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Biochemistry

Mammalian phospholipase D: Activation by ammonium sulfate and nucleotides

(phosphatidylcholine/G protein)

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ABSTRACT **Phospholipase D (PLD) associated with the rat kidney membrane was activated by guanine 5′-[γ-thio]triphosphate and a cytosol fraction that contained ADP-ribosylation factor. When assayed by measuring the phosphatidyl transfer reaction to ethanol with exogenously added radioactive phosphatidylcholine as substrate, the PLD required a high concentration (1.6 M) of ammonium sulfate to exhibit high enzymatic activity. Other salts examined were far less effective or practically inactive, and this dramatic action of ammonium sulfate is not simply due to such high ionic strength. Addition of ATP but not of nonhydrolyzable ATP analogue adenosine 5′-[β,γ-imido]diphosphate further enhanced the PLD activation ≈2- to 3-fold. This enhancement by ATP needed cytosol, implying a role of protein phosphorylation. A survey of PLD activity in rat tissues revealed that, unlike in previous observations reported thus far, PLD was most abundant in membrane fractions of kidney, spleen, and liver in this order, and the enzymatic activity in brain and lung was low.**

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PtdCho) in response to various external signals, including many growth factors (for review, see refs. 1 and 2). The mechanism of this enzyme activation has attracted considerable attention because the reaction may generate several lipid messengers or mediators such as diacylglycerol for transmembrane control of intracellular events (for review, see refs. 3–5). Phosphatidic acid, an immediate product of the PLD-catalyzed reaction, is proposed to serve as a mitogenic signal by itself (3). It is also suggested as a direct activator of protein kinase C (6) and phosphatidylinositol 4-kinase (7) or an inhibitor of GTPase-activating protein (8). PLD is associated tightly with particulate fractions, most likely membranous structures. With various assay methods, such as those with endogenously labeled phospholipids or with exogenously added radioactive phospholipids as substrates, attempts have been made to obtain cell-free preparations of PLD from several tissues and cell types, and the roles of GTP-binding regulatory proteins (G proteins) and protein phosphorylation in the activation of this enzyme have been proposed (for review, see refs. 9 and 10). It has recently been reported that in a cell-free system membrane-associated PLD is activated by small G proteins, such as ADP-ribosylation factor (11, 12), Rho (13, 14), or both (15). The enzymatic activity observed in cell-free preparations thus far obtained is, however, extremely low. An inhibitory protein factor has been recently found in bovine brain cytosol (16). Due to the complexity of this enzyme regulation and to the lack of an appropriate assay method of the enzyme, the mechanism of this enzyme activation has not been clarified.

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Studies in this laboratory have found that mammalian PLD associated with membranes of various tissues and cell types is nearly in a latent form but exhibits enormous enzymatic activity in high concentrations of ammonium sulfate, while maintaining its properties to respond to guanine nucleotide and a cytosol fraction that contains small G proteins including ADP-ribosylation factor. Such enzymatic activity is enhanced ≈2- to 3-fold by the addition of ATP. Although the mechanism of this salt effect remains unclear, this report will describe some kinetics of the PLD activation to facilitate further studies on the detailed properties, as well as the molecular mechanism, of the signal-induced activation of mammalian PLD.

MATERIALS AND METHODS

Materials and Chemicals. L-α-Dipalmitoyl[*palmitoyl*-1-^{14}C]phosphatidylcholine (^{14}C-PtdCho, 115 mCi/mmol; 1 Ci = 37 GBq) was purchased from DuPont/NEN. Phosphatidylethanol (PtdEtOH), a standard for TLC, was from Avanti Polar Lipids. Guanosine 5′-[γ-thio]triphosphate (GTP[γS]) and guanosine 5′-[β-thio]diphosphate (GDP[βS]) were from Boehringer Mannheim. Phorbol 12-myristate 13-acetate was a product of LC Services (Woburn, MA). Glass-backed silica gel 60 was purchased from Merck. (*p*-Amidinophenyl)methanesulfonyl fluoride hydrochloride (*p*-APMSF) was obtained from Wako Pure Chemical (Osaka). Monoclonal antibody 1D9 against ADP-ribosylation factor was from R. Kahn (National Cancer Institute, Bethesda).

