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SEPARATION AND IDENTIFICATION OF TWO PHOSPHATIDYLINOSITOL 4-KINASE ACTIVITIES IN BOVINE UTERUS

Yue-Sheng Li,[1] Forbes D. Porter,[2] Ruth M. Hoffman,[1] and Thomas F. Deuel[1,3]

Departments of [1]Medicine and [3]Biological Chemistry
Jewish Hospital at Washington University Medical Center
and
[2]Molecular Biology and Biochemistry Program
Washington University Medical School
St. Louis, Missouri 63110

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SUMMARY: Growth factor-activated second messenger pathways are mediated in part via breakdown products of phosphoinositides. We have separated two phosphatidylinositol (PtdIns) 4-Kinases from bovine uteri which appear to be regulated independently. The predominant type II enzyme previously was purified to apparent homogeneity; the type I enzyme has been purified ~ 1000 fold (specific activity, ~ 30 nmoles/mg/min). The type I and type II enzymes differ sharply in apparent Km for ATP and response to divalent cations. In contrast to type II enzyme, type I PtdIns kinase was resistant to inhibition by adenosine, inhibited by increasing concentrations of Triton X-100, and less stable to storage than type II enzyme at pH values below 6.5 and above 8.5. Type I PtdIns 4-kinase has an apparent molecular mass of ~ 200 kD and type II enzyme of ~ 80 kD. Using both enzymatic and chemical criteria, both enzymes specifically phosphorylated the fourth hydroxyl group of PtdIns. The results thus establish the presence of two distinct and separate enzymes catalyzing PtdIns 4-kinase activity with different physical, kinetic, and regulatory properties, suggesting an important site for the regulation of second messenger signals transducing the responsiveness of cells to growth factors.

The phosphoinositide second messenger system appears to mediate diverse signals initiated by a number of important polypeptides, including platelet derived growth factor (PDGF) (1, 2), bombesin (3), thrombin (4-6), bradykinin (6-8), vasopressin (9) and, under some conditions, epidermal growth factor (EGF) (6, 10-16). The first enzyme in the phosphoinositide pathway and thus a likely site for regulation is phosphatidylinositol (PtdIns) 4-kinase [EC 2.7.1.67], which catalyzes the phosphorylation of PtdIns in the 4 position to phosphatidylinositol 4-phosphate (PtdIns-4-P). Early results (17) suggested that more than one PtdIns kinase might mediate this activity. To date, however, a clear separation and initial characterization of different PtdIns kinases has not been presented.

In investigating the first unique enzyme in the biosynthesis of the inositol phosphates, we identified and separated two forms of PtdIns 4-kinase

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activity in bovine uterus. The predominant one, type II, has been purified to homogeneity (18). The second activity has now been separated, highly purified, and characterized; its properties have been compared with those of PtdIns previously reported (18-20) and those of the postulated 81-85 kD PtdIns 3-kinase (21-23).

MATERIALS AND METHODS

Materials: Mature bovine uteri were from Pelfreeze (Rosers, AR), silica gel 60 thin layer chromatography plates (0.25 mm thickness) were from Whatman, phosphatidylinositol (soybean), PtdIns-4-P, PtdIns 4,5-P_2, Triton X-100, and protease inhibitors were from Sigma. Octylglucoside (OG) was from Calbiochem and reduced Triton X-100 was from Aldrich. [gamma-^{32}P] ATP was from New England Nuclear, and Q-Sepharose Fast Flow, MonoQ and MonoS columns were from Pharmacia.

Extraction and Purification of PtdIns 4-Kinase Activities from Acetone Powders (Bovine Uterus): Acetone powders of mature bovine uteri were prepared as described previously (18) and extracted by agitation with 40 ml/g of 0.9 M NaCl, 20 mM Tris (pH 8.0), 5 mM ethylene glycol bis-(aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 0.15 mg/ml phenylmethylsulfonyl fluoride (PMSF), 10 mM benzamidine, 0.1 mg/ml soybean trypsin inhibitor, and 1 µg/ml aprotinin for 2 hr. at 4°C. After centrifugation (10,000xg for 30 min), the supernatant, containing the "type I" PtdIns kinase, was retained, whereas the "type II" PtdIns kinase required detergents for extraction from acetone powders or from the precipitate after the extraction of the type I enzyme in aqueous buffers. After extraction, it was purified as described before (18). All experiments comparing type I and II enzymes, unless otherwise noted, used extracts extensively dialyzed with buffer composed of 20 mM NaCl, 20 mM Tris (pH 8.0), 1 mM dithiothreitol (DTT) ("common buffer"), subsequently frozen with liquid nitrogen and stored at -70°C.

