycloheximide or sodium vanadate. Pretreatment with PMA had little effect on this response. These data indicate that it is likely that there are at least two mechanisms through which S6 kinase activity can be regulated, one of which apparently utilizes protein kinase C whereas the other(s) does not. Additional experiments show PMA-stimulated glucose transport was not attenuated by long-term incubation with phorbol ester, suggesting that another mechanism, which is not dependent on the presence of protein kinase C, maintains this response after the proposed down-regulation of the PMA receptor.**

Several transforming gene products and growth-factor receptors have tyrosine-specific protein kinase activity (ATP: protein-tyrosine *O*-phosphotransferase, EC 2.7.1.112) (1–3). Little is known, however, about the physiologically important cellular substrates of these enzymes despite prolonged investigations. The identification of such substrates is crucial for additional descriptions of the biochemistry of the cancer cell and of the regulatory events in normal cell division. As an alternative to direct identification of cellular proteins containing phosphotyrosine, we have investigated cellular enzymes that, because of their increased activity in transformed cells, are potential physiological substrates of transforming gene products or growth factor receptors. Alterations in transformed cells provide some information concerning enzymes of interest.

The expression of a transforming gene product, such as that of the Rous sarcoma virus (RSV) *src* gene, pp60$^{v\text{-}src}$, elicits in a cell a wide spectrum of phenotypic changes associated with malignancy. For example, normal cells in culture cease division and become quiescent when growth factors in serum are reduced or absent, whereas, under similar conditions, their transformed counterparts continue to divide. A biochemical marker for such quiescent cells is the unphosphorylated 40S ribosomal subunit protein S6. After the expression of pp60$^{v\text{-}src}$ or the addition of serum or tumor-promoting phorbol ester, S6 is rapidly phosphorylated on serine residues (4). It has been suggested that the phosphorylation of S6 plays a role in the regulation of protein synthesis (5–8). Thus, the study of protein kinases and/or phosphoprotein phosphatases acting on S6 and their regulation by transforming gene products and growth factor receptors offers the possibility of identifying additional tyrosine-specific protein kinase substrates.

As a first step in our studies, we have identified and carried out preliminary characterization of a protein kinase that phosphorylates S6 in 40S ribosomal subunits. We found that serum-deprived cells stimulated by pp60$^{v\text{-}src}$ expression or by serum or phorbol ester addition yield a chromatographically similar enzyme activity (9). Moreover, the stimulated S6 kinase activity is distinct from the cAMP-dependent protein kinase and protein kinase C (9). Phorbol 12-myristate 13-acetate (PMA) is known to bind to and activate protein kinase C (for review, see ref. 10); therefore, these results suggest that the S6 kinase activation in PMA-treated cells may be mediated by protein kinase C. Because our results show that the PMA-stimulated S6 kinase in chicken embryo fibroblasts (CEF) resembles the serum- and pp60$^{v\text{-}src}$-stimulated S6 kinases, protein kinase C may mediate both growth factor receptor and the *src* gene product S6 kinase activation. Growth-factor receptors also possess the ability to stimulate the production of the second messengers, inositol trisphosphate and diacylglycerol (11). Diacylglycerol also activates protein kinase C (10). Thus, one can imagine distinct stimuli resulting in the activation of protein kinase C, which then stimulates the S6 kinase activity. We have explored this possibility in this study.

The findings of Rozengurt and coworkers indicate that prolonged exposure of cells to phorbol ester results in down-regulation of protein kinase C activity and phorbol ester binding. Furthermore, they have demonstrated that PMA-stimulated phosphorylation of an M_r 80,000 protein is attenuated upon rechallenge with phorbol ester (12–15). This is consistent with other results (16–23) that indicate that prior treatment with phorbol ester attenuates many PMA-induced alterations upon subsequent exposure to the tumor promoter.

In this report we examined the capacity of a transforming gene product, pp60$^{v\text{-}src}$, or serum to stimulate S6 protein kinase activity in cells in which the activity of protein kinase C had been reduced by PMA treatment. The results are discussed with respect to the role of protein kinase C in activation of S6 kinase activity in CEF stimulated with PMA, with serum growth factors, or by expression of pp60$^{v\text{-}src}$. Furthermore, we briefly address the question of the role of PMA in the maintenance of phorbol ester-altered cellular metabolism, such as glucose transport (for review, see ref. 24), after the apparent down-regulation of protein kinase C.