Tissue Fractionation. Male Sprague–Dawley rats (6–8 weeks) were used throughout the study. All procedures were done at 0–4°C. Kidneys [5 g (wet weight)] were immediately removed, cut into small pieces, and mixed with 5 vol of buffer A (50 mM Hepes·NaOH, pH 7.4/1 mM $MgCl_2$/1 mM EGTA/0.25 M sucrose/10 μM *p*-APMSF) containing leupeptin at 20 μg/ml and aprotinin at 20 μg/ml. The tissue was homogenized with a Potter–Elvehjem Teflon/glass homogenizer. The homogenate was centrifuged for 10 min at 1000 × *g* to remove nuclei and unbroken tissues. The postnuclear fraction was centrifuged for an additional 60 min at 100,000 × *g*. The supernatant and pellet were used as cytosol and particulate fractions, respectively. For studies on tissue distribution of PLD, particulate fractions from various tissues were prepared similarly, as described above.

Preparation of Cytosol Factor. Cytosol proteins from the rat kidney were fractionated by ammonium sulfate (30–70% saturation), dissolved in 3 ml of buffer A, and dialyzed overnight against 1 liter of the same buffer. The dialysate was

Abbreviations: PLD, phospholipase D; PtdCho, phosphatidylcholine; G protein, GTP-binding regulatory protein; ^{14}C-PtdCho, L-α-dipalmitoyl[*palmitoyl*-1-^{14}C]PtdCho; PtdEtOH, phosphatidylethanol; GTP[γS], guanosine 5′-[γ-thio]triphosphate; GDP[βS], guanosine 5′-[β-thio]diphosphate; *p*-APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride.

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Table 1. Requirement of cytosol factor and ammonium sulfate for GTP[γS]-dependent PLD activation

	Ammonium sulfate	Fractions added	PtdEtOH produced, dpm	
			− GTP[γS]	+ GTP[γS]
Exp. 1	−	Particulate + CF	41 ± 9	41 ± 11
		Particulate alone	82 ± 25	99 ± 13
		CF alone	6 ± 2	8 ± 3
Exp. 2	+	Particulate + CF	261 ± 68	4923 ± 542
		Particulate alone	495 ± 89	825 ± 173
		CF alone	14 ± 4	3 ± 2

PLD was assayed under the conditions specified. In experiments 1 and 2, the enzyme was assayed without and with ammonium sulfate (1.6 M), respectively. Where indicated, particulate fraction (50 μg), cytosol factor (100 μg), and GTP[γS] (100 μM) were added. Each experiment represents the means ± SEMs of duplicate determinations from three separate experiments. CF, cytosol factor.

clarified by centrifugation for 30 min at 100,000 × *g* and subjected to gel filtration (Superdex 200, Pharmacia) with buffer A. The fraction capable of activating PLD in the presence of GTP[γS] appeared as a nearly single peak and was used as cytosol factor.

PLD Assay. The enzyme activity was assayed by measuring the formation of radioactive PtdEtOH by phosphatidyl transfer reaction of ^{14}C-PtdCho to ethanol as described (17). The reaction mixture (100 μl) contained 2 μmol of Hepes·NaOH (pH 7.4), 2 nmol of ^{14}C-PtdCho (13,750 dpm/nmol), 34.2 μmol of ethanol, 10 nmol of GTP[γS], 160 μmol of ammonium sulfate neutralized with ammonium hydroxide, cytosol factor, particulate fraction, and other ingredients as specified in each experiment. Practically, the reaction was started by adding the solution containing ^{14}C-PtdCho, ethanol, GTP[γS], ammonium sulfate, and other ingredients as specified to the enzyme solution containing particulate fraction and cytosol factor. After incubation for 20 min at 37°C, the reaction was stopped by the addition of 1 ml of ice-cold chloroform/methanol/HCl, 1:1:0.006, and mixed. After 0.5 ml of 0.1 M HCl/1 mM EGTA was added, the mixture was centrifuged for 10 min at 1000 × *g*. The lower, lipid-containing chloroform phase was collected and dried by centrifugation under vacuum. The residue was redissolved in 40 μl of chloroform/methanol, 95:5, containing an authentic sample of PtdEtOH (10 μg). The sample was spotted on a silica gel 60 TLC plate that had been activated by heating for 1 hr at 115°C just before use. The plate was developed with the upper phase of a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water, 13:2:3:10, as a solvent. The radioactivity of the spot corresponding to PtdEtOH was quantitated by using a Fujix Bio imaging analyzer, model BAS 2000 (Fuji). The radioactive PtdEtOH was produced only in the presence of ethanol during the incubation at the