The type I extract was purified with ammonium sulfate precipitation (30% saturation), chromatographed with a 500 ml Q-Sepharose Fast Flow column after dialysis against "common" buffer, and eluted with a 2 l gradient of 0.02-0.5 M NaCl. Active fractions containing PtdIns 4-kinase activity were dialyzed against 20 mM NaCl, 20 mM Tris pH 8.0, 1 mM DTT, 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA and loaded onto a MonoQ (HR16/10) column. After elution with a 180 ml gradient of 0.02-0.6 M NaCl, fractions containing the PtdIns 4-kinase were pooled, dialyzed against 20 mM NaCl, 20 mM Tris pH 7.0, 1 mM DTT, 10 mM OG, loaded onto a MonoS (HR5/5) column, and eluted with a 25 ml gradient of 0.02-0.45 M NaCl; at this point, the type I enzyme had been purified 1000 fold (specific activity ~ 30 nmole/mg/min). These fractions were used for characterization.

Assay of PtdIns 4-Kinase Activity: Proof of Product: Two assay systems were utilized in this study, unless otherwise noted. In the first assay (adapted from Bostwick, 24), 2% Triton X-100 was used for assay of type II enzyme and 0.25% Triton X-100 for assay of type I. A second assay system was performed as described (18); 1.0% Triton X-100 was used for assay of type II and 0.1% Triton X-100 for assay of type I, unless otherwise noted. All assays were done in duplicate or in triplicate. The chemical and enzymatic analysis of PtdIns 4-phosphate was performed as described by Majerus and co-workers (25-28).

Gel Exclusion Chromatography: Gel exclusion chromatography was performed on a 60 cm x 2.5 cm TSK 4000 column equilibrated with 150 mM NaCl, 50 mM sodium phosphate (pH 7.5), 0.1% Triton X-100 (d-r-Triton X-100) which had been deionized (by passage of a 10% v/v solution over amberlite MB-1) and reduced 4 mM deoxycholate and 1 mM DTT. The column was loaded with 2 ml samples and run at a flow rate of 1.0 ml/min.

RESULTS

Extraction of PtdIns 4-Kinase Activities: As noted in Methods (above), only limited PtdIns 4-kinase activity (type I, ~ 2%) was extracted from acetone powders of bovine uteri using 0.9 M NaCl containing aqueous buffers; a second aqueous extraction after the initial extraction failed to solubilize additional PtdIns 4-kinase activity. Subsequent extraction in 40 mM octylglucoside was required to release the bulk of PtdIns 4-kinase (type II), suggesting two distinct and separable PtdIns kinase activities in bovine uterus with different solubility properties. In addition to octylglucoside, Triton X-100 and deoxycholate were effective (data not shown) but polyols, including glycerol and ethylene glycol, were ineffective in solubilizing PtdIns 4-kinase activity.

It was first necessary to establish that the site of incorporation of ^{32}P into the inositol head group of PtdIns was identical for both type I and type II activities. The PtdIns-X-P products were purified by thin layer chromatography on oxalate-impregnated silica plates, extracted from the silica gel as described (29), incorporated into PtdIns/Ptd ethanolamine (4/1) unilamilar vesicles, and cleaved with sheep seminal vesicle phospholipase C, as described (26, 28). The soluble products were then chromatographed on a Partisil SAX column (27); both the type I and type II extracts produced an Ins-1,X-P_2 which comigrated with authentic Ins-1,4-P_2 (Fig. 1A and 1B). Subsequent treatment of these Ins-1,X-P_2 products with a purified Ins-1,4-P_2/Ins-1,3,4-P_3 1-Phosphatase (25, 29) resulted in the formation of a ^{32}P-labeled product which co-chromatographed with Ins-4-P (Fig. 1C and 1D). Based on the specificity of this 1-phosphatase, both type I and type II activities are therefore PtdIns 4-kinases. Authentic internal standards ruled out the possibility that a second type of product was lost during analysis.