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Abbreviations: RSV, Rous sarcoma virus; CEF, chicken embryo fibroblasts; ts-CEF, chicken embryo fibroblasts infected with a temperature-sensitive transformation mutant of RSV; PMA, phorbol 12-myristate 13-acetate; PHR, phorbol, inactive analog of PMA.

MATERIALS AND METHODS

Cell Culture and Protein Kinase Assay. CEF and CEF infected with NY72-4, a temperature-sensitive transformation mutant of RSV (ts-CEF), were cultured as described (9). Briefly, after seeding approximately 3–4 × 10^6 cells per 100-mm Costar culture dish, CEF or ts-CEF cultured at the nonpermissive temperature (41.5°C) were allowed to just reach confluence (2 days), after which the monolayers were incubated for an additional 24 hr with 8 ml of serum-free Dulbecco's modified Eagle's medium before preparation of cell-free lysates. For experiments involving pretreatment with phorbol ester, PMA (Pharmacia P-L Biochemicals, dissolved in dimethyl sulfoxide) was added to 100 ng/ml (final dimethyl sulfoxide concentration of 0.05–0.1%) for 4–60 hr prior to the experiment as described in each figure legend. The parent compound, phorbol (PHR, Pharmacia P-L Biochemicals), was used as the control. Preparation of cell-free lysates from confluent monolayers and assay of S6 protein kinase were performed as described (9).

Gel Electrophoresis. 40S ribosomal proteins used in kinase assays were resolved by $NaDodSO_4$/12% polyacrylamide gel electrophoresis as described by Laemmli (25). For analysis of S6 phosphorylation, Coomassie blue-stained gels were dried and exposed to DuPont Cronex-4 film or Kodak XAR-5 X-Omat film with DuPont Lightning-Plus intensifying screens. Radiolabeled S6 was identified and excised, and associated radioactivity was quantitated by liquid scintillation spectrometry.

Glucose Transport. Transport of 2-deoxy[^{3}H]glucose (New England Nuclear) into cultured CEF was assayed as described (26). Protein was determined by the method of Bradford (27) or Lowry *et al.* (28). Results are expressed in cpm per μg of protein.

RESULTS

Time-Dependent Stimulation of S6 Kinase Activity by PMA. Stimulation of S6 kinase activity in quiescent CEF by PMA was observed at concentrations as low as 5 ng per ml. In order to achieve consistent stimulation of S6 kinase activity with limited cell toxicity, 100 ng of PMA per ml was used in the following experiments. S6 kinase activity, as measured in cell extracts prepared at various times after addition of PMA to quiescent monolayers of CEF, was nearly maximal within 15 min and was maintained at this level beyond 1 hr. After 4 hr it declined to the basal level (Fig. 1). A second 1-hr PMA treatment of CEF previously exposed to PMA for 4 hr did not result in reactivation of S6 kinase activity (Fig. 1 *Left*, lane 7). Similar results were obtained when CEF were pretreated with PMA for longer periods of time (24–60 hr) prior to the experiment (Fig. 2). To determine the effects of PMA pretreatment on other transformation- and/or growth-related metabolic processes, experiments were performed after 24–48 hr of pretreatment.

Effect of PMA Pretreatment on the Stimulation of S6 Kinase Activity by Serum, pp60$^{v\text{-}src}$, Cycloheximide, or Vanadate. The effect of long-term PMA pretreatment on activation of the S6 kinase in response to serum or pp60$^{v\text{-}src}$ is demonstrated in Fig. 3. Addition of serum to phorbol-pretreated CEF for 1 hr (Fig. 3 *Left*, lane 3) resulted in a 6.6-fold stimulation of S6 kinase activity over the activity obtained in lysates from the control PHR-pretreated cells (Fig. 3 *Left*, lane 1). A lower level of serum-stimulated S6 kinase activity was obtained in CEF pretreated with PMA (Fig. 3 *Left*, lane 4). Similar results were obtained with pp60$^{v\text{-}src}$, where PMA pretreatment of ts-CEF transferred to 35°C for 1 hr resulted in S6 kinase activity that was partially attenuated (Fig. 3 *Right*, lane 4) compared with activity for PHR-pretreated ts-CEF transferred to 35°C (Fig. 3 *Right*, lane 3). Even the low level of S6 kinase activity in ts-CEF cultured at 41°C was reduced in PMA-pretreated cells (Fig. 3 *Right*, lane 2) in comparison to PHR-treated cells (Fig. 3 *Right*, lane 1). The data from several experiments showing the effects of PMA-induced desensitization on stimulation of S6 kinase activity are presented in Table 1.