Table 2. Effect of various salts on PLD activity

Addition	PtdEtOH produced, dpm
Ammonium sulfate	2956 ± 414
Sodium sulfate	1360 ± 272
Ammonium phosphate	628 ± 163
Sodium chloride	52 ± 11
Sodium phosphate	18 ± 4
None	41 ± 10

Particulate fraction (50 μg), cytosol factor (100 μg), GTP[γS] (100 μM), and various salts (1.6 M), as indicated, were assayed for PLD activity. Results are the means ± SEMs of duplicate determinations from three separate experiments.

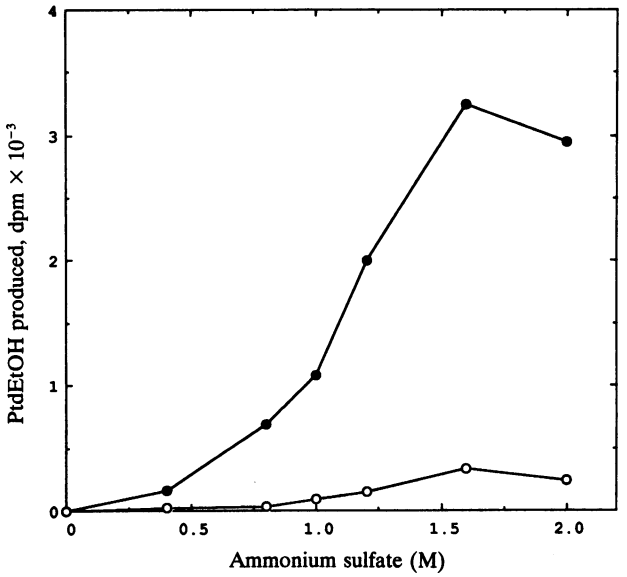

FIG. 1. Stimulation of PLD by GTP[γS] and ammonium sulfate. Particulate fraction (50 μg), cytosol factor (100 μg), and various concentrations of ammonium sulfate were assayed for PLD activity in the presence (●) or absence (○) of 100 μM GTP[γS]. Result is a representative of three separate experiments done in duplicate.

expense of phosphatidic acid and comigrated with an authentic sample upon two-dimensional TLC.

RESULTS

PLD Activation by Ammonium Sulfate. In the absence of ammonium sulfate PLD activity was nearly undetectable in rat kidney with the assay conditions specified above (Table 1). The enzymatic activity was uncovered by the addition of 1.6 M ammonium sulfate. As repeatedly documented elsewhere (11–14, 18, 19), PLD associated with particulate fraction was activated greatly by GTP[γS] and cytosol factor that contained small G proteins. The particulate fraction alone was almost

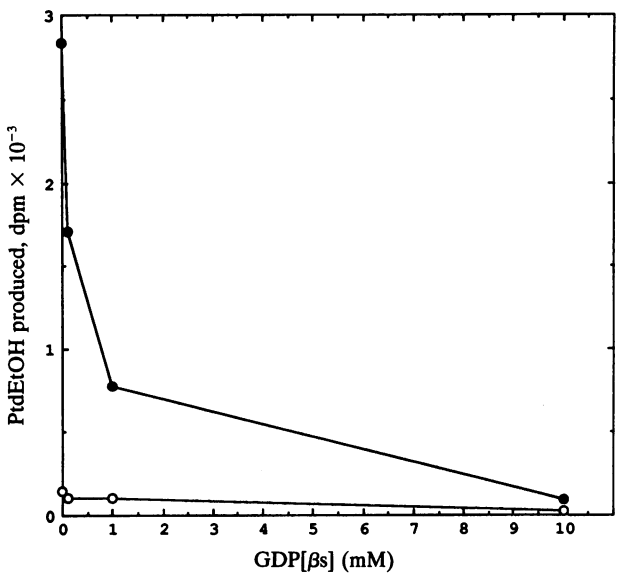

FIG. 2. Inhibitory action of GDP[βS] on GTP[γS]- and ammonium sulfate-induced PLD activation. Particulate fraction (50 μg), cytosol factor (100 μg), ammonium sulfate (1.6 M), and various concentrations of GDP[βS] were assayed for PLD activity in the presence (●) or absence (○) of GTP[γS] (100 μM). Result is a representative of three separate experiments done in duplicate.