Comparison of the Differentially Extracted PtdIns 4-Kinase Activities: Different criteria were used to establish that the two activities extracted from bovine uteri were separate and distinct proteins catalyzing the same PtdIns 4-kinase activity. The influence of increasing amounts of Triton X-100 in the assay buffer was strikingly different with type I and II enzymes (Fig. 2A). The type I activity was stimulated by low concentrations of Triton X-100 but inhibited as concentrations of Triton X-100 exceeded 0.2%. In contrast, type II activity was stimulated by concentrations of Triton X-100 up to 1%. In control experiments, pulse chase analysis with ^{32}P ATP established the absence of significant degradation of PtdIns-4-P in type I extracts treated with Triton X-100, and mixing experiments with types I and II failed to demonstrate an inhibitory activity stimulated by Triton X-100 in type I

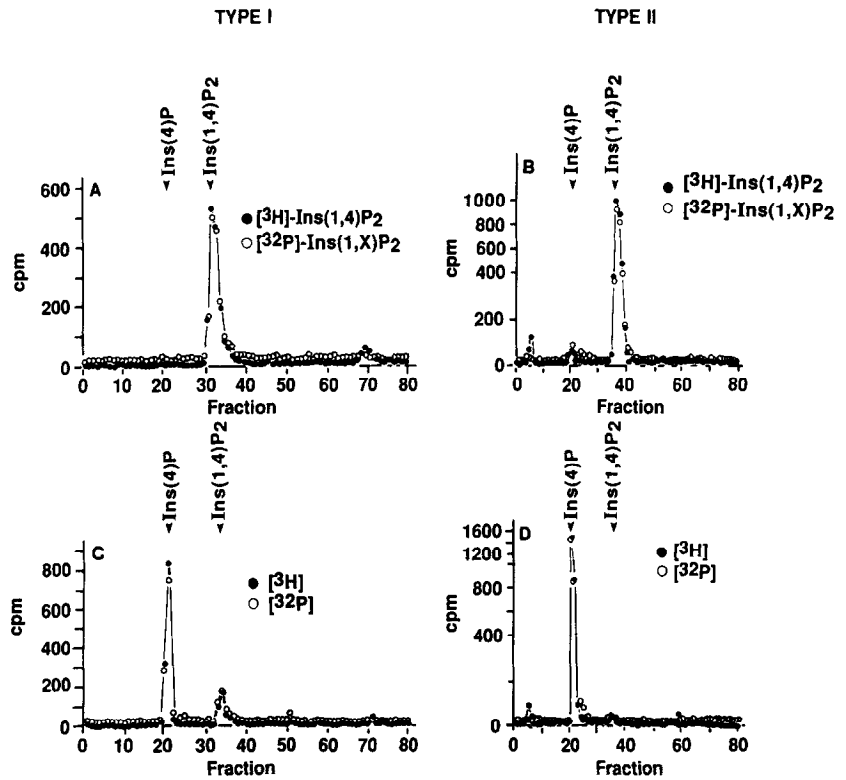

Figure 1. Determination of the site of phosphorylation by the type I and type II activities. (A) Partisil SAX chromatography of the type I phospholipase C product. (B) Partisil SAX chromatography of the type II phospholipase C product. (C) Partisil SAX chromatography of the type I polyphosphoinositol 1-phosphatase treated Ins-1,X-P_2. (D) Partisil SAX chromatography of the type II polyphosphoinositol 1-phosphatase treated Ins-1,X-P_2. Authentic ^{3}H-labled Ins-1,4-P_2 and ^{3}H-labeled Ins-4-P were used as internal standards in these experiments.

extracts. Substrate degradation was not stimulated by Triton X-100 nor did prior exposure of type I extracts to 40 mM octylglucoside influence the reaction (data not shown). Thus, the two different enzymatic activities identified by differential responsiveness to Triton X-100 were not artifactual responses to Triton X-100-activated PtdIns-4-P-degrading enzyme or a Triton X-100-activated inhibitor of PtdIns 4-kinase in type I extracts.

The stabilities of PtdIns 4-kinase activity extracted into aqueous salt buffers (type I) and into octylglucoside extracts (type II) were then compared after dialysis (4 hours) into identical buffers of different pH values. The enzymatic activity was assayed at pH 8.3 with optimal Triton X-100 concentrations for each enzyme preparation (Fig. 2B). Both extracts showed optimal stability at pH 7-8; however, type II activity was more stable than type I activity at pH values below 6.5 and above 8.5.

