# Communication

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# Phosphatidic Acid Is a Specific Activator of Phosphatidylinositol-4-phosphate Kinase*

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**The lipid dependence of phosphatidylinositol-4-phosphate (PIP) kinase purified from bovine brain membranes was investigated. In the assay used, PIP-Triton X-100 micelles containing the lipid to be tested were presented to the enzyme. Under these conditions, phosphatidic acid (PA) stimulated the enzyme activity in a concentration-dependent manner up to 20-fold when an equal molar ratio of PA to PIP was attained. Stimulation by PA was highly specific; other lipids including lyso-PA and dicetylphosphate had a relatively small effect. The activation by PA was completely suppressed by phosphatidylinositol 4,5-bisphosphate ($PIP_2$). To investigate the effect of PA on PIP kinase activity in natural membranes, endogenous PA was generated in rat brain synaptosomal plasma membranes by incubation with phospholipase D. Subsequent phosphorylation with [$\gamma$-$^{32}$P]ATP yielded an enhanced labeling of $PIP_2$ but not of PIP in these membranes. These results suggest that PIP kinase activity may be under control of PA levels in membranes. This may have important implications for the regulation of cellular responses by agonist-induced phosphoinositide turnover.**

The breakdown of phosphatidylinositol 4,5-bisphosphate ($PIP_2$)[1] by phospholipase C is a key step in hormone-regulated signal transduction (1–3). Cellular levels of $PIP_2$ are maintained by the sequential phosphorylation of phosphatidylinositol by phosphatidylinositol kinase and phosphatidylinositol-4-phosphate (PIP) kinase (1, 4). While the breakdown of $PIP_2$ and the role of the second messengers generated have been studied extensively (2, 3), little is known about the regulation of the kinases responsible for the synthesis of $PIP_2$ (4). In many instances, the activity of the enzymes related to phosphoinositide metabolism is modulated by lipids. So it is well known that protein kinase C is activated by diacylglycerol and phosphatidylserine (5, 6) as well as by $PIP_2$ (6) and inhibited by sphingosine (7). Phospholipase C is stimulated by phosphatidic acid (PA) (8, 9). Diacylglycerol kinase is stimulated by phosphatidylcholine and phosphatidylserine and inhibited by phosphatidylinositol (10). Different species of diacylglycerol kinase are either activated or inhibited by sphingosine (11). Stimulation by phosphatidylserine has been reported for PIP kinase from rat brain cytosol and from human erythrocyte membranes (12, 13). In a previous paper we reported the purification of a PIP kinase from bovine brain membranes (14). Here we have investigated the lipid dependence of this enzyme.

## EXPERIMENTAL PROCEDURES

*Materials*—All lipids and carrier lipid (Folch fraction I from bovine brain) were purchased from Sigma except phosphatidylinositol which was purified from yeast (15) and phosphatidylserine which was purified from bovine brain (16). Silica gel plates were from Merck (Darmstadt, Germany). ATP was purchased from Boehringer (Mannheim, Germany) and bovine serum albumin from Calbiochem. [$\gamma$-$^{32}$P]ATP (3,000 Ci/mmol) was obtained from Amersham (United Kingdom). Calcium-independent phospholipase D from *Streptomyces* sp. AA 586 was a kind gift of Dr. S. Imamura (see Ref. 17). Diacylglycerol kinase inhibitor R 59949 (see Ref. 29) was obtained from Janssen (Beerse, Belgium).

