Time-dependent Changes in Protein Kinase C Distribution and Disappearance in Phorbol Ester-treated Human Osteosarcoma Cells[1]

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ABSTRACT

To test directly whether protein kinase C activation is one of the required events leading to stimulation of prostaglandin production by bone cells, protein kinase C activity and prostaglandin E_2 release were measured in monolayer cultures of the clonal human osteosarcoma cell lines G-292 and SaOS-2 after exposure to phorbol myristate acetate (PMA). Both cell lines have specific receptors for PMA but only G-292 cells respond with increased prostaglandin E_2 production (M. A. Shupnik and A. H. Tashjian, Jr., J. Biol. Chem., 257: 12161–12164, 1982). The subcellular distribution of protein kinase C in both unstimulated osteosarcoma cell lines was similar; in an EDTA- and leupeptin-containing homogenization buffer, between 70 and 80% of the total enzyme activity was cytosolic. Short (<60 min) incubations with PMA induced marked decreases in cytosolic enzyme activity and parallel increases in particulate protein kinase C; thereafter, total measured cellular protein kinase C activity declined, mediated by decreases in both cytosolic and particulate protein kinase C specific activities. By 24 h cytosolic, particulate, and total protein kinase C activities were less than 10% of basal. Because the protein kinase C responses in both cell types were essentially the same, but only G-292 cells give a prostaglandin response to PMA, we conclude that protein kinase C activation by PMA is itself insufficient to stimulate prostaglandin E_2 production and that the lack of a prostaglandin response in SaOS-2 cells cannot be explained by lack of protein kinase C activation.

INTRODUCTION

The phospholipid- and Ca^{2+}-dependent protein kinase C has been implicated as a pivotal regulatory element in signal transduction, cellular regulation, and tumor promotion (1). Activation of protein kinase C requires the presence of low concentrations of calcium (approximately 100 nM), phospholipid (typically phosphatidylserine), and unsaturated 1,2-diacylglycerol, which is formed at the inner leaflet of the plasma membrane as a product of polyphosphatidylinositol hydrolysis (2). Protein kinase C has been localized to both the cytosol and membrane fractions in cells; its basal distribution between the two compartments varies from one cell type to another (3–5) and is also a function of the extraction procedure used for its measurement (6, 7). Translocation of the enzyme from the cytosol to the particulate fraction was first described by Kraft and Anderson (8) in parietal yolk sac cells treated with the phorbol ester, PMA,[4] and has since been demonstrated in many other cells using PMA as well as physiological regulators (9–12).

In bone in organ culture, both PMA (13) and EGF (14),

stimulate the synthesis of PGE_2 which acts to increase bone resorption. In turn, bone resorption is an important physiological process in plasma calcium homeostasis. The mechanisms by which PMA and EGF enhance PGE_2 production in bone have not been elucidated, although it is now recognized that the first step is the interaction of PMA or EGF with ligand-specific, high affinity-binding sites in or on the cells (15). Our previous studies demonstrated that EGF binding to SaOS-2 and G-292 cells was decreased by pretreatment with PMA, and we suggested that this action of PMA was mediated via activation of protein kinase C (15). On the other hand, we had no direct evidence for protein kinase C activation in these cells. Therefore, in order to determine directly whether activation of protein kinase C occurred in response to PMA, we have measured the enzymatic activity of this enzyme in these two clonal human osteosarcoma cell lines, and report here that PMA induces a rapid redistribution of protein kinase C from the cytosol to particulate fraction of the cells, followed by a progressive loss of total measured enzymatic activity. From these results we conclude that the lack of PGE_2 response in SaOS-2 cells treated with PMA is not due to the absence of a responsive form of protein kinase C, and that activation of protein kinase C is not sufficient for stimulation of PGE_2 synthesis in these cells.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (1000–3000 Ci/mmol) and [20-^{3}H]phorbol-12-myristate-13-acetate (10 Ci/mmol) were obtained from New England Nuclear (Boston, MA). ATP, histone HIII-S, 1,2-diolein, phosphatidylserine, dithiothreitol, leupeptin, Triton X-100, and D-glucosamine were from Sigma Chemical Co. (St. Louis, MO). DEAE-52-cellulose was from Whatman. PMA was purchased from LC Services (Woburn, MA); murine EGF was from Collaborative Research (Waltham, MA). Vasoactive intestinal peptide was obtained from Bachem, Inc. (Torrance, CA), and bovine parathyroid hormone was purchased from Wilson Laboratories (Chicago, IL). Ovalbumin was an ICN Pharmaceuticals' product (Cleveland, OH).