Incubation of cells with cycloheximide or vanadate stimulated S6 kinase activity (Fig. 4, lanes 5 and 7). Preliminary results suggest that in both cases this activation is proportional to the degree of inhibition of protein synthesis by these

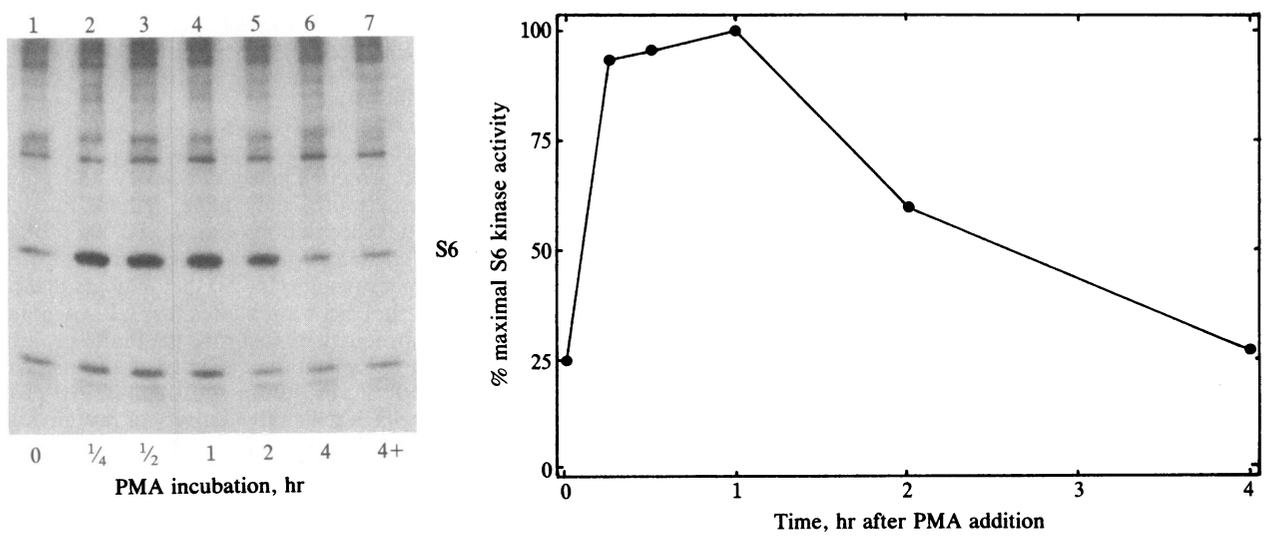

FIG. 1. PMA-stimulated activation of S6 kinase activity. (*Left*) Cell-free lysates were prepared from CEF incubated with 100 ng of PHR per ml for 1 hr (lane 1) or with 100 ng of PMA per ml for 15 min (lane 2), 30 min (lane 3), 1 hr (lane 4), 2 hr (lane 5), 4 hr (lane 6), and 4 hr plus a second addition of 100 ng of PMA per ml for 1 hr (lane 7). Aliquots (5 μl containing 2.3 μg of protein) were incubated with 40S ribosomal subunits (0.16 OD_{260} units) in the presence of 50 μM ATP (10 μCi of [γ-^{32}P]ATP per assay; 1 Ci = 37 GBq) and 15 mM $MgCl_2$ for 15 min at 30°C as described (9). Reactions were stopped by the addition of 5-times-concentrated electrophoresis sample buffer, and radiolabeled proteins were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. Final concentration of dimethyl sulfoxide was 0.05% (lanes 1–6) and 0.1% (lane 7). (*Right*) The S6 band was excised, and the radioactivity was determined. Data are plotted as % maximal S6 kinase activity obtained during the 4-hr time course after addition of 100 ng of PMA per ml. In these and other experiments, the incorporation of radiolabel in S6 ranged from about 1000 to 6000 cpm.

