COMPARATIVE PROPERTIES OF CONTROL AND ACTIVATED ENZYMES*

(Received for publication, January 13, 1992)

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We demonstrated previously tyrosine phosphorylation-dependent modulation of phospholipase C-γ1 (PLC-γ1) catalytic activity (Nishibe, S., Wahl, M. L., Hernandez-Sotomayor, S. M. T., Tonks, N. K., Rhee, S. G., and Carpenter, G. (1990) Science 250, 1253–1256). The increase in PLC-γ1 catalytic activity in A-431 cells occurs rapidly, with maximal activation 5 min after epidermal growth factor (EGF) stimulation. Certain other growth factors (fibroblast growth factor, platelet-derived growth factor) also stimulate PLC-γ1 catalytic activity, whereas insulin does not. A similar increase in PLC-γ1 specific activity (2–3-fold) was observed in both soluble (cytosol) and particulate (membrane) preparations from EGF-treated cells. Tyrosine-phosphorylated PLC-γ1 was detected in both cytosol and membrane fractions in lysates from EGF-treated A-431 cells, but the proportion of tyrosine-phosphorylated PLC-γ1 was higher in the cytosol (~60%) than in the membrane (~20%). Because a micellar concentration of the non-ionic detergent Triton X-100 allows detection of the tyrosine phosphorylation-dependent increase in PLC-γ1 catalytic activity in this assay, we evaluated the kinetic properties of PLC-γ1, immunoprecipitated from cytosol of control or EGF-treated cells, using substrate, phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P2), solubilized in Triton X-100 at various molar ratios. The behavior of the control enzyme differed from the EGF-activated enzyme with respect to both K_m and V_max. The control enzyme has a 7.5-fold higher K_m value than the activated enzyme (1.5 mM as compared with 0.22 mM). Activation by EGF is also a positive allosteric modifier of PLC-γ1-catalyzed PtdIns 4,5-P2 hydrolysis, i.e., the activated enzyme displayed apparent Michaelis-Menten kinetics, with a K_m of 0.6 mol fraction PtdIns 4,5-P2, whereas the control enzyme displayed sigmoidal kinetics with respect to PtdIns 4,5-P2 hydrolysis. At low substrate mol fractions (e.g. 0.07), the reaction velocity of the control enzyme was 4-fold lower than the activated enzyme. However, at a high substrate mol fraction (e.g. 0.33), the estimated maximal reaction velocities (V_max) for both forms of PLC-γ1 were equivalent. PLC-γ1 activity from both control and EGF-treated cells was stimulated by increasing nanomolar Ca^{2+} concentrations. Although the catalytic activity of PLC-γ1 from EGF-treated cells was greater than control PLC-γ1 at every Ca^{2+} concentration tested, the relative stimulation of activity was markedly greater at Ca^{2+} concentrations above ~300 nM.

EGF is a potent modulator of cell growth and other physiological functions (for review see Ref. 1). The EGF receptor is a cell surface glycoprotein with intrinsic, ligand-dependent tyrosine kinase activity (2). The capacity of this receptor to mediate cellular responses to EGF requires functional tyrosine kinase activity (for review see Refs. 1–3), suggesting that tyrosine phosphorylation of the receptor and/or exogenous substrates is crucial for signal transduction. A few cellular substrates for the tyrosine kinase activity of the EGF receptor have been identified and characterized in the attempt to define biochemical pathways that transduce the mitogenic signal. These substrate proteins include GTPase-activating protein, a regulator of ras GTPase activity (4, 5); phospholipase C-γ1 (PLC-γ1), a 145-kDa phospholipase C isozyme (for review see Ref. 6); and phosphatidylinositol 5-kinase (7).

PLC is the rate-limiting enzyme for phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P2) hydrolysis which generates the second messenger molecules inositol 1,4,5-trisphosphate (Ins 1,4,5-P3) and diacylglycerol. A family of PLC isoenzymes (for review see Ref. 8) mediates the generation of these second messenger molecules in response to hormones; however, the biochemical mechanisms of activation of specific PLC isoenzymes are not understood. Tyrosine phosphorylation of PLC-γ1 is believed to be involved in the mechanism by which tyrosine kinase-dependent growth factors stimulate PtdIns 4,5-P2 hydrolysis. Several groups have shown that the addition of EGF or platelet-derived growth factor (PDGF) to cells leads to rapid increases in the amount of phosphotyrosine and phosphoserine on PLC-γ1 (9–13). Transfection of PLC-γ1 cDNA into NIH/3T3 cells results in overexpression of PLC-γ1.

* This research was supported in part by Grant CA43720 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; aFGF, acidic fibroblast growth factor; PLC, phosphoinositide-specific phospholipase C; PtdIns 4,5-P2, phosphatidylinositol 4,5-bisphosphate; Ins 1,4,5-P3, inositol 1,4,5-trisphosphate; DMEM, Dulbecco's modified Eagle's medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethyleneglycol-bis(2-aminoethyl ether)-N,N'-tetraacetic acid.
γ1 protein and enhancement of PDGF-induced Ins 1,4,5-P3 generation (14). Multiple sites of tyrosine phosphorylation on PLC-γ1 have been identified for both in vitro (15) and in vivo (16) phosphorylations. The sites of growth factor-stimulated serine phosphorylation have not yet been reported. Other PLC isoenzymes, β-1 and δ, are not substrates for the EGF receptor tyrosine kinase (11, 17). Although many experiments suggested that cellular PLC activity increases after growth factor treatment of cells, only recently has evidence been produced to demonstrate that tyrosine phosphorylation per se affects PLC-γ1 catalytic activity (18, 32, 33).

We reported increased catalytic activity of PLC-γ1 in immunoprecipitates recovered from EGF-treated A-431 cells and demonstrated that in vitro tyrosine phosphorylation of PLC-γ1 by purified EGF receptor activates and dephosphorylation by phosphotyrosine-specific protein phosphatase deactivates the enzyme (18). We now demonstrate stimulation of PLC-γ1 catalytic activity by EGF, PDGF, and acidic fibroblast growth factor (aFGF); characterize the subcellular localization and tyrosine phosphorylation of activated PLC-γ1 in 293 cell lysates; and, in a PtdIns 4,5-P2 Triton X-100 mixed micelle assay, the kinetic parameters of control and activated enzymes.