Cell Culture. *Mycoplasma*-free cells were cultured in 100-mm Falcon plastic dishes and used at, or near, confluence. Medium was always changed 24 h prior to beginning an experiment. The clonal line SaOS-2 was obtained from Dr. J. Fogh of the Sloan-Kettering Memorial Cancer Center and was maintained in minimal essential medium containing 10% fetal bovine and 5% horse serum. G-292 cells (clone A141B1) from the American Type Culture Collection (Rockville, MD) were grown in Ham's F10 medium supplemented with 15% horse and 2.5% fetal bovine serum in a humidified atmosphere of 5% CO_2:95% air.

Partial Purification of Protein Kinase C. Cells were washed twice with phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) and were detached from the dishes into this buffer by gentle scraping with a rubber policeman. They were pelleted by centrifugation (2 min at 250 × g) and disrupted in 400 μl of column buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 2 mM dithiothreitol) containing 100 μg/ml leupeptin by repeated aspiration through a 25-gauge needle attached to a 1-ml plastic syringe. For direct determination of total protein kinase C activity referred to as "measured total protein kinase C," cells were broken in homogenization medium containing 1% Triton X-100. Otherwise, homogenates were sedimented at 12,000 × g for 10 min in a Beckman

microcentrifuge to yield cytosol and particulate fractions. The pellet was taken up in column buffer containing 1% Triton X-100, and, like the homogenate preparation, sonicated for 15 s in a bath ultrasonicator, and incubated for 1 h on ice. The supernatant resulting from a centrifugation at 12,000 × g for 10 min at 4°C contained detergent-solubilized protein kinase C. This homogenate and particulate preparation as well as the original cytosol fraction of the enzyme were separated from inhibitors and/or phosphatase activity by DEAE-cellulose chromatography (8, 16–18). Samples were loaded onto individual columns (500-μl capacity) preequilibrated with column buffer. Following three washes, each with 1 ml of column buffer, protein kinase C was eluted into one fraction of 1.4 ml with column buffer containing 80 mM NaCl. These conditions ensured maximal recovery of protein kinase C from both control and PMA-treated cells. The kinase was stored at 4°C and generally was assayed within 24 h of its preparation.

Protein Kinase C Activity. Protein kinase C activity was assayed by measuring the incorporation of ^{32}P from $[\gamma$-^{32}P]ATP into HIII-S histone under conditions where histone phosphorylation was proportional to enzyme concentration, by modification of a described procedure (19). The standard assay mixture (250 μl) contained 20 mM Tris buffer, pH 7.5, 100 μg of histone, 1.5 mM CaCl$_2$, 5 mM magnesium acetate, 2.5 nmol $[\gamma$-^{32}P]ATP (3–4 × 10^5 cpm/nmol), 25 μg phosphatidylserine, and 2.5 μg 1,2-diolein. The lipid suspension (50 μl/assay) was prepared just prior to use by evaporating aliquots to dryness under nitrogen (the stocks of phosphatidylserine and 1,2-diolein were stored in 95:5 chloroform:methanol and 100% chloroform, respectively) and sonicating them into suspension in 20 mM Tris-HCl, 6 mM magnesium acetate for 30 s at room temperature. The reaction was started by the addition of sample (5–25 μg protein in a volume contributing 200 nmol EDTA) and was terminated after 10 min at 30°C by the addition of 0.3 ml ice-cold 10% trichloroacetic acid. The precipitate was collected on Millipore HAWP filters (0.45 μm) by vacuum filtration (4 washes, each of 2 ml with 10% trichloroacetic acid containing 1 mM EDTA and 10 mM sodium phosphate), dissolved in Aquasol scintillation fluor, and radioactivity was counted (6). Protein kinase C is reported as ^{32}P incorporation in the presence of 1.5 mM added Ca^{2+}, phosphatidylserine, and diolein, minus that measured in the presence of 0.8 mM added EDTA, 1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, and phosphatidylserine. Kinase activity in the presence of chelators was less than 10%, the value found in the presence of phosphatidylserine, diolein, and Ca^{2+}, and was barely higher (by 5%), than the assay blank in which column buffer replaced enzyme-containing samples. All values represent the mean from at least two separate culture dishes with an average difference between duplicates of <±5%. Protein was measured on duplicate samples by the Bradford assay using ovalbumin as standard (20).