*Assay of PIP Kinase*—Lipid mixtures consisting of PIP (16 nmol) and various amounts of other lipids were dried from a chloroform solution under $N_2$. These mixtures were then exposed for 2 h to the vacuum of a lyophilizer to remove the last traces of chloroform. After suspension in buffer (0.12 ml) consisting of 50 mM Tris/HCl (pH 7.4), 0.333 M sucrose, 0.133% polyethylene glycol 20,000, 0.167% Triton X-100, 150 mM NaCl, and bovine serum albumin (0.67 mg/ml) the lipids were sonicated with a Branson probe sonifier (output, 45 watts) under $N_2$ for 3 min (5 s on, 10 s off) on ice. Sonication was carried out for brief periods to prevent lipid degradation (18). Phosphorylation of PIP was carried out in glass tubes in an incubation medium (final volume of 50 $\mu$l) containing final concentrations of 50 mM Tris/HCl (pH 7.4), 100 mM NaCl, 15 mM $MgCl_2$, 1 mM EGTA, 80 $\mu$M PIP, bovine serum albumin (0.4 mg/ml), 0.25 M sucrose, 0.1% polyethylene glycol 20,000, 0.1% Triton X-100, and 50 $\mu$M [$\gamma$-$^{32}$P] ATP (1.5 $\mu$Ci/assay). After preincubation for 5 min at 30 °C, the reaction was started by addition of the enzyme solution (10 $\mu$l) and continued for 8 min at 30 °C. $PIP_2$ formation was linear with protein concentration and time for at least 10 min. All assays were conducted in triplicate. The reaction was stopped by addition of 3 ml of chloroform:methanol:concentrated HCl (200:100:0.75, by volume). A mixture of carrier lipids consisting of phosphatidylinositol, PIP, $PIP_2$, and phosphatidylserine (40–200 nmol each) was added, and a two-phase separation was induced by addition of 0.6 M HCl (0.6 ml) followed by vortexing. After centrifugation, the lower phase was collected and washed twice with 1.5 ml of chloroform, methanol, 0.6 M HCl (3:48:47, by volume). The lower phase was dried under a stream of $N_2$ at 50 °C, and the lipid residue was redissolved in 60 $\mu$l of ice-cold chloroform:methanol:water (75:25:2, by volume) by vortexing. An aliquot (30 $\mu$l) was applied on an oxalate-impregnated thin layer chromatography plate (19), and the lipids were separated by developing the plate in chloroform:acetone:methanol:acetic acid:water (40:15:15:12:7.5, by volume) for 60 min. Lipids were visualized by iodine staining. After sublimation of the iodine, the area containing [$^{32}$P]$PIP_2$ was scraped from the plate and counted by liquid scintillation spectrometry after the addition of 3 ml of Xylofluor.

*PIP Kinase Activity in Synaptosomal Plasma Membranes*—Synaptosomal plasma membranes were prepared as described (20) and stored at −20 °C at a concentration of 5 mg/ml protein in 10 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, and 1 mM EGTA (buffer A) containing 10% glycerol. These membranes (10 $\mu$g of protein) were preincubated in 10 $\mu$l of buffer A for 3 min at 30 °C and then incubated

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[1] The abbreviations used are: $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; PIP, phosphatidylinositol 4-phosphate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