**EXPERIMENTAL PROCEDURES**

**Materials**—EGF was isolated from mouse submaxillary glands as described previously (19). PDGF (recombinant human PDGF-BB homodimer) and aFGF were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). PDIns 4,5-P2 ammonium salt was obtained from Boehringer Mannheim. PDIns 4,5-P2 was obtained from Du Pont-New England Nuclear. n-Octyl β-D-glucopyranoside (octyl glucoside) and Pansorbin/Staphyloccoccus aureus cells were obtained from Calbiochem. Nitrocellulose was obtained from Schleicher & Schuell. 125I-goat anti-mouse IgG was obtained from ICN Radiochemicals (Irvine, CA). Fetal calf serum and calf serum were products of Gibco. Plastic culture dishes (35, 60, and 100 mm) and the cell scrapers were purchased from Costar (Cambridge, MA). Plastic culture plates (245 × 245 mm) were obtained from USA Scientific (Ocala, FL). Teflon homogenizer was obtained from Wheaton Scientific (Millville, NJ). BCA protein assay reagent was produced by Pierce Chemical Co.

**Culture**—A-431 cells, human foreskin fibroblasts, and NIH/3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% (v/v) fetal calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) fetal calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcultured by plating 106 cells/cm2. DMEM plus 10% (v/v) calf serum and grown for 3-4 days until 80% confluent. Human fibroblast NIH/3T3 fibroblasts were subcul...
micellar concentration of 240 μM). These substrate solutions were sonicated again as above. Aliquots of the sonicated substrate solutions were then used for PLC assay or gel filtration chromatography.

**Gel Filtration Chromatography**—Ptd[3H]Ins 4,5-P2 Triton X-100 stock substrate solutions (30 μl) at 1:2, 1:4, 1:8, or 1:16 molar ratios plus 10 mM ATP (1.5 μl) were added to a column (60 × 0.9 cm) packed with Sephacryl S-300 gel filtration matrix, pre-equilibrated with running buffer (containing 35 mM NaH2PO4, pH 6.8, 70 mM KCl, 0.5 mM EGTA, and 240 μM Triton X-100). The buffer was run at a flow rate of 0.13 ml/min, and 1.0-ml fractions were collected. The elution of protein standards, Triton X-100, and ATP from the column was monitored with a UV absorbance meter (280 nm). The elution of Ptd[3H]Ins 4,5-P2 was monitored by measuring the radioactivity in each fraction of each column. The column was calibrated using blue dextran 2000 (elution volume 15 ml), thyroglobulin (18 ml), ferritin (20.5 ml), catalase (23 ml), aldolase (24 ml), bovine serum albumin (25.5 ml), ovalbumin (27 ml), chymotrypsinogen A (30 ml), ribonuclease A (32 ml), and ATP (37 ml).

**RESULTS**

**Formation of Activated PLC-γ1 in Intact Cells**—Treatment of A-431 cells with EGF yields a detectable increase in the formation of inositol phosphates and the tyrosine phosphorylation of PLC-γ1 within 1 min of stimulation (22, 23). To determine whether the catalytic activity of PLC-γ1 is similarly increased, we evaluated the time course of PLC-γ1 activation in EGF-treated and control A-431 cells, using PLC-γ1 immunoprecipitates of solubilized extracts and the in vitro PLC assay (Fig. 1). When cells were incubated with EGF at 37°C, increased PLC-γ1 catalytic activity was detected within 1.0 min and reached a maximum of 5-fold at 5 min after the addition of EGF. PLC-γ1 activity recovered from EGF-treated cells then decreased significantly (~25%) between 10 and 10 min after stimulation but remained elevated (3.1-fold) for at least 60 min. Activation of PLC-γ1 by the addition of EGF to cells maintained at 4°C was slower than at 37°C but continued to increase for 60 min. At the end of the 4°C time course (60 min), EGF treatment of A-431 cells increased PLC-γ1 activity 4.3-fold, similar to the level of activation achieved with a reaction mixture containing 100 μM Ptd[3H]Ins 4,5-P2, 5 mM octyl glucoside, and 0.80 mM free Ca2+ (18).

**Detection** of phospholipase C activity in intact cells can be extended to other growth factors and cell lines, we evaluated the capacity of several growth factors to influence the in vitro catalytic activity of PLC-γ1 (Table I). NIH/3T3 and human foreskin fibroblasts were stimulated with PDGF, αFGF, EGF, or insulin. After solubilization of cell proteins, PLC-γ1 was immunoprecipitated, and PLC-γ1 activity was assayed. PDGF (BB homodimer) increased PLC-γ1 catalytic activity approximately 3-fold in both cell lines. αFGF activated PLC-γ1 about 2-fold in NIH/3T3 cells but did not detectably activate PLC-γ1 in human fibroblasts. EGF activated PLC-γ1 about 20% in human fibroblasts but did not activate PLC-γ1 in NIH/3T3 cells which have very low numbers of EGF receptors (24). Insulin did not affect PLC-γ1 activity in either cell line. These data support the hypothesis that there is a general but variable, capacity for mitogenic polypeptides to generate an intracellular signal by phosphorylation and activation of PLC-γ1 in different cell types. The different capacities of PDGF, αFGF, and EGF to elicit a detectable response may reflect differences in the phosphorylation of PLC-γ1, perhaps caused by differences in receptor concentration on the cell surface. Insulin, a nonmitogenic polypeptide, is incapable of signaling by this pathway even when the receptor is present at high concentration on the cell surface (24).

**Detergent Requirement for Detection of PLC-γ1 Activation**—Of the many factors that influence the catalytic activity detected in a PLC assay, the detergent for solubilization of the substrate appears to be particularly important to the detection of tyrosine phosphorylation-dependent modulation of PLC-γ1 activity. Although PLC-γ1 activation by tyrosine phosphorylation was not detected in our initial studies, which employed substrate solubilized in either octyl glucoside (16) or deoxycholate (15), we subsequently observed phosphorylation-dependent changes in PLC-γ1 activity when Triton X-100 was used (18), alone or together with octyl glucoside, to prepare the substrate. Fig. 2 demonstrates the critical influence of Triton X-100 concentration in the PLC assay, when the substrate (Ptd[3H]Ins 4,5-P2) is solubilized in the commonly used detergents.