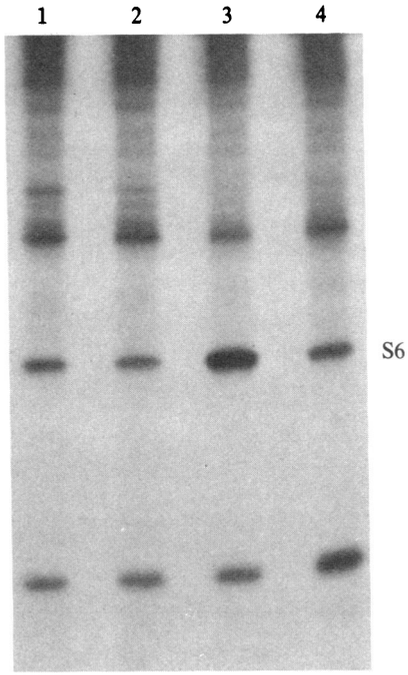

FIG. 2. Effect of long-term PMA treatment on stimulation of S6 kinase activity by additional PMA. After CEF were subcultured for 24 hr, PMA or PHR (100 ng/ml) was added to the culture medium for 36 hr. The medium was then changed to serum-free Dulbecco's modified Eagle's medium containing 100 ng of PHR or PMA per ml for an additional 24 hr. Cell-free lysates were then prepared 1 hr after a third addition of PMA or PHR (100 ng/ml; final concentration of dimethyl sulfoxide was 0.1%). S6 kinase activity was determined as described (ref. 9; Fig. 1). Lanes: 1, PHR-pretreated CEF plus 1 hr of PHR; 2, PMA-pretreated CEF plus 1 hr of PHR; 3, PHR-pretreated CEF plus 1 hr of PMA; 4, PMA-pretreated CEF plus 1 hr of PMA.

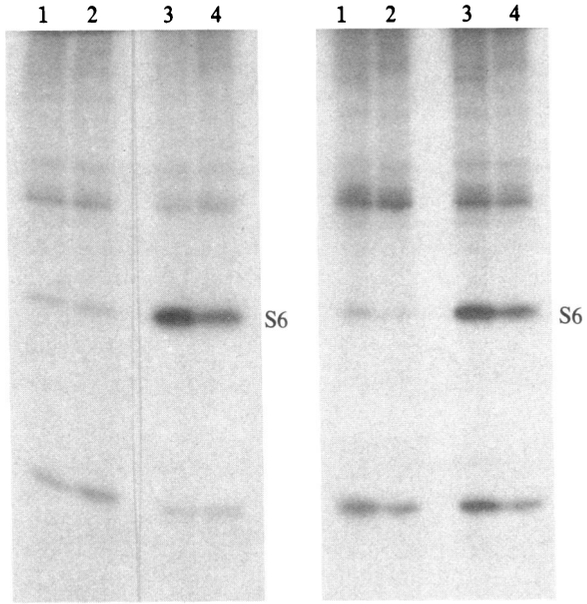

FIG. 3. Effect of long-term PMA pretreatment on stimulation of S6 kinase activity by serum or by pp60$^{v\text{-}src}$. Uninfected CEF (*Left*) and ts-CEF (*Right*) were cultured, and lysates were prepared as described in Fig. 2, except that the total pretreatment time was 48 hr. (*Left*) Lanes: 1, PHR-pretreated CEF; 2, PMA-pretreated CEF; 3, PHR-pretreated CEF plus 10% calf serum for 1 hr; 4, PMA-pretreated CEF plus 10% calf serum for 1 hr. (*Right*) Lanes: 1, PHR-pretreated ts-CEF cultured at 41°C; 2, PMA-pretreated ts-CEF cultured at 41°C; 3, PHR-pretreated ts-CEF transferred to 35°C for 1 hr; 4, PMA-pretreated ts-CEF transferred to 35°C for 1 hr.

agents. In the experiments described here, serum-deprived cells showed approximately 50% reduction in the rate of protein synthesis, whereas incubation of cells with cycloheximide or sodium vanadate as described in Fig. 4 resulted in more than 90% inhibition of protein synthesis. At present we cannot rule out inhibition of tyrosine phosphoprotein phosphatases as another possible mechanism for the modulation of S6 kinase activity by cycloheximide or vanadate. Stimulation of S6 kinase activity by cycloheximide or vanadate (Fig. 4, lanes 5 and 7) was unaffected by PMA pretreatment (Fig. 4, lanes 6 and 8), whereas PMA-stimulated activation of S6 kinase activity in this experiment was inhibited.