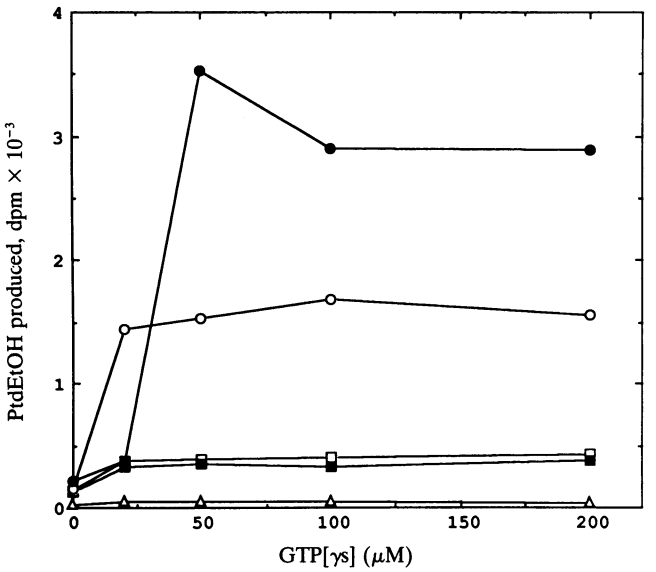

FIG. 3. Enhancement of PLD by ATP. Particulate fraction (50 μg) and various concentrations of GTP[γS] were assayed for PLD activity with or without cytosol factor (100 μg), ammonium sulfate (1.6 M), and ATP (500 μM) as indicated. Result is representative of three separate experiments done in duplicate. ●, With cytosol factor, ATP, and ammonium sulfate; ○, with cytosol factor and ammonium sulfate; □, with ATP and ammonium sulfate; △, with cytosol factor and ATP; ■, with ammonium sulfate.

insensitive to GTP[γS]. In the presence of cytosol factor and ammonium sulfate, the formation of PtdEtOH was linear with particulate fraction up to 50 μg of protein per tube, and the reaction proceeded linearly with time up to 50 min. Half-maximal activity was obtained at 42 μM dipalmitoyl-PtdCho. Stimulation of PLD by cytosol factor was linear up to 40 μg and saturated with 100 μg of protein per tube. The enzyme reacted with PtdCho and, to a lesser extent, phosphatidylethanolamine (one third of the initial velocity for PtdCho). Phosphatidylserine and phosphatidylinositol were inert as substrates. This dramatic salt effect was not simply due to the high ionic strength. Sodium sulfate and ammonium phosphate were far less effective in a wide range of concentrations (Table 2). Sodium phosphate and sodium chloride showed practically no effect.

Guanine Nucleotides. It is worth noting that ammonium sulfate could not substitute for GTP[γS] or for cytosol factor, and this salt and GTP[γS] were synergistic with each other for PLD activation (Fig. 1). The enzyme activation by GTP[γS] was counteracted by GDP[βS] (Fig. 2). Neither GTP, ATP, nor aluminum fluoride was capable of substituting for GTP[γS], consistent with the previous observations (11–15) that small G proteins are needed for PLD activation.

Table 3. Subcellular distribution PLD in rat kidney

Subcellular fraction	Specific activity,* pmol/min·mg	Total activity,* nmol/min (%)
Homogenate	454	35.5 (100)
Pellet (1000 × *g*)	963	7.5 (21)
Pellet (100,000 × *g*)	975	14.0 (39)
Supernatant (100,000 × *g*)	22	1.0 (3)

Subcellular fractions of rat kidney were prepared as described. Each fraction (50 μg), saturated amount (100 μg) of cytosol factor, ammonium sulfate (1.6 M), and GTP[γS] (100 μM) were assayed for PLD activity. Result is representative of three separate experiments done in duplicate.

*Specific activity and total activity of PLD from 1000 × *g* and 100,000 × *g* pellets were underestimated because of endogenous phospholipids.

