Received 4/1/85; revised 8/7/85; accepted 3/3/86.

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2 The abbreviations used are: PE, phorbol ester; DO, diolein; EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; PK-C, protein kinase C; PMA, phorbol-12-myristate-13-acetate; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; IL-2, interleukin 2; DG, diacylglycerol.

INTRODUCTION

The interaction of tumor-promoting PE with their receptors on EL4 mouse thymoma cells leads to production of large amounts of IL-2 (1, 2). The time course for IL-2 production (1) and the isolation of IL-2 mRNA from PE-treated cells (3, 4) suggest that IL-2 production requires a transcriptional event. mRNA produced in PE-treated EL4 cells can be translated in vitro into biologically active IL-2 (4). PE-stimulated IL-2 production thus appears to require transfer of a signal from the initial PE-receptor interaction to the nucleus to initiate transcription.

Accumulating evidence (5–9) indicates that the PE receptor is probably identical with PK-C, a calcium-dependent kinase which is stimulated by DG and phospholipids (10–12). PK-C has been purified to varying extents by a number of methods, and PE receptor activity is observed to copurify (5–7, 13). PE can bind directly to purified PK-C in a calcium- and phospholipid-dependent manner (7) and can substitute for DG in stimulating PK-C (8). Treatment of intact cells with PE results in redistribution of PK-C from the cytosol to the membrane, presumably resulting in activation of PK-C (14, 15).

We are testing the hypothesis that PK-C substrates are mediators of PE action in EL4 cells. A second line of EL4 cells has PE receptors/PK-C, also redistributed to the membrane in response to PE (14, 15), but does not produce IL-2 in response to PE (2). Thus, these two lines can be compared in an effort to identify those events which may have a role in IL-2 production. In this study we have begun to characterize cytosolic proteins in these two lines which can serve as PK-C substrates in vitro, with the eventual goal of determining which of these phosphoproteins are affected by PE in vivo.

MATERIALS AND METHODS

Materials. PS was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). DO and PMA were purchased from Sigma Chemical Co. (St. Louis, MO). Lipids were stored in chloroform at −20°C; for each experiment they were combined, dried under N2 gas, and sonicated on ice in 20 mM Tris, pH 7.5, using an Ultrasonics sonicator equipped with a microtip (10). 32Pi5ATP, 0.15–1.0 Ci/mmole, was obtained from the Diabetes Research Center Core Laboratory at the University of Virginia, where it was synthesized by the method of Johnson and Walseth (16).

Acrylamide, bisacrylamide, SDS molecular weight standards, and other chemicals for gels were obtained from Bio-Rad Laboratories, Richmond, CA. Ampholines and glycerol were obtained from LKB Laboratories (Rockville, MD). Ultrapure urea was obtained from Schwarz-Mann (Spring Valley, NY).

Cell Culture and Cytosol Preparation. EL4 cells were maintained in suspension culture as described (9). Cytosol was obtained from homogenates of EL4 cells in buffer containing 20 mM Tris, pH 7.5, 0.33 M sucrose, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM benzamidine, and leupeptin, 25 µg/ml, after centrifugation for 45 min at 100,000 × g, and stored at −70°C until use.

In Vitro Phosphorylation. Cytosol was used as the source of PK-C and its potential substrates for in vitro phosphorylation. Reactions were initiated by adding to cytosol a reaction mix containing [32P]ATP (20 µCi for one-dimensional, or 0.1 mCi for two-dimensional gels), 5 mM magnesium acetate, 10 µM ATP, and concentrations of CaCl2, PS, DO, and PMA as indicated. CaCl2 concentrations indicated in the text represent added CaCl2 and not necessarily free Ca2+. Reactions contained 2–5 µg EGTA since cytosol was prepared in buffer containing 0.5 mM EGTA. Cytosol was usually diluted 40–50 times in 20 mM Tris in order to add the same amount of protein (100 µg) in each reaction. Samples were incubated for 3 min at 30°C, and reactions were stopped by addition of 0.5 volume of 3 × Laemmli buffer (6% SDS; 30% glycerol; 15% 2-mercaptoethanol) or 1.5 volume of acetone:NaOH (10:6:6:6, v/v) as indicated.