In Fig. 2A, Ptd[3H]Ins 4,5-P2 was solubilized in octyl glucoside, and increasing concentrations of Triton X-100 (0–24 mM) were included in the assay. The final concentration of octyl glucoside in the assay is 5 mM. A substantial and differential influence of Triton X-100 on the activity of PLC-

![Fig. 1](image-url)

**FIG. 1.** Time course of formation of activated PLC-γ1 in EGF-stimulated A-431 cells. A-431 cells (60-mm dish) were treated with (●) or without (○) EGF (300 ng/ml) at 0°C (●) or 37°C (○, ○). After the indicated periods of time, cells were washed rapidly with ice-cold Ca2+-, Mg2+-free phosphate buffered saline and solubilized with 50 μl of homogenization buffer containing 16 mM Triton X-100. PLC-γ1 activity, immunoprecipitated from 100 μg of solubile protein extracts, was measured (average of duplicate assays) in the presence of 200 μM Ptd[3H]Ins 4,5-P2, 5 mM octyl glucoside, and 0.80 mM Triton X-100, and 1.0 μM free Ca2+ (18).

**Table 1**

| Additions    | PLC-γ1 activity (pmol/min/dish) |
|--------------|---------------------------------|
| None         | 28.6                            |
| PDGF (50 ng/ml) | 91.4                          |
| EGF (50 ng/ml)  | 60.2                            |
| Insulin (100 ng/ml) | 26.1                           |
| EGF (50 ng/ml)  | 27.4                            |
was detected. Low concentrations of Triton X-100 (up to 0.24 mM) increased the PLC-γ1 activity from both control and EGF-treated cells. Thus, EGF activation of PLC-γ1 was not detectable in the assay containing this concentration of deoxycholate, a concentration used in previous studies of PLC-γ1 activity (15, 39).

Activated PLC-γ1 in Cytosolic and Membrane Fractions—
Before growth factor stimulation, the majority of PLC-γ1 is present in the soluble (cytosol) fraction of cell lysates, whereas a smaller portion is detected in the particulate (membrane) fraction (26). After EGF stimulation, a redistribution of PLC-γ1 to the membrane fraction has been documented (26). In that study, cells were gently lysed (through a needle) in a homogenization buffer containing divalent cation (Mg²⁺). In the present study, cell lysates were prepared by vigorous homogenization in a buffer containing a chelator (EGTA) and no divalent cation. A-431 cells were treated with or without EGF for 5 min at 37 °C, lysed, and the cytosol and membrane fractions separated by centrifugation. After solubilization of protein in the membrane fraction with Triton X-100, PLC-γ1 was quantitatively immunoprecipitated from the cytosol and membrane fractions with specific monochlonal antibodies (20). The relative PLC-γ1 protein content in the immunoprecipitates was quantified after gel electrophoresis by anti-PLC-γ1 immunoblot analysis, and the PLC-γ1 catalytic activity was quantified by PLC assay.

Fig. 3 demonstrates a comparison of the fractionation of PLC-γ1 from control and EGF-treated cells with regard to the subcellular location of protein and enzyme activity, the relative PLC-γ1 tyrosine phosphorylation in each subcellular fraction, and specific enzyme activity in each fraction. Under these homogenization conditions, approximately one-third of the PLC-γ1 protein was present in the particulate (membrane) fraction, and two-thirds was located in the soluble (cytosol) fraction from both control and EGF-treated cells (Fig. 3A). Assay of PLC activity in PLC-γ1 immunoprecipitates from each fraction revealed a similar distribution: approximately two-thirds of total cell lysate PLC-γ1 activity was in the soluble fraction, and one-third remained in the particulate fraction (Fig. 3B). Although the relative proportion of PLC-γ1 activity in membrane and cytosol fractions was similar for control and EGF-treated cells, the absolute PLC-γ1 activity was 2-3-fold greater in the EGF-treated preparation. When PLC-γ1 activities were normalized for the amount of total protein present in each fraction (soluble or particulate) to yield apparent PLC-γ1 specific activities for each fraction, a 2-3-fold increase in PLC-γ1 specific activity was observed in both the soluble and particulate fractions of lysates obtained from EGF-stimulated cells (Fig. 3C).

Because PLC-γ1 present in both particulate and soluble fractions from cell lysates is activated after EGF treatment, we measured the proportion of PLC-γ1 protein that is phosphorylated on tyrosine in each fraction (Fig. 3D). Cytosol (and solubilized) membrane fractions were adsorbed to excess anti-phosphotyrosine matrix. After recovery of nonadsorbed and specifically adsorbed proteins from the matrix, the relative PLC-γ1 protein content in each sample was quantified, after gel electrophoresis, by anti-PLC-γ1 immunoblot analysis. In unstimulated A-431 cells, a small fraction (2-3%) of PLC-γ1 present in both the soluble and the particulate fractions specifically adsorbed to the anti-phosphotyrosine matrix. In EGF-treated cells, approximately 20% of the particulate PLC-γ1, and 54% of the soluble PLC-γ1 bound to anti-phosphotyrosine. In the soluble fraction, this represents the

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**Fig. 2. Influence of Triton X-100 concentration on PLC-γ1 activity from A-431 cells treated with or without EGF.** A-431 cells treated with (●) or without (○) EGF (300 ng/ml) for 5 min at 37 °C were lysed, and PLC-γ1 was immunoprecipitated from the cytosol fraction as described under "Experimental Procedures." Aliquots of PLC-γ1 immunoprecipitates (from 100 μg of cytosol protein) were added to reaction mixtures containing various concentrations of Triton X-100. Ptd[3H]Ins 4,5-P₂ was resuspended in 50 mM (w/v) octyl glucoside (panel A), 0.80 mM (v/v) Triton X-100 (panel B), or 12.5 mM (w/v) deoxycholate (panel C). The final concentrations of detergent, attributable to the initial substrate preparations, in the reaction mixtures were 5 mM (octyl glucoside), 0.08 mM (Triton X-100) and 2.4 mM (deoxycholate). The Ptd[3H]Ins 4,5-P₂ final concentration was 200 μM and the free Ca²⁺ 1.0 μM. The reaction velocity was measured (average of duplicate assays) and plotted versus the final (additive) Triton X-100 concentration in the reaction mixture.