Adenine Nucleotides. The PLD activation by cytosol factor, GTP[γS], and ammonium sulfate was enhanced further by ATP addition (Fig. 3). Nonhydrolyzable ATP analogue, adenosine 5′-[β,γ-imido]diphosphate, was inactive. This enhancement of PLD by ATP was not observed in the absence of cytosol and was prevented by a protein kinase inhibitor, such as genistein. The results suggest a potential role of protein phosphorylation in PLD activation.

Enzyme Distribution. When assayed in the presence of a saturated amount of cytosol factor as well as GTP[γS] and ammonium sulfate, PLD was recovered in the 1000 × *g* and 100,000 × *g* pellets, and practically no activity was found in the soluble fraction (Table 3). Although previous studies have shown that PLD is most abundant in the brain and lung (for review, see refs. 1 and 20), a survey of enzyme activity in various rat tissues revealed that kidney and spleen contained large quantities of PLD (Fig. 4). The liver and thymus contained significant quantities, but the enzyme activity in the brain and lung was very low.

DISCUSSION

High concentration of ammonium sulfate unmasks PLD in tissue extracts and enables us to detect the enzyme with a

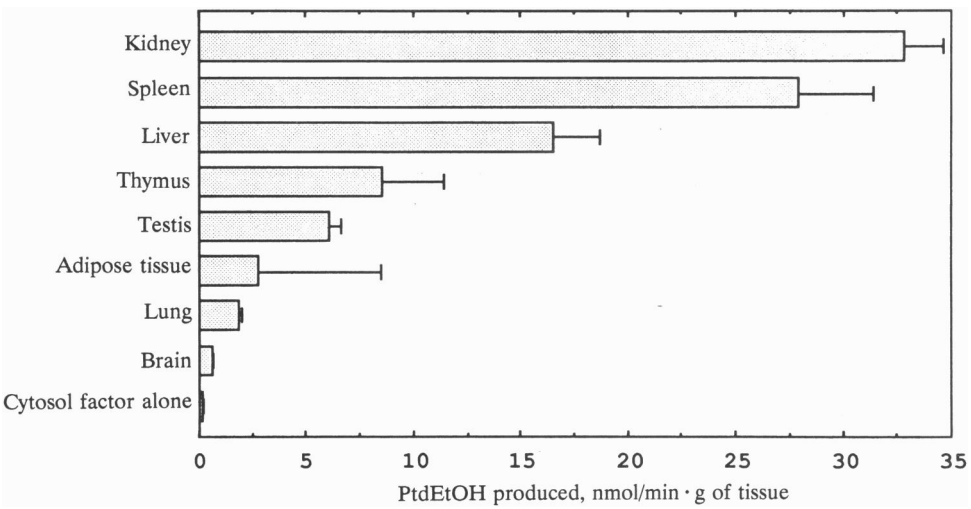

FIG. 4. Tissue distribution of PLD. Particulate fractions (50 μg) from various rat tissues, a saturable amount (100 μg) of cytosol factor from rat kidney, ammonium sulfate (1.6 M), and GTP[γS] (100 μM) were assayed for PLD activity. Results are the means ± SEMs of duplicate determinations from three separate experiments.

specific activity several hundred times higher than that observed under the assay conditions used by previous investigators. PLD has been studied mainly with brain, lung, adipose tissue, endothelial cells, HL-60 cells, and neutrophils (for review, see refs. 1 and 20). Thus far, lung, brain, HL-60 cells, and neutrophils were shown to be rich sources. The specific activity of PLD in the brain homogenate was 8 pmol/min per mg of protein, and a similar value was reported for HL-60 cell extracts (for review, see ref. 20). Under the assay conditions described in the present report, the specific activity of PLD in particulate fraction from kidney was 4.1 nmol/min per mg of protein. Although the mechanism of action of ammonium sulfate remains unknown, it is clear that PLD is ubiquitously distributed among various tissues. Unlike previous observations, the spleen and kidney are the richest sources, whereas the lung and brain show low enzymatic activity.

In mammalian tissues two types of membrane-associated PLD have been reported: one is dependent on oleic acid (21–23), and the other is activated by small G proteins (11–15). In the present studies, the addition of oleate showed no effect. It is presently unknown whether the oleate-dependent PLD activity is low in rat tissues or oleate effect is masked under high concentrations of ammonium sulfate.