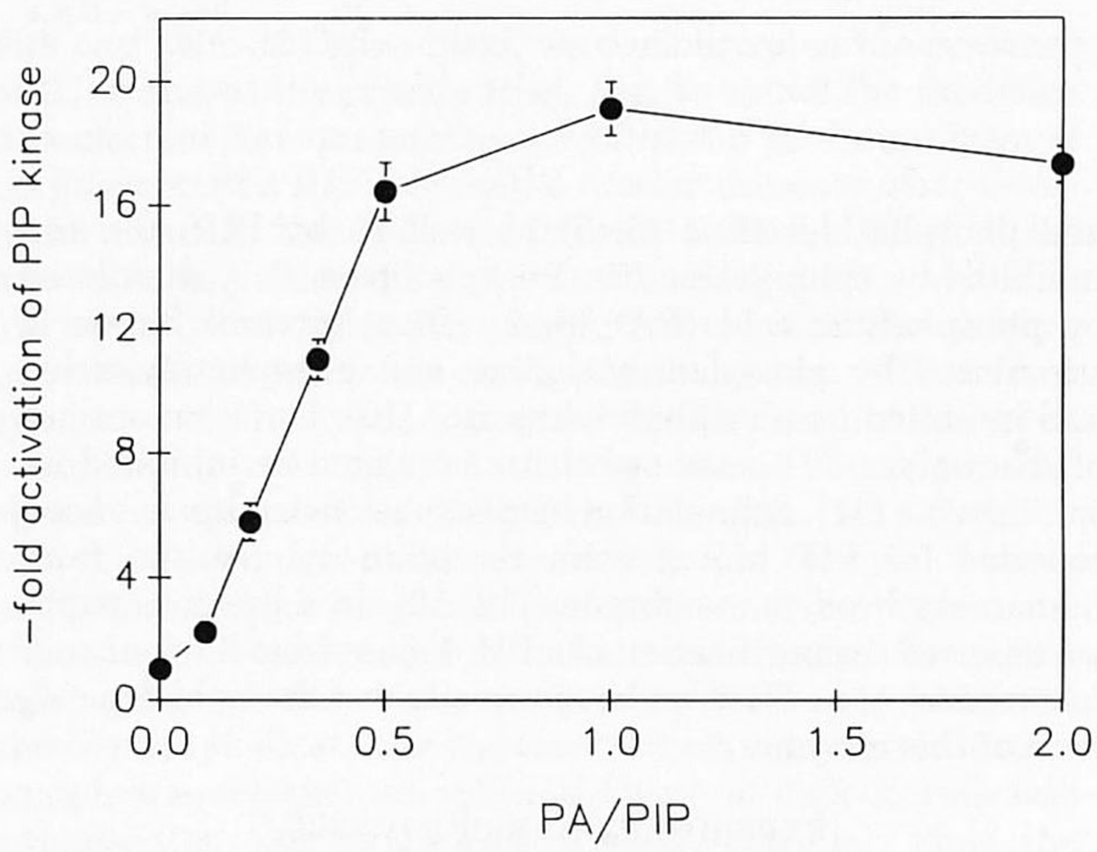


FIG. 1. **Stimulation of PIP kinase by PA.** Various amounts of PA were added to the assay containing 80 μM PIP. The molar ratio of PA/PIP was varied from 0.1 to 2. PIP kinase activity was assayed as described under "Experimental Procedures." Data are mean ± S.E. based on two experiments in triplicate ($n = 6$).

for 5 min at 30 °C with 0.25 units of phospholipase D added in 10 μl of buffer A in the presence or absence of diacylglycerol kinase inhibitor R 59949. [γ-$^{32}$P]ATP (3 μCi/assay) in buffer A (5 μl) was added to the membranes, and phosphorylation was carried out for 15 s at 30 °C. Final concentrations (total volume of 25 μl) were 7.5 μM ATP, and, when added, $10^{-6}$ or $10^{-5}$ M inhibitor R 59949. The reaction was stopped by the addition of 3 ml of chloroform:methanol:concentrated HCl (200:100:0.75, by volume). Extraction and separation of the lipids was carried out as described for the PIP kinase assay. $^{32}$P-Labeled incorporation was visualized by autoradiography (exposure time, 16 h).

*Other Methods*—PIP kinase was purified from bovine brain membranes as described (14). The protein content of synaptosomal plasma membranes was determined according to the method of Bradford (21) using bovine serum albumin as a standard.

## RESULTS AND DISCUSSION

We have determined the effect of lipids on the enzymatic activity of PIP kinase purified from bovine brain membranes. This PIP kinase phosphorylates PIP specifically at the 5′-position.[2] The lipids presented to the enzyme were mixed with an excess of Triton X-100 to minimize the effect of the lipid substrates on the character of the micellar interface.

The effect of PA on the activity of PIP kinase is shown in Fig. 1. The molar ratio of PA:PIP in the assay was varied between 0.1 and 2 at a constant amount of PIP (80 μM). Already at a molar ratio of 0.1, a 2-fold increase in PIP kinase activity was observed. Stimulation was optimal at a molar ratio of 1, resulting in a 20-fold higher activity. To exclude the possibility that the stimulation was due to the presence of a contaminant in the PA preparation, PA was submitted to an additional purification step on a silica gel-high performance liquid chromatography column. This, however, had no effect on the observed stimulation by PA (data not shown).