The substrate requirements of each enzyme for catalytic activity and the possible regulatory role of adenosine as an inhibitor were also examined.

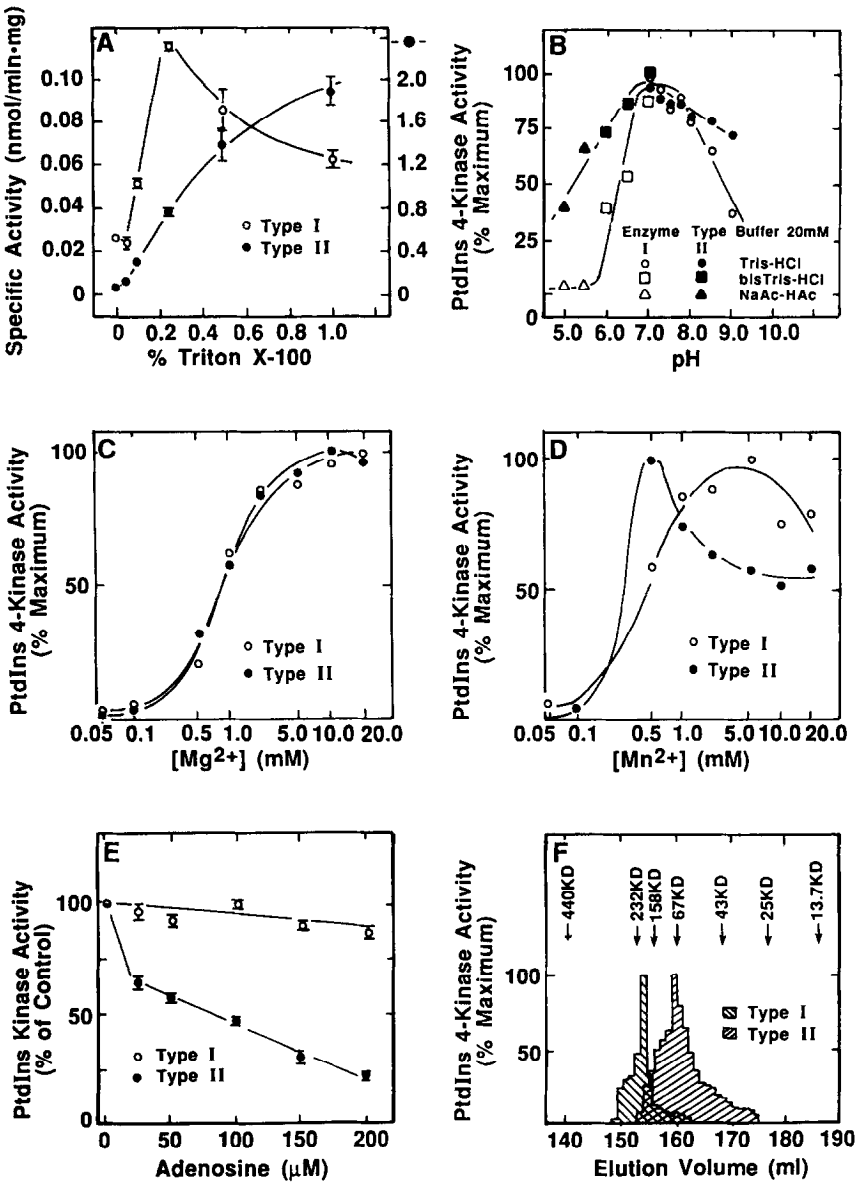

Figure 2. Comparison of two PtdIns 4-kinase activities in bovine uterus. (A) The effect of Triton X-100 on two PtdIns 4-kinase activities. The Bostwick (24) assay was used. (B) The pH stability of two PtdIns 4-kinase activities. The Bostwick (24) assay was used. (C and D) Divalent cation [Mg^{2+}, (C); Mn^{2+}, (D)] requirements of two PtdIns 4-kinase activities. (E) Inhibition of two PtdIns 4-kinase activities by adenosine. The experiments were performed as described (18) except that the concentration of ATP was 50 μM, and adenosine was added as indicated. (F) Gel filtration of the two PtdIns 4-kinase activities. The experiments were performed as described in Methods. The elution position of various standards is also shown. All data points are the mean of three assays.