Prostaglandin E$_2$ measurements. The PGE$_2$ produced by the cells and released into the culture medium was measured by radioimmunoassay using anti-PGE$_2$ (13, 15), and was expressed as the mean ng per ml medium from triplicate cultures.

RESULTS

Distribution of Protein Kinase C in SaOS-2 and G-292 Cells. Because protein kinase C activity has not been described previously in human bone cells, the optimal conditions for the measurement of this enzymatic activity in human osteosarcoma cells were established. Protein kinase C activity was markedly enhanced by calcium in the presence of phosphatidylserine and diolein in each cell line in both the cytosol and particulate fractions. Maximal activation was obtained with about 0.5 mM added to Ca^{2+} and no further increase in kinase activity was observed by raising the calcium to 1.5 mM (data not shown). The protein kinase C from SaOS-2 cells had a specific activity at least twice that in G-292 cells or several other non-osteosarcoma cell lines tested. Thus SaOS-2 cells had cytosol and particulate protein kinase C activity levels of, respectively, 116 and 49 pmol ^{32}P incorporated in 10 min per μg protein, com-

pared to 44 and 14 in G-292 cells. The following other cell lines GH$_4$C$_1$ (rat pituitary); HIT (hamster islet transformed); rMTC 44-2 (rat medullary thyroid carcinoma), and HL-60 (human promyelocytes) cells had cytosol and particulate protein kinase C activity levels of 33 and 27, 26 and 15, 14 and 11, and 10 and 6 pmol ^{32}P incorporated in 10 min per μg protein, respectively.

The distribution of protein kinase C activity was a function of the calcium concentration in the homogenization medium; addition of 1 mM CaCl$_2$ favored association of the enzyme with the membrane fraction (data not shown), results which were similar to those described in GH$_4$C$_1$ cells by Fearon and Tashjian (6). As our studies were directed primarily at detecting redistribution of cytosolic protein kinase C activity to the particulate fraction during treatment of intact cells, extracts were always prepared in the presence of 2 mM EDTA. Protein kinase C activity levels did not appear to be modified as a function of cell growth; values were the same in the logarithmic and the stationary phases of growth; nor was the kinase activity altered when cell growth (assessed by cell protein) was reduced by 50% in the course of a 6-day incubation with 5 mM D-glucosamine (data not shown), an agent which is known to exert a noncytotoxic growth inhibitory effect in human malignant epithelial cells (21).

PMA-induced Changes in Protein Kinase C Activity and Distribution. A brief exposure of intact cells to PMA (less than 60 min) led to a rapid decrease in cytosolic protein kinase C activity which was accompanied by an equally rapid increase in activity in the particulate fraction; total protein kinase C activity remained unchanged during this 1-h period (Fig. 1). With more prolonged incubation, the cytosolic levels declined further but were no longer compensated by an increase in particulate pro-
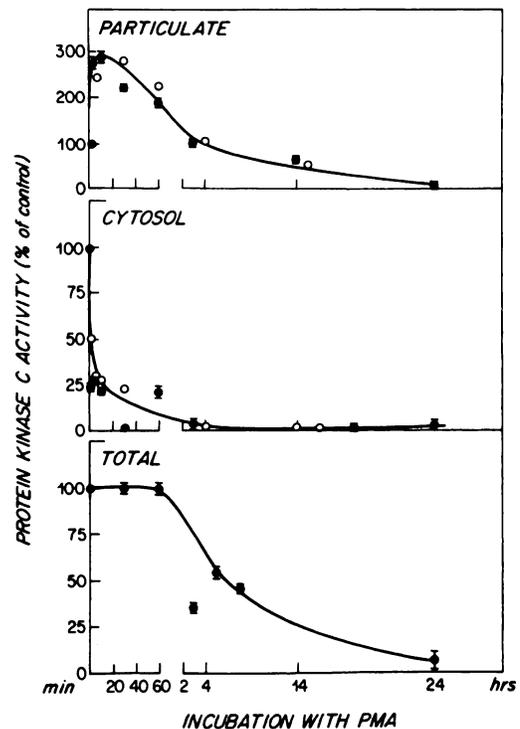