γ1 immunoprecipitated from EGF-treated and control cells was detected. Low concentrations of Triton X-100 (up to approximately 0.24 mM) increased the PLC-γ1 activity detected in the immunoprecipitates from both EGF-treated and control cells. Higher concentrations of Triton X-100 (0.48–2.4 mM) produced a selective decrease in the PLC-γ1 activity from control cells. Thus, a 2-5-fold increase in PLC-γ1 activity from EGF-treated cells relative to PLC-γ1 activity from control cells is detectable at Triton X-100 concentrations above ~0.24 mM.

Fig. 2B shows the results of a similar assay, performed with substrate solubilized in Triton X-100 (without octyl glucoside). Low concentrations of Triton X-100 (0.08–0.48 mM) decreased the activity of PLC-γ1 from both EGF-treated and control cells. However, at higher concentrations of Triton X-100 (>0.48 mM) a differential influence on enzyme activity was observed again. Thus, a 2-6-fold activation of PLC-γ1 from EGF-treated cells relative to control cells was detectable at higher Triton X-100 concentrations.

In Fig. 2C, Ptd[3H]Ins 4.5-P₂ was solubilized in deoxycholate, the detergent most commonly used for in vitro PLC assays, and PLC-γ1 activity was measured in the presence of increasing concentrations of Triton X-100 (0–2.4 mM). In the presence of 2.4 mM deoxycholate, Triton X-100, at concentrations above 0.16 mM, produced equivalent decreases of PLC-γ1 activity from both control and EGF-treated cells (panel C). Thus, EGF activation of PLC-γ1 was not detectable in the assay containing this concentration of deoxycholate, a concentration used in previous studies of PLC-γ1 activity (15, 39).
fraction of PLC-\(\gamma\)1 molecules containing phosphorytosine. PLC-\(\gamma\)1 in the particulate fraction may adsorb to the anti-phosphotyrosine matrix directly or indirectly as a result of association with the EGF receptor (10). However, we have found very little PLC-\(\gamma\)1 co-precipitating with the EGF receptor in A-431 cells under a variety of isolation conditions (data not shown). If 20% of the PLC-\(\gamma\)1 molecules in the particulate fraction contain phosphoryotosine, it is surprising that the degree of activation is similar to that observed in the cytosol fraction, in which 50% of the PLC-\(\gamma\)1 molecules are tyrosine-phosphorylated. Several possible explanations for this difference exist, including: 1) unrecognized activating factors in the membrane fraction or 2) differences in the phosphorylation of individual tyrosine residues (32).

#### Gel Chromatographic Analysis of Substrate Micelles

Our previous experiments (Fig. 2) indicate that the mode of presentation of the substrate phospholipid to the enzyme has a significant influence on the detection on the tyrosine phosphorylation-dependent modulation of PLC-\(\gamma\)1 activity in vivo. Because the differential influence of Triton X-100 on the activity of control and phosphorylated PLC-\(\gamma\)1 is observed only with Triton X-100 concentrations greater than its nominal critical micelle concentration (0.24 mM), detection of PLC-\(\gamma\)1 activation in vitro may require presentation of PtdIns 4,5-P\(_2\) as a mixed micelle with Triton X-100. We began the evaluation of the mechanistic basis of the apparent PLC-\(\gamma\)1 activation in this assay with gel chromatographic analysis of PtdIns 4,5-P\(_2\)-Triton X-100 micelles followed by kinetic analysis of hydrolysis of the substrate by control and activated PLC-\(\gamma\)1.

PtdIns 4,5-P\(_2\) containing a tracer amount of Ptd\([^{3}H]\)Ins 4,5-P\(_2\) was solubilized in a buffer solution containing 240 \(\mu\)M Triton X-100 (the nominal critical micelle concentration) plus additional Triton X-100 to produce micellar molar fractions (PtdIns 4,5-P\(_2\)/Triton X-100) of molar fractions 0.35, 0.2, 0.11, and 0.07. After sonication to provide a uniform dispersion of PtdIns 4,5-P\(_2\) in Triton X-100 micelles, each solution was applied to Sephadryl S-300 gel filtration matrix. We observed that for all PtdIns 4,5-P\(_2\)-Triton X-100 molar ratios, both Ptd\([^{3}H]\)Ins 4,5-P\(_2\) and Triton X-100 eluted as single peaks with similar elution volumes (data not shown). At each ratio, elution of PtdIns 4,5-P\(_2\) and Triton X-100 occurred at a volume (23-24 ml) similar to that observed for catalase (23 ml, \(M_t\) \(\sim\)232,000) and aldolase (24 ml, \(M_t\) \(\sim\)158,000) and slightly before elution of Triton X-100 (24-25 ml) in the absence of PtdIns 4,5-P\(_2\). Essentially all of the Ptd\([^{3}H]\)Ins 4,5-P\(_2\) was recovered at micellar molecular sizes.

The coelution of PtdIns 4,5-P\(_2\) and Triton X-100 from the gel filtration column suggests that the detergent and phospholipid substrate combine to form a mixed micelle. Alternatively, they may individually form micelles that coincidentally have similar sizes. Because the elution volume does not vary detectably with the molar ratio of substrate to detergent, the size of the presumed mixed micelles appears to be invariant.

#### Kinetic Analysis of PLC-\(\gamma\)1 in a Mixed Micelle Assay

Neither the mechanism of PLC-\(\gamma\)1 hydrolysis of PtdIns 4,5-P\(_2\) nor the role of tyrosine phosphate in PLC-\(\gamma\)1 activation in vivo or in vitro is well understood. Because the presence of Triton X-100 allows a clearly measurable difference between the in vivo activities of control and tyrosine-phosphorylated PLC-\(\gamma\)1, we evaluated and compared the kinetic parameters of control and activated (by treatment of intact cells with EGF) PLC-\(\gamma\)1 using a Ptd\([^{3}H]\)Ins 4,5-P\(_2\)-Triton X-100 mixed micelle assay.