Gel Electrophoresis. For slab gel electrophoresis, protein (30 µg of each sample) was separated on a 10% acrylamide gel with a 3% stacking gel by established methods (17). PI Gel Electrophoresis was performed under...
RESULTS

Calcium and Phospholipid Dependence of Substrate Phosphorylation. In order to optimize conditions for in vitro phosphorylation of PK-C substrates, the dependence of cytosolic protein phosphorylation on calcium and phospholipid concentrations was determined (Figs. 1 and 2). Cytosol was incubated with 20 \( \mu \)Ci of \([32P]ATP\), magnesium acetate, ATP, and CaCl\(_2\) and/or phospholipids in the concentrations indicated in "Materials and Methods" and electrophoresed on a 10% acrylamide gel. In Fig. 1A, the profile of PK-C substrates, visualized by autoradiography, and their calcium sensitivity are illustrated. In particular, proteins with molecular weights of 18,000–20,000, 36,000, 40,000, 45,000, 53,000, 67,000, 70,000, 84,000, and 92,000 showed calcium-dependent and phospholipid-stimulated phosphorylation. A Mr 60,000 band, which incorporated equal amounts of \( ^{32}P \) in each condition and thus is not a PK-C substrate, served as a benchmark protein. The Mr 27,000 protein which is prominent in this experiment varied in labeling intensity between experiments and may represent a cell cycle variant.

The maximal effects seen at 50 \( \mu \)M CaCl\(_2\) are illustrated in Fig. 1B. Phosphatidylserine (PS) and dioleoyl phosphatidylcholine (DO) each stimulated incorporation of \( ^{32}P \) into PK-C substrates at concentrations of 50 \( \mu \)M CaCl\(_2\). In the presence of 50 \( \mu \)M CaCl\(_2\), both PS and PMA enhanced labeling of the Mr 53,000 protein, taken from the experiment in Fig. 1A. \( ^{32}P \) incorporation was linear from 10–100 \( \mu \)M CaCl\(_2\); addition of phospholipids caused a shift of this curve to the left, in the 10–100 \( \mu \)M CaCl\(_2\) range.

In the presence of 50 \( \mu \)M CaCl\(_2\), both PS and PMA enhanced labeling of the Mr 53,000 protein in a concentration-dependent manner (Fig. 2). In cytosol (○), 10 \( \mu \)M PMA stimulated \( ^{32}P \) incorporation 30% over CaCl\(_2\) plus PS, 10 \( \mu \)g/ml, and PS, 150 \( \mu \)g/ml, produced a 40% stimulation over CaCl\(_2\) alone. PK-C can be partially purified from cytosol by binding cytosol proteins to a PS affinity column in the presence of calcium and then eluting PK-C by adding EGTA (19). We were interested in determining whether any proteins eluted with PK-C by this method (not shown) are PK-C substrates. The Mr 53,000 protein was present in this partially purified PK-C preparation.
and the PS and PMA stimulation of $^{32}$P incorporation essentially paralleled that in cytosol (Fig. 2, A). Maximal stimulation occurred at the same PS and PMA concentrations, with slightly higher stimulation over CaCl$_2$ alone (40% for 10 nM PMA and 60% for PS 150 μg/ml). The other major PK-C substrates with molecular weights of 18,000–20,000, 36,000, 67,000, 70,000, 84,000, and 92,000 were also present in this partially purified PK-C (not shown). Since column fractions were used, samples contained considerably less protein than cytosol, and this is reflected in lower $^{32}$P incorporation.

Substrates for PK-C in EL4 Cytosol from PE-responsive and nonresponsive Cells. In Fig. 3 potential PK-C substrates in cytosol from PE-responsive and -nonresponsive EL4 cells are compared. Bands corresponding to phosphoproteins which were consistently phospholipid stimulated were cut, and $^{32}$P radioactivity was determined. The quantitative results from the experiment in Fig. 3 are given in Table 1. Equal amounts of cytosol protein from PE-responsive or -nonresponsive cells were used, and equal amounts of protein were loaded onto each lane. As illustrated in Fig. 3 and Table 1, a striking difference between responsive and nonresponsive cells is observed in the ability of the $M_r$ 45,000 protein to be phosphorylated in the presence of PS and DO or PS and PMA. The $M_r$ 45,000 protein was a PK-C substrate in 12 of 13 experiments using cytosol from PE-sensitive cells, but in none of 12 experiments using cytosol from PE-insensitive cells. $M_r$ 36,000 and 40,000 proteins were relatively poorer PK-C substrates in PE-insensitive cell cytosol than in sensitive cell cytosol.