The effect of other phospholipids on PIP kinase activity was tested as well. These lipids were: phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine, diacylglycerol, phosphatidylglycerol, and lyso-PA. In the assay, the amount of lipids used was the same as for PIP (80 μM). As shown in Table I, the activation of PIP kinase by PA is a highly specific. The only other lipid that gave a substantial activation (5-fold) was phosphatidylserine. This is in agreement with previous reports (12, 13). Other

[2] A. Moritz and B. Payrastre, unpublished results.

TABLE I

*Stimulation of PIP kinase by various lipids*

In the assay, mixed Triton X-100 micelles containing equimolar amounts of PIP and the lipid to be tested (both 80 μM) were used. The PIP kinase activity was assayed as described under "Experimental Procedures." Data are mean ± S.E. based on two experiments in triplicate ($n = 6$).

| Lipid added | $PIP_2$ formed | Stimulation |
|---|---|---|
| | *pmol/min* | *-fold* |
| None | 1.20 ± 0.02 | 1.00 ± 0.02 |
| Phosphatidic acid | 24.5 ± 1.2 | 20.4 ± 1.0 |
| Phosphatidylserine | 5.95 ± 1.15 | 4.96 ± 0.96 |
| Phosphatidylinositol | 3.30 ± 0.35 | 2.75 ± 0.29 |
| Phosphatidylglycerol | 2.22 ± 0.13 | 1.85 ± 0.11 |
| Lyso-phosphatidic acid | 2.31 ± 0.17 | 1.92 ± 0.14 |
| Dicetylphosphate | 1.32 ± 0.03 | 1.10 ± 0.03 |
| Phosphatidylethanolamine | 2.37 ± 0.24 | 1.97 ± 0.20 |
| Phosphatidylcholine | 1.28 ± 0.01 | 1.06 ± 0.01 |
| Diacylglycerol | 1.18 ± 0.04 | 0.98 ± 0.03 |

negatively charged phospholipids (*i.e.* phosphatidylinositol and phosphatidylglycerol) had a relatively small effect on the enzyme activity. This shows that the activation by PA is not merely an effect of negative charge. A striking observation is that lyso-PA exerts only a 2-fold stimulatory effect on the enzyme. In addition, the PA analog dicetylphosphate does not influence PIP kinase activity. These results strongly suggest that the interaction between PA and PIP kinase is very specific, possibly involving a binding site for PA. The stimulatory effect of PA on PIP kinase activity was also observed when both PA and PIP were incorporated in bilayers of phosphatidylcholine (results not shown). Magnesium, the only metal ion necessary for enzyme activity, is present in the assay at a much higher concentration than that of PA. In addition, traces of calcium present will be complexed by 1 mM EGTA. Hence metal chelation by PA can be ruled out as an explanation for the effects of the lipid on PIP kinase activity. Furthermore, it may be assumed that lyso-PA will not differ greatly from PA in its cation binding properties, yet its effect on PIP kinase activity is very low compared with that of PA (see above).

To test whether the stimulation of PIP kinase by PA is influenced by $PIP_2$ through product inhibition, various amounts of $PIP_2$ were added to the assay under conditions where the activation of the enzyme by PA was optimal (PA and PIP present in a molar ratio of 1, see Fig. 1). The molar ratio of $PIP_2$:PIP was varied from 0.1 to 1. As shown in Fig. 2, already at a molar ratio of 0.2, PIP kinase activity is decreased for more than 50%. When $PIP_2$ and PIP are present in a molar ratio of 1, the stimulatory effect of PA is completely suppressed.