PLC-\(\gamma\)1 was quantitatively immunoprecipitated from the cytosol of untreated and EGF-treated A-431 cells. Western blot analysis confirmed that a similar amount of PLC-\(\gamma\)1 was recovered in the immunoprecipitates regardless of EGF treatment (data not shown). Control experiments verified that the hydrolysis of Ptd\([^{3}H]\)Ins 4,5-P\(_2\) in this assay was linear with time (0-30 min) and protein concentration (10-100 \(\mu\)g) for both control and activated enzyme preparations (data not shown). To evaluate the influence of substrate concentration, the concentration of Ptd\([^{3}H]\)Ins 4,5-P\(_2\) was varied from 100 to 800 \(\mu\)M and the concentration of Triton X-100 from 100 (above the critical micelle concentration) to 12,800 \(\mu\)M. This produced molar ratios of Ptd\([^{3}H]\)Ins 4,5-P\(_2\) to Triton X-100 ranging from 0.33 to 0.07. The concentrations of Ptd\([^{3}H]\)Ins 4,5-P\(_2\) and Triton X-100 were experimentally chosen to allow...
Fig. 4. Kinetic analysis of control and activated PLC-γ1. A-431 cells treated without or with EGF (500 ng/ml) for 5 min at 37°C were lysed, and PLC-γ1 was immunoprecipitated from the cytosol fraction as described under "Experimental Procedures." Aliquots of PLC-γ1 immunoprecipitates (from 50 µg of cytosol protein) were added to reaction mixtures containing Ptd[3H]Ins 4,5-P2 (100, 200, 400, and 800 µM) and micellar Triton X-100 (at substrate mol fractions of 0.33, 0.2, 0.11, 0.07 for each substrate concentration), 1.0 µM free Ca++, and incubated at 35°C for 7.5-30 min. The reaction velocity was measured (average of duplicate assays) and the data plotted in the double reciprocal form, (velocity)$^{-1}$ versus (bulk substrate concentration)$^{-1}$ for each substrate mol fraction, 0.33 (●), 0.20 (○), 0.11 (●), and 0.07 (△) for control (panel A) or activated (panel B) PLC-γ1. The relative velocities and slope values were calculated for each line in panels A and B, and these values (−)EGF (○), (+)EGF (●), were plotted versus the reciprocal of the substrate mol fraction in panels C and D, respectively.

Table II

| Kinetic parameters of PLC-γ1 isolated from control and EGF-stimulated cells |
|---------------------------------------------------------------|
| Constant | Control | EGF-stimulated | Fold change |
| $V_{max}$ (nmol/min/mg) | 5.5 | 5.5 | 1.0 |
| $S_{0.5}$ (mol fraction)$^a$ | 0.95 | 0.6 | 1.6 |
| $K_c$ (mM) | 1.50 | 0.2 | 7.5 |

$^a$ $V_{max}$ for the control enzyme was estimated by extrapolation of the line in Fig. 4C.

$^b$ $S_{0.5}$ is equivalent to the $K_n$ for EGF-activated enzyme.

EGF-activated PLC-γ1, $K_n$ was determined to be 0.6 mol fraction, and $K_c$ was determined to be 0.22 mM. The plot of intercept or slope versus mol fraction for the control enzyme deviated from linearity (Fig. 4, C and D). This deviation from linearity suggested that the kinetics for the control enzyme are more complex than the activated enzyme. Evaluation of kinetic parameters (Table II) for control PLC-γ1 is difficult from the secondary analyses plots because the relationship of slope or intercept versus mol fraction was not linear. At a substrate mol fraction of 0.07, there is a 4-fold difference in reaction velocities of control and activated enzymes. As the mol fraction increases, the lines converge so that at 0.33 substrate mol fraction the reaction velocities are equivalent (Fig. 4C). A Hill plot of the data for the control enzyme allowed the estimation of the substrate concentration where half-maximal activity is observed, or $S_{0.5}$ (0.95 mol fraction PtdIns 4,5-P2; data not shown). $K_c$ for the control enzyme was estimated from the double reciprocal plots in Fig. 4A, with $K_c$ being equivalent to the $-1/x$ value of the point at which all lines converge. $K_c$ was determined to be approximately 1.5 mM.

A double reciprocal plot of the reaction velocity versus mol fraction also deviated from linearity for the control enzyme (Fig. 5A). This behavior suggests that the control enzyme displays apparent allosteric kinetics, whereas the EGF-acti-
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FIG. 5. Kinetic analysis of control and activated PLC-γ1. A431 cells were treated with or without EGF. PLC-γ1 was immunoprecipitated from the cytosol fractions, and enzyme activities were measured as described in Fig. 4. The data were plotted as the double reciprocal of (reaction velocity)‘‘ versus (mol fraction)” at each bulk substrate concentration, 100 μM (●), 200 μM (○), 400 μM (●), 800 μM (▲) for control (panel A) or activated (panel B) PLC-γ1. The inset in panel A shows the data transformed using the Hill equation.

vated enzyme does not (Fig. 5B). A Hill plot of the data in Fig. 5A (inset) revealed that the cooperativity index did not change as the substrate concentration increased, therefore allosterism cannot be overcome by increasing the substrate concentration. Kinetic parameters calculated from Fig. 5B (EGF-activated enzyme) were similar to those calculated from Fig. 4B (data not shown).

Comparison of PLC-γ1 Activity in Membrane and Cytosol Fractions—The data in Fig. 4 were obtained using PLC-γ1 from the cytosol fraction of control and EGF-treated cells. Because a portion (~33%) of PLC-γ1 is present in the membrane fraction and is activated in EGF-treated cells (Fig. 3), we directly compared the activity of PLC-γ1 from membrane and cytosol fractions, using increasing concentrations of Ptd[3H]Ins 4,5-P2 at a 0.33 mol fraction. At this relative substrate concentration, PLC-γ1 from cytosol of control and activated PLC-γ1 had similar relative velocities (Fig. 4). A double reciprocal plot of the reaction velocities versus the substrate bulk concentration is shown in Fig. 6. Extrapolation of the data revealed similar relative velocities (~5.00 nmol/min·mg) values for each enzyme preparation in this assay. These data suggest that in this assay PLC-γ1 activity recovered from membrane fraction performs similarly to PLC-γ1 activity recovered from the cytosol fraction. Extrapolation also revealed that membrane and cytosolic enzyme from control or EGF-treated cells intersected the x axis at a common point.