In the presence of GTP[γS], ATP plus Mg^{2+} enhanced further PLD activity (Fig. 4). It has been reported that PLD may be activated by stimulation of the purinergic receptor (24, 25). The effect of ATP observed in the present study, however, is likely mediated through protein phosphorylation, because nonhydrolyzable ATP analogue adenosine 5′-[β,γ-imido]-diphosphate did not show any effect. Phorbol 12-myristate 13-acetate was inert. However, in another set of experiments with streptolysin O-permeabilized HL-60 cells, phorbol 12-myristate 13-acetate significantly enhanced the GTP[γS]-dependent PLD activation in the presence of ATP plus Mg^{2+} (26).

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1. Billah, M. M. & Anthes, J. C. (1990) *Biochem. J.* **269,** 281–291.
2. Exton, J. H. (1990) *J. Biol. Chem.* **265,** 1–4.
3. Boarder, M. R. (1994) *Trends Pharmacol. Sci.* **15,** 57–62.
4. Liscovitch, M. & Cantley, L. C. (1994) *Cell* **77,** 329–334.
5. Nakamura, S. & Nishizuka, Y. (1994) *J. Biochem. (Tokyo)* **115,** 1029–1034.
6. Stasek, J. E., Jr., Natarajan, V. & Garcia, J. G. N. (1993) *Biochem. Biophys. Res. Commun.* **191,** 134–141.
7. Moritz, A., Graan, P. N. E., Gispen, W. H. & Wirtz, K. W. A. (1992) *J. Biol. Chem.* **267,** 7207–7210.
8. Tsai, M.-H., Yu, C.-L., Wei, F.-S. & Stacey, D. W. (1989) *Science* **243,** 522–525.
9. Nishizuka, Y. (1992) *Science* **258,** 607–614.
10. Nishizuka, Y. (1995) *FASEB J.* **9,** 484–496.
11. Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C. & Sternweis, P. C. (1993) *Cell* **75,** 1137–1144.
12. Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O. & Hsuan, J. J. (1994) *Science* **263,** 523–526.
13. Bowman, E. P., Uhlinger, D. J. & Lambeth, J. D. (1993) *J. Biol. Chem.* **268,** 21509–21512.
14. Malcolm, K. C., Ross, A. H., Qiu, R.-G., Symons, M. & Exton, J. H. (1994) *J. Biol. Chem.* **269,** 25951–25954.
15. Singer, W. D., Brown, H. A., Bokoch, G. M. & Sternweis, P. C. (1995) *J. Biol. Chem.* **270,** 14944–14950.
16. Geny, B., Paris, S., Dubois, T., Franco, M., Lukowski, S., Chardin, P. & Marie, F. R. (1995) *Eur. J. Biochem.* **231,** 31–39.
17. Kobayashi, M. & Kanfer, J. N. (1987) *J. Neurochem.* **48,** 1597–1603.
18. Anthes, J. C., Wang, P., Siegel, M. I., Egan, R. W. & Billah, M. M. (1991) *Biochem. Biophys. Res. Commun.* **175,** 236–243.
19. Olson, S. C., Bowman, E. P. & Lambeth, J. D. (1991) *J. Biol. Chem.* **266,** 17236–17242.
20. Cockroft, S. (1992) *Biochim. Biophys. Acta* **1113,** 135–160.
21. Chalifa, V., Möhn, H. & Liscovitch, M. (1990) *J. Biol. Chem.* **256,** 17512–17519.
22. Massenburg, D., Han, J.-S., Liyanage, M., Patton, W. A., Rhee, S. G., Moss, J. & Vaughan, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11718–11722.
23. Okamura, S. & Yamashita, S. (1994) *J. Biol. Chem.* **269,** 31207–31213.
24. El-Moatassim, C. & Dubyak, G. R. (1992) *J. Biol. Chem.* **267,** 23664–23673.
25. El-Moatassim, C. & Dubyak, G. R. (1993) *J. Biol. Chem.* **268,** 15571–15578.
26. Inoue, H., Shimooku, K., Akisue, T. & Nakamura, S. (1995) *Biochem. Biophys. Res. Commun.* **210,** 542–548.