Proteins were also analyzed by two-dimensional electrophoresis, which provides pI and enhanced resolution of proteins. Fig. 4 depicts a typical Coomassie blue stained gel, and autoradiograms are shown in Fig. 5. Many of the PK-C substrates shown as bands in Figs. 4A and 3 are shown here (as indicated by arrows): $M_r$ 92,000 (pI 7.0–7.4); 70,000 (pI 6.4); 53,000 (pI 5.4–5.6); 45,000 (pI 6.8); 40,000 (pI 5.8); and 18,000–20,000 (pI 5.0–5.2). The $M_r$ 92,000 protein appears to be multiply phosphorylated, and the $M_r$ 53,000 band appears to be a set of at least three proteins.
The experiment illustrated in Fig. 3 was quantitated by cutting bands and determining \( ^{32}P \) radioactivity. Background \( ^{32}P \) radioactivity (50 cpm) was subtracted from each value.

### Table 1 Substrates for Ca\(^{2+}\) and Ca\(^{2+}\)/phospholipid-dependent kinases in EL4 cytosol

The concentration of PMA which maximally stimulated \(^{32}P\) incorporation into the Mr 53,000 protein was 10 nM (Fig. 2), which is close to the concentrations of PMA which are half maximal for competition with \([3H]phorbol dibutyrate\) binding (10 nM) and for stimulation of IL-2 production (5 nM) in EL4 cells (2). Although the qualitative pattern of phosphoproteins observed was quite reproducible, some variation in the degree of protein phosphorylation was observed between experiments using cytosols from different flasks of cells (not shown). This is not surprising since the cells have not been synchronized, and therefore protein synthesis, processing, and the basal state of phosphorylation may be different. As indicated in Table 1, some substrates were not seen in every experiment. The Mr 18,000—20,000 proteins were particularly variable in their ability to be phosphorylated in a phospholipid-dependent manner.

[Table 1 Substrates for Ca\(^{2+}\) and Ca\(^{2+}\)/phospholipid-dependent kinases in EL4 cytosol]

| Band | \( M_r \) | PE resistant | | PE sensitive | |
|------|-----------|-------------|----------------|----------------|
| A    | (no Ca\(^{2+}\)) | | (Ca\(^{2+}\)) | |
| B    | (Ca\(^{2+}\), PS, DO) | | (Ca\(^{2+}\), PS) | |
| C    | (Ca\(^{2+}\), PS, PMA) | | (Ca\(^{2+}\), PS, PMA) | |
| D    | (no Ca\(^{2+}\)) | | (Ca\(^{2+}\)) | |
| E    | (Ca\(^{2+}\), PS, DO) | | (Ca\(^{2+}\), PS) | |
| F    | (Ca\(^{2+}\), PS, PMA) | | (Ca\(^{2+}\), PS, PMA) | |
| G    | (Ca\(^{2+}\), PS) | | (Ca\(^{2+}\), PS) | |
| H    | (Ca\(^{2+}\), PS, DO) | | (Ca\(^{2+}\), PS) | |
| I    | (Ca\(^{2+}\), PS, PMA) | | (Ca\(^{2+}\), PS, PMA) | |
| J    | (Ca\(^{2+}\), PS) | | (Ca\(^{2+}\), PS) | |
| K    | (Ca\(^{2+}\), PS, DO) | | (Ca\(^{2+}\), PS) | |
| L    | (Ca\(^{2+}\), PS, PMA) | | (Ca\(^{2+}\), PS, PMA) | |
| M    | (Ca\(^{2+}\), PS) | | (Ca\(^{2+}\), PS) | |
| N    | (Ca\(^{2+}\), PS, DO) | | (Ca\(^{2+}\), PS) | |
| O    | (Ca\(^{2+}\), PS, PMA) | | (Ca\(^{2+}\), PS, PMA) | |

* Numbers in parentheses, number of times observed per total number of experiments.

Fig. 4. Coomassie blue-stained proteins in EL4 cytosol. Cytosol (100 μg) was incubated for 3 min at 30°C with 10 μM ATP containing 0.1 mCi of \(^{32}P\)ATP, 5 mM magnesium acetate, CaCl\(_2\) (50 μM), and phospholipids (PS, 96 μg/ml, and DO, 3.2 μg/ml). A sample containing 50 μg of protein was electrophoresed on a 10% slab gel after isoelectric focusing as described in "Materials and Methods."