It was also investigated whether PA has an effect on the endogenous PIP kinase activity of natural membranes. To this end, synaptosomal plasma membranes were incubated with phospholipase D in order to increase the endogenous PA content. Incubation of these treated membranes with [γ-$^{32}$P] ATP resulted in an enhanced labeling of $PIP_2$, while the labeling of PIP remained constant as compared with untreated membranes (Fig. 3, *lanes A* and *B*). Determination of $^{32}$P radioactivity by scintillation counting confirmed that, as a result of the phospholipase D treatment, the labeling of $PIP_2$ was more than 2-fold enhanced whereas the labeling of PIP was not affected (data not shown). Labeling of PA was also increased, probably due to phosphorylation of diacylglycerol formed by phosphohydrolase-mediated breakdown of PA generated in the membranes. The presence of an active PA phosphohydrolase in synaptosomal membranes has been reported (22). The presence of the diacylglycerol kinase inhibi-

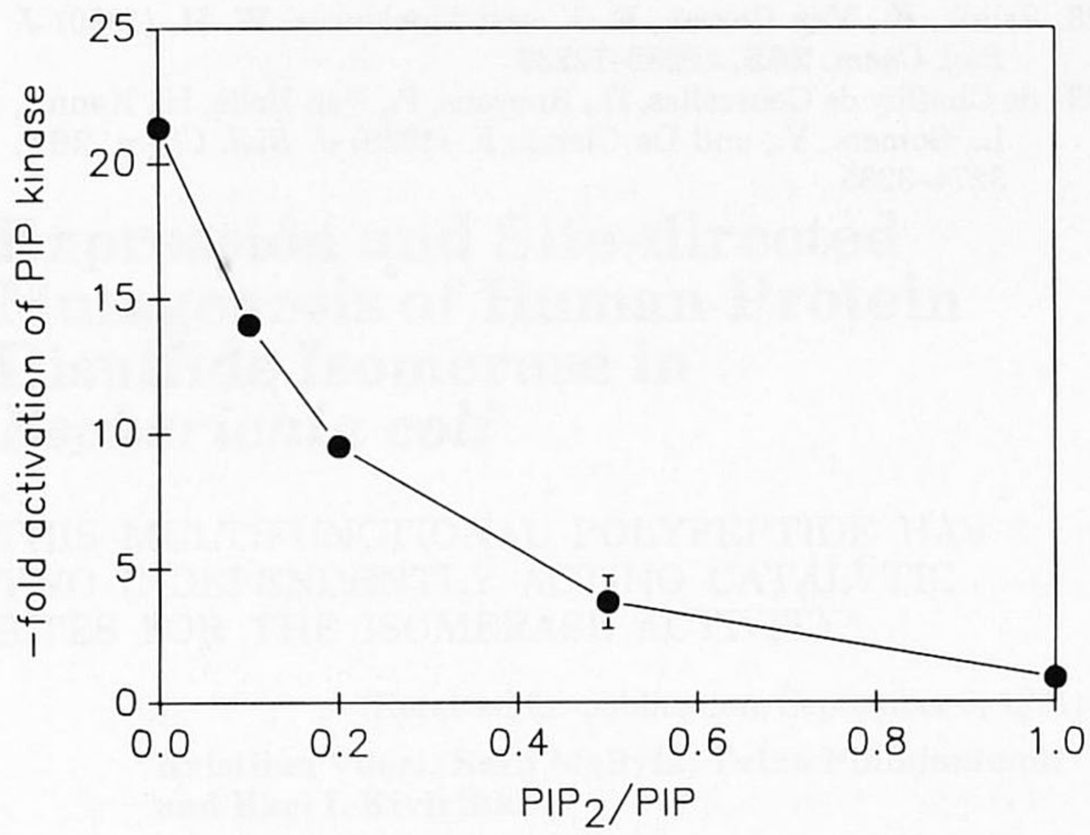


FIG. 2. **Inhibition of PA-stimulated PIP kinase by $PIP_2$.** Various amounts of $PIP_2$ were added to the assay containing equimolar amounts of PA and PIP (both 80 $\mu$M). The molar ratio of $PIP_2$/PIP was varied from 0.1 to 1. PIP kinase activity was assayed as described under "Experimental Procedures." Data are mean ± S.E. based on two experiments in triplicate ($n = 6$).