Both type I and type II activities require divalent cations for catalytic activity; both type I and type II activities demonstrated a similar dependence on Mg^{2+} concentration (Fig. 2C). The optimum Mn^{2+} concentrations

required for maximum catalytic activity, however, differed significantly; type I extracts were optimum at a broad Mn^{2+} concentration range of 1-20 mM, while type II extracts were optimally active at a Mn^{2+} concentration of 0.5 mM and sharply inhibited at concentrations of Mn^{2+} above 0.5 mM (Fig. 2D). Substantial differences also were noted in the Km for ATP. The type I activity had an apparent Km of ~ 250 μM for ATP, while the purified type II activity had an apparent Km of ~ 18 μM for ATP (data not shown). Also, types I and II PtdIns 4-kinase activities could be differentiated by sensitivity to inhibition by adenosine; type I activity was resistant to inhibition by adenosine, whereas type II activity was inhibited by increasing concentrations of adenosine (Fig. 2E).

In addition to differences in the physical properties noted above, the two PtdIns kinase activities demonstrated differences in molecular mass when analyzed with gel exclusion chromatography. In a buffer containing 4 mM deoxycholate and 0.1% d-r-Triton X-100, the type I activity migrated with an apparent molecular mass of ~ 200 kDa, while the type II activity migrated with an apparent molecular mass of ~ 80 kDa (Fig. 2F). The broader pattern of peak elution than the ideal for both types I and II suggested that both enzymes were differentially aggregated during gel exclusion chromatography.

DISCUSSION

The polyphosphoinositides are an important subset of membrane lipids which have important roles in signal transduction. Agonist-induced phosphalipase C cleavage of phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P_2) results in the formation of inositol 1,4,5-trisphosphate, which functions as a second messenger to increase cytoplasmic Ca^{2+} concentrations (30), and in the formation of 1,2-diacylglycerol, which activates protein kinase C (31). PtdIns 4-kinase is the first unique enzyme in this important pathway and thus a likely site for regulation.

Harwood and Hawthorne (17) first proposed the possibility of multiple PtdIns kinases in rat liver. Recently, multiple forms of PtdIns kinase activities were suggested to reside in mouse 3T3 fibroblasts (19) and in bovine brain (20). Furthermore, investigators found a PtdIns kinase activity in immunoprecipitates of the polyoma middle T/$pp60^{c\text{-}src}$ complex (21), and in immunoprecipitates from lysates of PDGF-stimulated Balb/c 3T3 fibroblasts with antiphosphotyrosine antisera (22). Subsequently, this PtdIns kinase was shown to phosphorylate PtdIns on the third but not the fourth hydroxyl to form PtdIns 3-P (23).

We now describe the separation and characterization of two PtdIns 4-kinase activities from bovine uteri. These two activities differ in apparent molecular mass and with respect to stability at different pHs, in sensitivity

to Triton X-100, in utilization of Mn^{2+}, in the Km for ATP, and in inhibition by adenosine. The predominant type II enzyme shares properties with the type II activity described but not purified from 3T3 fibroblasts (19) and from bovine brain (20). The relationship of the type I activity to those activities described previously, however, has not been established, which suggests that the uterine type I PtdIns 4-kinase is a unique protein species. It is most similar to the brain type III enzyme (20) in size, in inhibition by adenosine, and in having a high apparent Km for ATP. The two enzymes differ, however, in the requirement for Mg^{2+} and in sensitivity to Triton X-100.

Importantly, the differences in the two enzymes we have reported identify multiple sites for potential regulation. The type II PtdIns 4-kinase is sensitive to adenosine and, by virtue of the low Km for ATP (18 μM), would be less likely to be strongly inhibited by low concentrations of other nucleoside triphosphates or susceptible to alterations in the adenine nucleotide pool. Through its striking sensitivity to low concentrations of Mn^{2+}, the type II enzyme likely is highly responsive to fluctuations in divalent cation concentrations. It is perhaps important also that the type I enzyme would be sensitive to modulations of the adenine nucleotide pool, perhaps particularly so during periods of high demand such as in periods of rapid DNA replication.