Fig. 1. PMA-induced redistribution of protein kinase C activity in SaOS-2 cells. Experiments were initiated by replacing the culture medium by fresh preequilibrated medium lacking or containing PMA (160 nM) at zero time. Protein kinase C activity is given as percentage of control; means from triplicate cultures (●); bars, SE. Total refers to measurements on solubilized cellular homogenates. O, values obtained in separate experiments from single culture dishes.

tein kinase C activity. By 24 h of incubation with PMA, protein kinase C activity in the homogenate (total measured activity), cytosol, and particulate fractions in SaOS-2 cells were each less than 10% of control values. Essentially the same results as those shown in Fig. 1 were obtained when protein kinase C was expressed as the sum of the activities measured in cytosol and particulate fractions. Thus, after a 60-min treatment with PMA, protein kinase C activities per μg SaOS-2 cell protein were 18, 191, and 100% of control for the cytosol, particulate, and total measured lysate fractions, respectively, and 12, 173, and 93% of control ^{32}P incorporated in 10 min per total cell extract. The corresponding percentage of control values for 24 h-treated cells were 4, 27, and 11, compared to 11, 32, and 17, respectively, for the total summed subcellular fractions measured as ^{32}P incorporated in 10 min per total cell extract. Total measured lysate activity was generally 20–25% less than the sum of the activities measured in the cytosol plus particulate fractions in both control and PMA-treated cells. When G-292 cells were treated with PMA their cytosol and particulate protein kinase C activities were, respectively, 30 ± 11 and 587 ± 75% of control after 30 min, and 7 ± 5 and 34 ± 8% of control after 24 h (means of duplicate cultures ± range). Prostaglandin release over the 24-h exposure to PMA was negligible (<0.2 ng/ml) in SaOS-2 cells, and was 2.1 and 8.5 ng/ml in control and treated G-292 cells, respectively.

Actions of Several Physiological Ligands on Protein Kinase C Activity. SaOS-2 cells also have functional receptors for EGF, parathyroid hormone, and vasoactive intestinal peptide (22). Concentrations of these ligands which are at least as great as those known to act biochemically on these cells and to stimulate bone resorption in mouse calvaria *in vitro* (23), *i.e.*, 8.3 nM, 26 nM (24), and 100 nM (23), respectively, did not induce protein kinase C translocation at times (15 s to 60 min) when PMA was highly effective. We conclude that rapid protein kinase C activation does not occur in SaOS-2 cells in response to EGF, parathyroid hormone, or vasoactive intestinal peptide.

DISCUSSION

PMA (160 nM), which evokes enhanced production of PGE_2 in G-292 but not in SaOS-2 cells, induced similar changes in protein kinase C distribution and activity in both human osteosarcoma cell lines.

The unstimulated forms of cytosol and particulate protein kinase C in both cell types had similar Ca^{2+}, phospholipid, and diolein requirement characteristics, which are typical of the calcium-phospholipid protein kinase activities described in other cells (2, 6, 11, 16). The basal subcellular distribution of the enzyme was also similar, and comparable to that found in the other cell lines tested which were homogenized in the presence of chelating agents to reduce the loose association of the cytosolic form of the enzyme with membranes. In the basal state, the mean summed total activity values (mean ± SE) in SaOS-2 cells were 5.1 ± 0.22 μmol ^{32}P/10 min/total protein for the cytosolic activity and 2.2 ± 0.34 units for the particulate fraction; the summed protein kinase C activity thus obtained was 7.3 μmol ^{32}P/10 min/total protein. The direct measurement of total protein kinase C activity in homogenate preparations yielded a somewhat lower value for total measured activity of 5.8 ± 0.7 μmol ^{32}P/10 min/total protein. By choosing the sum of the activities measured in the cytosol plus particulate fractions as total activity rather than the underestimated total measured activity, we conclude that at least 70% of basal total protein kinase C activity is in the cytosolic compartment in both SaOS-2 and G-292 cells. The lower recovery of total