Stimulation of PLC-γ1 Activity by Ca2+—The catalytic activity of all mammalian PLC isozymes is critically dependent on the concentration of Ca2+ (8). We examined the Ca2+-dependent stimulation of the catalytic activity of PLC-γ1 immunoprecipitated from the cytosol of control and EGF-treated A-431 cells using 200 μM Ptd[3H]Ins 4,5-P2 at a substrate:detergent molar fraction of 0.33. We observed (Fig. 7A) that both control and activated PLC-γ1 are stimulated by Ca2+ concentrations in the nanomolar range. PLC-γ1 from EGF-treated cells demonstrated a maximal activity of ~1.9 nmol/min·mg in this assay, whereas the control enzyme maximal activity was ~0.6 nmol/min·mg. Although PLC-γ1 from EGF-treated cells displays higher catalytic activity at each Ca2+ concentration than the control enzyme, the degree of activation is greater with Ca2+ concentrations higher than ~300 nM. The data in Fig. 7A were reevaluated with regard to the relative stimulation (percent maximal) of activity at each Ca2+ concentration. Fig. 7B demonstrates that the half-maximal stimulation of control enzyme was achieved with a Ca2+ concentration of ~56 nM, whereas activated PLC-γ1 required ~316 nM Ca2+ for half-maximal stimulation. When the experiment was performed with a mol fraction of 0.2, a similar result was obtained (data not shown).
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**Fig. 7.** Ca^{2+} stimulation of control and activated PLC-γ1. A-431 cells treated without (○) or with (●) EGF (600 ng/ml) for 5 min at 37 °C were lysed, and PLC-γ1 was immunoprecipitated from the cytosol fraction as described under “Experimental Procedures.” Aliquots of PLC-γ1 immunoprecipitates (from 50 μg of cytosol protein) were added to reaction mixtures containing Ptd[3H]Ins 4,5-P_2 (200 μM) and micellar Triton X-100 at a substrate:detergent mol fraction of 0.33 and incubated at 35 °C for 15–30 min. The free Ca^{2+} concentration (0.000, 0.032, 0.065, 0.100, 0.210, 0.430, 1.00, or 100 μM) of each assay was adjusted by varying the ratio of Ca^{2+} to EGTA (33). The reaction velocity was measured (average of duplicate assays) and the data plotted as velocity versus log (free Ca^{2+} concentration) in panel A or percent maximal velocity versus log (free Ca^{2+} concentration) in panel B.

**DISCUSSION**

Using a Triton X-100-based PLC assay, we have characterized several parameters of PLC-γ1 activation. The rapid time course of PLC-γ1 activation demonstrated in Fig. 1 is indistinguishable from the time course of EGF-stimulated PLC-γ1 tyrosine phosphorylation documented previously (9). Other growth factors known to induce PLC-γ1 tyrosine phosphorylation likewise rapidly increase PLC-γ1 catalytic activity (Table I). Importantly, these data show that PLC-γ1 catalytic activation is not restricted to EGF-stimulated A-431 cells (a carcinoma that overexpresses the EGF receptor) but occurs in nontransformed cells expressing normal complements of growth factor receptors. The capacity to detect tyrosine phosphorylation-dependent modulation of PLC-γ1 in this assay requires the presence of Triton X-100 at a micellar concentration (Fig. 2) and is compatible with the presence of octyl glycoside but not the ionic detergent deoxycholate, although a variety of deoxycholate concentrations and conditions have not been tested.

Although previous studies have characterized various PLC activities in vitro (34–41), little information is available, particularly in comparison with studies of phospholipase A_2 (27–31), regarding either the mechanism of PLC-mediated hydrolysis of PtdIns 4,5-P_2 or the influence of any hormonally mediated activation steps upon this reaction mechanism. Our analysis attempts to follow the theory and experimental conditions outlined by Dennis and his colleagues in their investigations of the mechanism of phospholipase A_2 catalytic activities (27–30). The primary goals in this study are to evaluate the kinetic parameters of control and activated PLC-γ1 under our experimental conditions and to assess the influence of tyrosine phosphorylation on the catalytic activity of this enzyme. To our knowledge, this is the first example of such a study for a tyrosine kinase substrate (other than the autophosphorylation activity of receptor tyrosine kinases).

The kinetic analysis was performed with PLC-γ1 immunoprecipitated from the soluble fraction of cell lysates. We have termed this PLC-γ pool “cytosolic” as an operational designation. Because a substantial portion of total cellular PLC-γ1 remains in the particulate ("membrane") fraction of cell lysates (Fig. 3, A and B), we compared its activity with the cytosolic PLC-γ1. Although the tyrosine phosphorylation of PLC-γ1 tightly associated with the membrane is significantly less than the tyrosine phosphorylation of soluble PLC-γ1 (Fig. 3D), both fractions have higher activity in EGF-treated than control cells (Fig. 3C). Comparison of PLC-γ1 from control and EGF-treated cell lysates suggests that activated enzyme from cytosolic or membrane preparations performs similarly in the assay and likewise for the control enzyme (Fig. 6).

We performed the kinetic analysis of PLC-γ1 activities to evaluate the basis for the differential rate of hydrolysis of substrate by control and activated PLC-γ1 using a mixed micelle assay system. In this assay system, V_max is equivalent to maximal velocity at infinite mol fraction of PtdIns 4,5-P_2, K_m is the concentration that yields half-maximal velocity, and K_i is equivalent to the dissociation constant for PLC-γ1 binding to the micelle. The reaction velocity of both activated and control PLC-γ1 increased as bulk substrate concentration in the reaction mixture increased from 100 to 800 μM (Fig. 4, A and B). The substrate bulk concentration-dependent increase in catalytic activity was observed at each PtdIns 4,5-P_2 ratio tested (Fig. 4, A and B). Our analysis of the kinetic data included not only double reciprocal analysis (Fig. 4, A and B, and Fig. 5, A and B) but also secondary analysis of the apparent kinetic constants (Fig. 4, C and D). For an ordered reaction the y intercept of Fig. 4C is equivalent to 1/V_max and the slope of the line is equivalent to K_m/V_max with K_m having units of "mol fraction" in this case. The slope of the lines in Fig. 4D is equivalent to K_m/K_iV_max, with K_i representing an affinity constant and having units of "nm." From the experimentally observed values in Fig. 4, C and D, substitution yields a V_max of 5.5 nmol/min/mg, K_i value of 0.22 mm, and K_m value of 0.6 mol fraction for activated PLC-γ1 (Table II). Because the secondary analysis of the control enzyme deviated from linearity, it is difficult to determine the kinetic parameters. Examination of the secondary plot of intercept versus mol fraction (Fig. 4C) revealed that the line for control enzyme approached that of the EGF-activated enzyme. It is possible that the V_max of the control enzyme may be the same as the activated enzyme. Determining the exact V_max for the control enzyme experimentally is difficult because at mol fractions greater than 0.33 the exact nature of the micelle is not known and may no longer be uniform. When the data from Fig. 4A were transformed using the Hill equation, it was possible to determine an S_0.5 for the control enzyme. This parameter was determined to be 0.95 mol fraction of PtdIns 4,5-P_2. K_i was evaluated from the double reciprocal plot (Fig. 4A). It is equivalent to 1/x value of the point at which all lines intersect, this point being 1.5 mM. From our kinetic analysis, EGF stimulation of PLC-γ1 activity resulted in a 7-fold reduction in the K_i (Table II). Kinetic analysis also revealed that EGF stimulation of PLC-γ1 activity had a positive cooperative effect on enzyme association.
with PtdIns 4,5-P_2 mixed micelles.