Effect of Long-Term PMA Treatment on Glucose Transport. As shown in Fig. 5, the level of glucose transport in uninfected CEF pretreated with 100 ng of PMA per ml for 36 hr remained unchanged for another 16 hr after subsequent addition of PMA, whereas glucose transport in PHR-pretreated cells slowly increased to the maximal rate by 4–8 hr after addition of PMA, confirming previously reported kinetic data (29). Similarly, pretreatment of ts-CEF cultured at 41.5°C with 100 ng of PMA per ml for 36 hr resulted in glucose transport activity that remained unchanged for an additional 16 hr after transfer of cultures to the permissive temperature for pp60$^{v\text{-}src}$ expression (35°C). However, upon temperature-shift of PHR-pretreated ts-CEF from 41.5°C to 35°C, glucose transport activity reached the same rate as seen in ts-CEF (41.5°C) pretreated with PMA. The time required to achieve maximal glucose transport (4–8 hr) was similar to that described in CEF infected with other temperature-sensitive transformation mutants of RSV (30, 31). It should be noted that glucose transport in ts-CEF treated with PMA and cultured at 41.5°C or 35°C was greater than that observed in uninfected CEF incubated with PMA. These results are consistent with the report of Bissell *et al.* (32) and are apparently due to the biochemical activity of pp60$^{v\text{-}src}$ encoded by temperature-sensitive transformation mutants in cells growing at the nonpermissive temperature. Thus, in contrast to stimulation of S6 kinase activity by PMA, which is rapid and transitory, glucose transport rose slowly and was maintained at the maximal level up to 48 hr after PMA addition. Moreover, there was no further stimulation by

Table 1. Effect of PMA pretreatment on stimulation of S6 kinase activity

Second stimulation*	Exp.	Fold stimulation	
		PHR-pretreated	PMA-pretreated
PMA (100 ng/ml)	1	3.8	1.3
	2	2.2	0.8
	3	1.9	1.1
	4	4.0	1.0
Serum (10%)	1	6.6	4.0
	2	5.7	3.6
	3	4.5	3.7
	4	10.9	4.9
pp60$^{v\text{-}src}$	1	4.1	2.5
	2	3.6	2.9
	3	4.1	2.8
	4	3.5	2.7

Pretreatment with PHR or PMA was for 6–24 hr as described in *Materials and Methods* and Fig. 3.

*PMA and serum treatments were with uninfected CEF. Activation of pp60$^{v\text{-}src}$ was accomplished by transfer of ts-CEF to 35°C. ts-CEF cultured at 41.5°C were used as controls. All treatments were for 1 hr.

†Fold stimulation was calculated by using cells pretreated with 100 ng of PHR per ml/hr followed by another 1-hr treatment of 100 ng of PHR per ml as the control.