measured protein kinase C activity in homogenates may reflect incomplete solubilization, the presence of a protein kinase C inhibitor in the homogenate [as described in brain cytosol (25)], or the presence of protease which, despite addition of leupeptin, converts protein kinase C to a form which is no longer Ca^{2+} and phospholipid dependent and which is, therefore, not measured by our procedure for determining protein kinase C activity (see below).

PMA induced two distinct phases of change in protein kinase C activity and subcellular distribution in osteosarcoma cells. First, the initial rapid loss (within 10 minutes) in the activity of the cytosolic component with no change in total protein kinase C activity can most readily be explained by quantitative intracellular translocation from the cytosolic to the particulate fraction of the cell, since total protein kinase C activity was essentially conserved. This rapid phase of protein kinase redistribution has been reported in many cells (5, 8, 10, 26). The second stage we describe involved a gradual decline in total measured and calculated enzyme activity, with an estimated half-life of loss of 6–7 h. This result is similar to the disappearance rate of protein kinase C activity reported in phorbol-12,13-dibutyrate-treated 3T3 cells (27), but is higher than the 40% decrease in protein kinase C activity described for GH$_3$ cells exposed for 24 h to 400 nM PMA (28). Prolonged exposure to phorbol ester has also been found to cause disappearance of protein kinase C activity in 3T3-L1 (29) and mouse epidermal HEL 37 cells (30).

In a series of experiments designed to examine the reversibility of the PMA-induced loss of total protein kinase C activity, we treated cells for 24 h with either 160 or 20 nM PMA, then washed the cultures extensively, and incubated the cells for an additional 24 to 72 h in medium lacking PMA. Little (<40%) or no recovery of protein kinase C activity was observed by the end of the second incubation. To determine the effectiveness of the washing procedure, we incubated cells with [^{3}H]PMA (160 nM for 24 h) and then measured the residual cell-associated radioactivity before and after the wash procedure. We found that 91 ± 4% of the cell-associated radioactivity was removed by washing. However, the 9% residual PMA content of the cells could still have been sufficient to prevent recovery of protein kinase C activity during the second incubation.

The mechanism by which the tumor promoter causes changes in protein kinase C subcellular distribution and loss of total activity is not clear. Results obtained in GH$_3$ cells using a polyclonal antibody to immunoprecipitate protein kinase C indicated that PMA induces translocation of the enzyme protein to the membrane, followed by an accelerated rate of degradation of the membrane-associated protein kinase (28). To date, a rat brain Ca^{2+}-dependent neutral protease (7) and a human neutrophil neutral proteinase (31) have been shown to catalyze the limited proteolysis of protein kinase C to a phospholipid- and Ca^{2+}-independent M_r 50,000 protein (protein kinase M). The latter kinase elutes from DEAE-cellulose at a higher salt concentration than protein kinase C (32), and would not be measured by using our present experimental protocol. Evidence favoring this mechanism for the observed disappearance of protein kinase C was obtained from studies on human platelets; PMA caused translocation of protein kinase C to the membranes and simultaneously increased activity of a Ca^{2+}, phospholipid-independent activity in both the soluble and particulate fraction of the cell (33). Additional experiments are required to test whether this explanation for the loss of protein kinase C on prolonged exposure to PMA also applies to human osteosarcoma cells.

Our primary aim in initiating these studies was to determine whether protein kinase C activation was a prerequisite for PGE₂ production in human osteosarcoma cells. As PMA induces similar changes in protein kinase C subcellular distribution and activity in SaOS-2 and G-292 cells, but only the latter give a PGE₂ response, we conclude that the lack of response in SaOS-2 cells cannot be explained by lack of protein kinase C activation.

ACKNOWLEDGMENTS

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