In this paper we attempted to analyze PLC-γ1 kinetics using the ordered sequential model outlined by Dennis and co-workers for phospholipase A_2 (27-31). Inherent in this model are the assumptions that the transition of the enzyme to the interface of the micelle occurs before the hydrolysis of the substrate and that the binding sites for the micelle and the binding site for the substrate are independent and noncooperative. For the EGF-activated enzyme, the assumptions apparently hold true, but for the control enzyme the curves of reaction velocity versus mol fraction of PtdIns 4,5-P_2 appear sigmoidal in nature. Therefore, that the binding site for the micelle and the binding site for PtdIns 4,5-P_2 are independent and noncooperative is not true. The sigmoidal behavior displayed by the control enzyme could result from a greater sensitivity to Triton inhibition than the activated enzyme. It is also possible that the apparent cooperativity is caused by the binding of PtdIns 4,5-P_2 to a nonsubstrate site on the enzyme or that PLC-γ1 is a multisubunit or multizymal enzyme. The apparent cooperativity resulting from the binding of lipid to a nonsubstrate site on the protein has been seen with many membrane-associated proteins (29, 42, 43), although it is not clear why the activated enzyme does not display some degree of cooperativity. The possibility of PLC-γ1 being a multisubunit or multimembrane protein is intriguing. If this is the case, then not only does tyrosine phosphorylation shift the equilibrium of PLC-γ1 so that it is now more likely to associate with micelles by lowering the K, 7-fold (1.5 to 0.22 mM), but it can act as a positive allosteric modifier of PLC-γ1 activity. The allosteric behavior of PLC-γ1 may explain why no difference in activity caused by tyrosine phosphorylation is seen when pure liposomes containing a high mol fraction of PtdIns 4,5-P_2 are used (15, 33). This hypothesis may also explain why a difference in activity resulting from tyrosine phosphorylation is seen when PLC-γ1 activity is assayed in the presence of profilin (33). Profilin binding to PtdIns 4,5-P_2 effectively lowers the PtdIns 4,5-P_2 concentration in the liposome (48). At this effectively lowered PtdIns 4,5-P_2 concentration the control enzyme cannot bind PtdIns 4,5-P_2 as readily as the activated enzyme and therefore displays an overall lower rate of PtdIns 4,5-P_2 hydrolysis.

A major consequence of hormone-stimulated PLC activity is an increase in the intracellular free Ca\(^{2+}\) level. Whereas Ca\(^{2+}\) affects the activities of many cellular proteins, it is also an important regulator of PLC activities. We demonstrated previously that EGF stimulation of PLC activity in A-431 cells is biphasic (23). The initial phase is brief, correlates with induction of PLC-γ1 tyrosine phosphorylation, and occurs in the absence of extracellular Ca\(^{2+}\). The second phase of EGF-stimulated inositol phosphate formation is prolonged and, despite high levels of PLC-γ1 tyrosine phosphorylation, occurs only in the presence of extracellular Ca\(^{2+}\). Other research has documented a biphasic rise in intracellular Ca\(^{2+}\) concentration in EGF-treated cells which depended largely, but not entirely, on the presence of extracellular Ca\(^{2+}\) (44, 46-48). The precise relationships between Ca\(^{2+}\) mobilization and regulation of PLC-γ1 activity in EGF-stimulated A-431 cells remain to be clarified. We investigated the influence of Ca\(^{2+}\) concentrations on PLC-γ1 activities in the mixed micelle assay. The catalytic activity of PLC-γ1 from EGF-treated cells was greater than control PLC-γ1 activity at each Ca\(^{2+}\) concentration tested. However, at the higher Ca\(^{2+}\) concentrations (above \(~300\) nM) the relative stimulation of activated PLC-γ1 was markedly enhanced. It is possible that this result reflects catalytic activation of a portion of the PLC-γ1 protein molecules which requires a high Ca\(^{2+}\) concentration. Alternatively, at the higher Ca\(^{2+}\) concentrations, activated PLC-γ1, but not control PLC-γ1, may bind with higher affinity to the substrate mixed micelles. This interpretation would be consistent with our interpretation of kinetic data in Fig. 4 that Triton X-100 does not affect activated PLC-γ1 association with mixed micelles. (Note that the kinetic analysis was performed with a Ca\(^{2+}\) concentration of \(~1.0\) μM.) Finally, the in vitro Ca\(^{2+}\) stimulation of activated PLC-γ1 is consistent with our previous observations that extracellular Ca\(^{2+}\) is required to maintain the second phase of EGF stimulation of PLC activity in A-431 cells.

Acknowledgments—We thank Deirdre Sanchez for technical assistance and Sue Carpenter for preparation of the manuscript and figures.