Fig. 5. Substrates for PK-C in EL4 cytosol. Gels of samples containing CaCl\(_2\) or CaCl\(_2\) and phospholipids as in Fig. 3 were autoradiographed using Kodak XAR-5 film. Arrow at Mr 50,000, pi 6.5 protein indicates benchmark protein; other arrows indicate proteins which exhibited increased phosphorylation in a calcium-dependent, phospholipid-stimulated manner, i.e., PK-C substrates.

have begun to characterize several in vitro PK-C substrates in the cytosol of EL4 cells. Phosphorylation of each of these proteins was calcium dependent in the range of 1–100 μM and stimulated by phospholipids (Fig. 1). High calcium concentrations (500 μM and above) inhibited \(^{32}P\) incorporation and reduced the phospholipid stimulation. High concentrations of calcium have been shown to inhibit PK-C activity (14, 21). Alternatively, calcium-dependent phosphatases activated at high concentrations of calcium may be present. Proteolysis of PK-C to a fully active form, not stimulated by phospholipids,

which has been shown to occur at high calcium concentrations (22), may also be responsible for the loss of phospholipid stimulation.

The ability of PMA to directly stimulate phosphorylation of proteins which showed enhanced phosphorylation with phospholipids is of particular importance. PMA can directly bind to (7) and stimulate PK-C (8). Since a great deal of evidence now supports the identity of PK-C and the PE receptor, the ability of PMA to stimulate phosphorylation of proteins in vitro provides strong evidence that these are PK-C substrates.

Although the qualitative pattern of phosphoproteins observed was quite reproducible, some variation in the degree of protein phosphorylation was observed between experiments using cytosols from different flasks of cells (not shown). This is not surprising since the cells have not been synchronized, and therefore protein synthesis, processing, and the basal state of phosphorylation may be different. As indicated in Table 1, some substrates were not seen in every experiment. The Mr 18,000–20,000 proteins were particularly variable in their ability to be phosphorylated in a phospholipid-dependent manner. Proteins with molecular weights of 25,000 and 32,000 which were not phosphorylated in the experiment of Fig. 3 were also PK-C substrates but were highly variable.
Another important finding of these studies is the substantial copurification of PK-C and several of its substrates. Copurification of substrates has been shown to occur with several enzymes (23, 24). The copurification of PK-C and its substrates by passing cytosol over a PS affinity column is particularly interesting. In this case substrates in the purified preparation may be tightly bound to PK-C during the PS column binding and elution, or they may bind to the PS column, like PK-C, in a calcium-dependent manner. Similar calcium-dependent binding of PK-C and its substrates to membranes in vivo would have important implications for the interaction of PK-C and intracellular calcium. Recently, several studies have implicated a synergistic relationship between effects of PK-C and free intracellular calcium. PE can replace DG, generated during the PI response to many hormones and neurotransmitters, in stimulating PK-C. DG or PE acts synergistically with a rise in free Ca$^{2+}$ to raise glycogen phosphorylase activity in rats (25), evoke secretion from platelets (26), stimulate insulin release from pancreatic islets (27), and stimulate mitogenesis in bovine lymphocytes (28). One possible mechanism for this synergism may be that, in addition to promoting the binding of PK-C to membranes, calcium enhances or stabilizes the binding of one or more of the PK-C substrates rendering them readily accessible to the kinase. It will be interesting to determine whether the subcellular location of any PK-C substrates is affected by treatment of intact EL4 cells with PE.

The finding of differences in the ability of at least three proteins to serve as PK-C substrates in PE-sensitive and -insensitive cell cytosol is particularly exciting. The $M_r$ 45,000 protein, and probably also the $M_r$ 36,000 and 40,000 proteins, may represent critical links in the response of EL4 cells to phorbol esters. If one of these proteins is a mediator for the PE/PK-C-stimulated initiation of IL-2 production, deficient phosphorylation or absence of the protein in PE-resistant cells may be a mechanism for the defect in these cells. Our ultimate goal is to identify these proteins. Several PK-C substrates which have already been identified in other systems include: myelin basic protein (29); eukaryotic initiation factor (30); receptors for insulin, somatomedin C, and epidermal growth factor (31, 32); histone proteins (33); retinoid binding proteins (34); vinculin (35); and glycogen synthase (36, 37). Also, several investigators have begun to characterize proteins, as yet unidentified, which serve as PK-C substrates both in vivo and in vitro (38–43). Progress toward identification of some of these proteins will be made as antibodies to suspected candidates become available. Some possible candidates for the substrates we have described here include phosphorylase b ($M_r$ 97,400) and retinoic acid binding proteins ($M_r$ 18,000–20,000). These studies are being extended to intact EL4 cells.

ACKNOWLEDGMENTS

We would like to express our appreciation to Dr. James C. Garrison for his contributions and many helpful discussions and to Hannah Anderson for her help in maintaining cultures of EL4 cells.

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Substrates for Protein Kinase C in Cytosol of EL4 Mouse Thymoma Cells

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