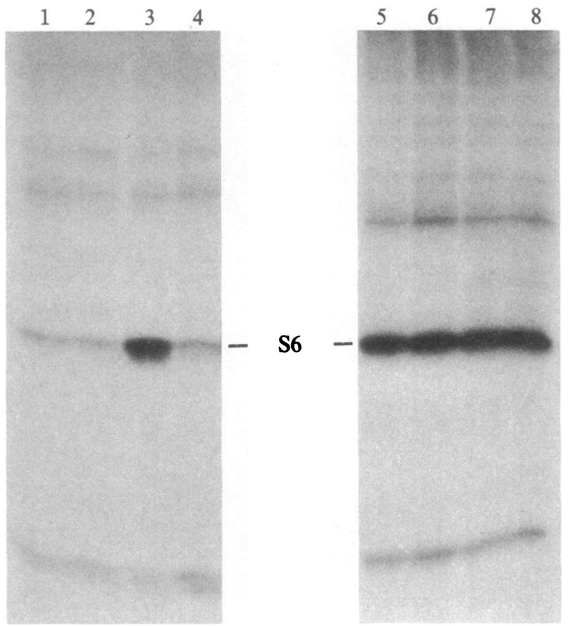

FIG. 4. Cycloheximide- and vanadate-stimulated S6 kinase activity and the effect of PMA pretreatment. Uninfected cells were cultured, and experiments were performed as described in Fig. 3. Lanes: 1, PHR-pretreated CEF plus 1 hr of PHR; 2, PMA-pretreated CEF plus 1 hr of PHR; 3, PHR-pretreated CEF plus 1 hr of PMA; 4, PMA-pretreated CEF plus 1 hr of PMA; 5, PHR-pretreated CEF plus 1 hr of 500 μM cycloheximide; 6, PMA-pretreated CEF plus 1 hr of 500 μM cycloheximide; 7, PHR-pretreated CEF plus 1 hr of 1 mM vanadate; 8, PMA-pretreated CEF plus 1 hr of 1 mM vanadate.

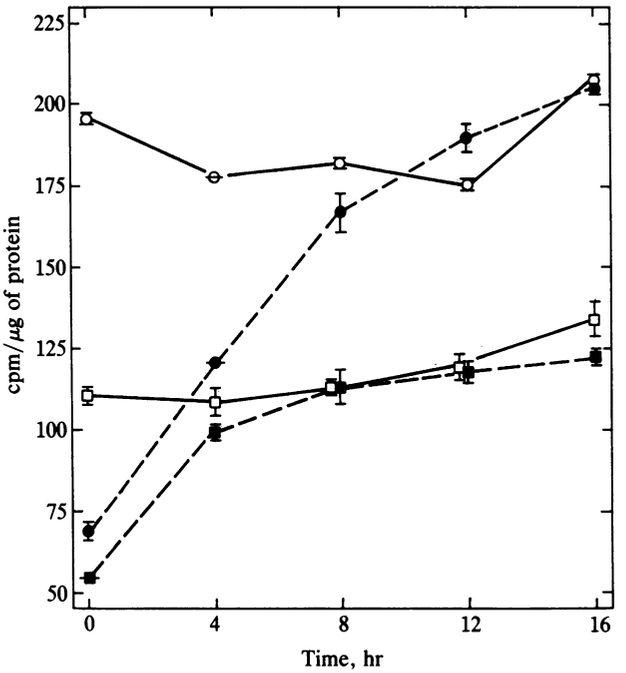

FIG. 5. Effect of long-term PMA pretreatment on glucose transport. CEF and ts-CEF were cultured and pretreated with PMA (24 hr in serum-depleted medium plus 12 hr in serum-free medium). At zero hour PMA was added to CEF, or ts-CEF were transferred from 41.5°C to 35°C, and 2-deoxyglucose transport was measured in duplicate at various times as described (26). ■, PHR-pretreated CEF incubated with PMA (100 ng/ml); □, PMA-pretreated CEF incubated with PMA; ●, PHR-pretreated ts-CEF transferred from 41.5°C to 35°C; ○, PMA-pretreated ts-CEF transferred to 35°C.

additional PMA or by expression of pp60$^{v\text{-}src}$ after shift of ts-CEF to 35°C.

DISCUSSION

Phosphorylation of 40S ribosomal protein S6 in quiescent, serum-deprived CEF can be stimulated by incubation of these monolayers with serum or the tumor promoter (PMA) at the appropriate dosages or by the expression of pp60$^{v\text{-}src}$ (4). These three diverse stimuli also stimulate a similar protein kinase activity that is specific for S6 in the 40S ribosomal subunit (9). These results suggested that the biochemical pathway(s) influenced by treatment of cells with each of these stimuli appears to converge at or before the point where the ribosomal protein S6 kinase activity is regulated. The results presented here represent an initial attempt to define the pathways leading to the stimulation of S6 kinase activity. We have made use of the observations by Rozengurt and coworkers (long-term treatment of cells with PMA results in the down-regulation of protein kinase C; ref. 13) to investigate the role of the PMA receptor in the regulation of S6 kinase activity. These studies have led to several observations: (*i*) activation of S6 kinase activity by PMA was strongly inhibited by prior treatment with the phorbol ester; (*ii*) in contrast, activation of S6 kinase activity by serum growth factors or pp60$^{v\text{-}src}$ was only partially attenuated by PMA pretreatment, implying that there is a mechanism for modulating S6 kinase activity independent of protein kinase C; and finally (*iii*) some effects of PMA treatment on cell metabolism were unaltered long after the proposed down-regulation of protein kinase C, raising questions about the role of this enzyme in these processes.