REFERENCES

1. Carpenter, G., and Wahl, M. I. (1990) in Handbook of Experimental Pharmacology (Sporn, M. B., and Roberts, A. B., eds) Vol. 95, pp. 69-171, Springer-Verlag, Heidelberg
2. Carpenter, G., and Cohen, S. (1990) J. Biol. Chem. 265, 7709-7712
3. Ullrich, A., and Schlessinger, J. (1990) Cell 61, 203-212
4. Molloy, C. J., Bettaro, T. P., Fleming, T. P., Marshall, M. S., Gibbs, J. B., and Aaronson, S. A. (1989) Nature 342, 711-714
5. Ellis, C., Moran, M., McCormick, F., and Pawson, T. (1990) Nature 348, 377-381
6. Wahl, M. I., Nishibe, S., and Carpenter, G. (1989) Cancer Cells 1, 101-107
7. Bjoere, J. D., Chen, T.-O., Antczak, M., Kung, H.-J., and Fujita, D. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3816-3820
8. Rhee, S. G., Suh, P.-G., Ryu, S.-H., and Lee, S. Y. (1988) Science 244, 546-550
9. Wahl, M. I., Nishibe, S., Suh, P.-G., Rhee, S. G., and Carpenter, G. (1988) Proc. Natl. Acad. Sci. U. S. A. 86, 1565-1572
10. Margolis, B., Rhee, S. G., Felder, S., Mervic, S., Lyon, R., Levitzki, A., Ulrich, A., Zilberstein, A., and Schlessinger, J. (1989) Cell 57, 1101-1107
11. Meisenhelter, J., Suh, P.-G., Rhee, S. G., and Hunter, T. (1989) Cell 67, 1109-1122
12. Wahl, M. I., Olashaw, N. E., Nishibe, S., Rhee, S. G., Pledger, W. J., and Carpenter, G. (1989) Mol. Cell. Biol. 9, 2934-2943
13. Morrison, D. K., Kaplan, D. R., Rhee, S. G., and Williams, L. T. (1990) Mol. Cell. Biol. 10, 2399-2406
14. Mochizuki, B., Zilberstein, A., Franks, C., Felder, S., Kremer, S., Ulrich, A., Rhee, S. G., Skorecki, J., and Schlessinger, J. (1990) Science 248, 607-610
15. Kim, J. W., Sim, S. S., Kim, U-H, Nishibe, S., Wahl, M. I., Carpenter, G., and Rhee, S. G. (1990) J. Biol. Chem. 265, 3940-3943
16. Wahl, M. I., Nishibe, S., Kim, J. W., Kim, H., Rhee, S. G., and Carpenter, G. (1989) J. Biol. Chem. 264, 3944-3948
17. Nishibe, S., Wahl, M. I., Rhee, S. G., and Carpenter, G. (1989) J. Biol. Chem. 264, 10355-10368
18. Nishibe, S., Wahl, M. I., Hernandez-Sotomayor, S. M. T., Tonks, N. K., Rhee, S. G., and Carpenter, G. (1990) Science 250, 1253-1256
19. Savage, C. R., and Cohen, S. (1972) J. Biol. Chem. 247, 7609-7611
20. Suh, P.-G., Ryu, S. H., Choi, W. C., Lee, K.-Y., and Rhee, S. G. (1989) J. Biol. Chem. 264, 14497-14504
21. Frackleton, A. R., Jr., Ross, A., and Eisen, H. (1983) Mol. Cell. Biol. 3, 1353-1360
22. Wahl, M. I., Daniel, T. O., and Carpenter, G. (1988) Science 241, 966-970
23. Wahl, M., and Carpenter, G. (1988) J. Biol. Chem. 263, 7581-7580
24. Nishibe, S., Wahl, M. I., Wedegaertner, P. B., Kim, J. W., Rhee, S. G., and Carpenter, G. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 424-428
25. Veil, T. J., Beguinot, L., Voss, W. C., Willingham, M. C., Merzino, F. T., Pastan, I., and Lowy, D. R. (1987) Science 238, 1488-1410
26. Toderud, G., Wahl, M. I., Rhee, S. G., and Carpenter, G. (1990) Science 249, 296-299
27. Dennis, E. A. (1974) J. Supramol. Struct. 2, 682–694
28. Deems, R. A., Eaton, B. R., and Dennis, E. A. (1975) J. Biol. Chem. 250, 9013–9020
29. Hendrickson, H. S., and Dennis, E. A. (1984) J. Biol. Chem. 259, 5734–5739
30. Robson, R. J., and Dennis, E. A. (1983) Acc. Chem. Res. 16, 251–258
31. Dennis, E. A. (1983) In The Enzymes (Boyer, P., ed) 3rd Ed., Vol. 16, pp 307–353, Academic Press, New York
32. Kim, H. K., Kim, J. W., Zilberstein, A., Margolis, B., Kim, J. G., Schlessinger, J., and Rhee, S. G. (1991) Cell 65, 435–441
33. Goldschmidt-Clermont, P. J., Kim, J. W., Machesky, L. M., Rhee, S. G., and Pollard, T. D. (1991) Science 251, 1231–1233
34. Tsien, R. Y. (1980) Biochemistry 19, 2386–2404
35. Irvine, R. F., Letcher, A. J., and Dawson, R. M. C. (1984) Biochem. J. 218, 177–185
36. Hofmann, S. L., and Majerus, P. W. (1982) J. Biol. Chem. 257, 6461–6469
37. Wilson, D. B., Brosa, T. E., Hofmann, S. L., and Majerus, P. W. (1984) J. Biol. Chem. 259, 11718–11724
38. Hofmann, S. L., and Majerus, P. W. (1982) J. Biol. Chem. 257, 14359–14364
39. Ryu, S. H., Cho, K. S., Lee, K.-Y., Suh, P.-G., and Rhee, S. G. (1987) J. Biol. Chem. 262, 12511–12518
40. Banno, Y., Yada, Y., and Nozawa, Y. (1988) J. Biol. Chem. 263, 11459–11465
41. Fukui, T., Lutz, R. J., and Lowenstein, J. M. (1988) J. Biol. Chem. 263, 17730–17737
42. Ridgway, N. D., and Vance, D. E. (1988) J. Biol. Chem. 263, 16864–16871
43. Sandermann, H., and Gottwald, B. A. (1983) Biochim. Biophys. Acta 732, 332–335
44. Wheeler, L. A., Goodrum, D. D., and Sachs, G. (1990) J. Membr. Biol. 118, 77–91
45. Goldschmidt-Clermont, P. J., Machesky, L. A., Baldessare, J. J., and Pollard, T. D. (1990) Science 247, 1575–1578
46. Pandiella, A., Malgaroli, A., Meldolesi, J., and Vincenti, L. (1987) Exp. Cell Res. 170, 175–185
47. Hepler, J. R., Nakahata, N., Lowenberg, T. W., DiGuiseppi, J., Herman, B., Earp, H. S., and Harden, T. K. (1987) J. Biol. Chem. 262, 2951–2956
48. Tilly, B. C., Van Paridon, P. A., Varlaan, L. De Laat, S. W., and Moolenaar, W. H. (1988) Biochem. J. 252, 857–863