PtdIns 4-kinase is the first enzyme in a complex pathway leading directly to products of major importance in the transduction of signals from growth factors and other agonists leading to Ca^{++} mobilization, activation of protein kinase C, and prostaglandin biosynthesis. Two types of PtdIns 4-kinase, each with distinct physical and catalytic properties, provide at least one level of independent regulation of these pathways and thus the potential to modulate the levels of signal transduction.

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REFERENCES

1. Berridge, M.J., Heslop, J.P., Irvine, R.F. and Brown, K.D. (1984) Biochem. J. 222, 195-201.
2. Escobedo, J.A., Keating, M.T., Ives, H.E., and Williams, L.T. (1988) J. Biol. Chem. 263, 1482-1487.
3. Takuwa, N., Takuwa, Y., Bollag, W.E., and Rassmussen, H. (1987) J. Biol. Chem. 262, 182-188.
4. Paris, S. and Pouyssegur, J. (1986) EMBO 5, 55-60.
5. Carney, D.H., Scott, D.L., Bordon, E.A., and LaBelle, E.F. (1985) Cell 42, 479-488.
6. L'Allemain, G., and Pouyssegur, J. (1986) FEBS Lett. 197, 344-348.

7. Tilly, B.C., van Paridon, P.A., Verlaan, I., Wirtz, K.W.A., De Laat, S.W., and Moolenaar, W.H. (1987) Biochem. J. 244, 129-135.
8. Lambert, T.L., Kent, R.S., and Whorton, A.R. (1986) J. Biol. Chem. 261, 15288-15293.
9. Vicentini, L.M., and Villereal, M.S. (1984) Biochem. Biophys. Res. Comm. 123, 663-670.
10. Pike, L.J., and Eakes, A.T. (1987) J. Biol. Chem. 262, 1644-1651.
11. Walker, D.H., and Pike, L.J. (1987) Proc. Natl. Acad. Sci. USA 84, 7513-7517.
12. Macara, I.G. (1986) J. Biol. Chem. 261, 9321-9324.
13. Besterman, J.M., Watson, S.P., and Cuatrecasas (1986) J. Biol. Chem. 261, 723-727.
14. Taylor, D., Uhing, R.J., Blackmore, P.F., Prpic, V., and Exton, J.H. (1985) J. Biol. Chem. 260, 2011-2014.
15. Olashaw, N.E., and Pledger, W.J. (1988) J. Biol. Chem. 263, 1111-1114.
16. Johnson, R.M., and Garrison (1987) J. Biol. Chem. 262, 17285-17293.
17. Harwood, J.L., and Hawthorne, J.N. (1969) Biochim. Biophys. Acta. 171, 75-88.
18. Porter, F.D., Li, Y.S., and Deuel, T.F. (1988) J. Biol. Chem. 263, 8989-8995.
19. Whitman, M., Kaplan, D., Roberts, T., and Cantley, L. (1987) Biochem. J. 247, 165-174.
20. Endemann, G., Dunn, S.N., and Cantley, L.C. (1987) Biochemistry 26, 6845-6852.
21. Courtneidge, S.A., and Heber, A. (1987) Cell 50, 1031-1037.
22. Kaplan, D.R., Whitman, M., Schaffhausen, B., Pallas, D.C., White, M., Cantley, L., and Roberts, T.M. (1987) Cell 50, 1021-1029.
23. Whitman, M. Downes, C.P., Keeler, M. Keller, T., and Cantley, L. (1988) Nature 322, 644-646.
24. Bostwick, J.R., and Eichberg, J. (1981) Neurochemical Res. 6, 1053-1065.
25. Inhorn, R.C., and Majerus, P.W. (1987) J. Biol. Chem. 262, 15946-15952.
26. Hofmann, S.L., and Majerus, P.W. (1982) J. Biol. Chem. 259, 6461-6469.
27. Auchus, R.J., Kaiser, S.L., and Majerus, P.W. (1987) Proc. Natl. Acad. Sci. USA 84, 1206-1209.
28. Wilson, D.B., Bross, T.E., Hofmann, S.L., and Majerus, P.W. (1984) J. Biol. Chem. 259, 11717-11724.
29. Inhorn, R.C., Bansal, V.S., and Majerus, P.W. (1987) Proc. Natl. Acad. Sci. USA 84, 2170-2174.
30. Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315-321.
31. Nishizuka, Y. (1984) Nature 308, 693-698.