Recently, we identified S6 kinase activity in cultured CEF that is activated by treatment with PMA but that *in vitro* is clearly distinct from the phospholipid- and Ca^{2+}-dependent protein kinase C (9). Here we report that S6 kinase activity was rapidly activated (within 15 min) after treatment with PMA but, by 4 hr after the initial PMA treatment, declined to the level observed in untreated CEF. Exposure of CEF to a single PMA treatment (4–60 hr in these experiments) strongly inhibited subsequent stimulation of S6 kinase activity by PMA. Rozengurt and coworkers have recently reported that PMA pretreatment of cultured cells results in down-regulation of: (*i*) a M_r 80,000 phosphoprotein phosphorylation (14), (*ii*) phorbol 12,13-dibutyrate (PMA analog) receptors (12) and (*iii*) protein kinase C activity (13). Thus, our findings suggest that the apparent down-regulation of protein kinase C by long-term PMA pretreatment also results in a significant attenuation of S6 kinase activity in PMA-treated CEF.

The ability of PMA to down-regulate the protein kinase C activity as well as its ability to attenuate the PMA-stimulated activation of S6 kinase activity provides a tool to analyze the role of the PMA receptor (protein kinase C) in controlling the pleiotropic nature of PMA action. In addition, we can investigate the potential involvement of protein kinase C in the action of other stimulatory agents such as growth factors or oncogene products. Such involvement is predicted based on observations that phosphatidylinositol turnover is stimulated in RSV-transformed cells and in cells incubated with various growth factors (33, 34) and that this metabolism can result in the generation of the second messengers, inositol trisphosphate and diacylglycerol (34). Diacylglycerol presumably activates protein kinase C (10). As anticipated, our results show that activation of S6 kinase activity in CEF treated with serum growth factors or in CEF that express RSV pp60$^{v\text{-}src}$ can be partially attenuated by long-term PMA pretreatment; however, the degree of inhibition was clearly less than that observed with PMA treatment of desensitized cells. Thus, other mechanisms for the activation of S6 kinase activities by serum growth factors or pp60$^{v\text{-}src}$ are apparently utilized in addition to the potential modulation of S6 kinase activity by the activation of protein kinase C. The notion that

the activation of S6 kinase(s) can occur independently of protein kinase C is further supported by the finding that PMA pretreatment had a small effect on the stimulation of S6 kinase activity by cycloheximide or sodium vanadate.

Finally, it should be noted that markers that respond slowly to PMA, such as elevated 2-deoxyglucose transport (Fig. 4; ref. 29), cell-surface fibronectin, and cell morphology (for review, see ref. 24), are maintained long after down-regulation of the phosphorylation of the M_r 80,000 protein and the proposed disappearance of protein kinase C activity in PMA-treated cells (13). At least two possible mechanisms could account for this phenomenon: (*i*) PMA may activate a series of processes that, once activated, no longer require the phospholipid- and Ca^{2+}-dependent protein kinase; or (*ii*) PMA-activated protein kinase C may be modified, for example by proteolysis, and thereby rendered unrecognizable both in terms of size and its dependence on Ca^{2+} and phospholipid. This form of the enzyme may then regulate a second set of biochemical events. In support of this latter hypothesis, polyclonal antibodies to protein kinase C have recently been generated that recognize a cross-reacting protein of M_r 67,000 that has been suggested to be a Ca^{2+}- and phospholipid-independent protein kinase (35, 36). Understanding the regulation of S6 kinase activity will require additional experimentation and should provide information concerning the biochemical events involved in regulating cell growth and metabolism.

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