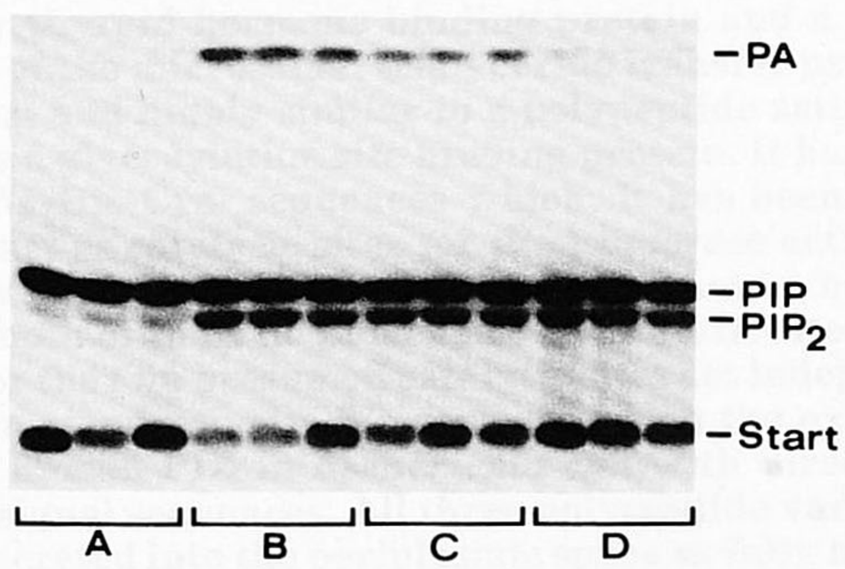


FIG. 3. **Stimulation of PIP kinase in synaptosomal plasma membranes by endogenous PA.** Synaptosomal plasma membranes were incubated with phospholipase D in the presence or absence of the diacylglycerol kinase inhibitor R 59949 and subsequently phosphorylated with [$\gamma$-$^{32}$P]ATP. Lipids were extracted, separated by thin layer chromatography, and $^{32}$P incorporation was visualized by autoradiography (exposure time, 16 h). *Lanes A,* control incubation; *lanes B,* incubation with phospholipase D; *lanes C,* incubation with phospholipase D in the presence of $10^{-6}$ M R 59949; *lanes D,* incubation with phospholipase D in the presence of $10^{-5}$ M R 59949.

tor R 59949 during the incubation of the membranes with phospholipase D greatly reduced the labeling of PA without affecting the labeling of $PIP_2$, showing that these two phosphorylation reactions were not related (Fig. 3, *lanes C* and *D*). The enhanced labeling of $PIP_2$ and PA on incubation with phospholipase D cannot be ascribed to endogenous lipid kinase activities of the enzyme preparation used. In assays of diacylglycerol kinase and PIP kinase activity, the phospholipase D preparation did not show any phosphorylation activity (data not shown).

Our results suggest that the activity of PIP kinase can be modulated by the PA content in membranes. A possible physiological role for such a modulation may be linked to alterations in membrane PA levels after hormonal activation of cells. This, however, remains to be established. A hormone-induced generation of PA has been reported to occur by phosphorylation of diacylglycerol after phospholipase C-catalyzed hydrolysis of phosphoinositides (23) or through activation of phospholipase D (24). Newly formed PA could then enhance the turnover of $PIP_2$ through activation of PIP kinase, providing a positive feedback mechanism which could sustain a hormonal response. $PIP_2$ is able to suppress the stimulation of PIP kinase by PA very effectively (see Fig. 2). Hence, prolonged synthesis of $PIP_2$ by PIP kinase should only be possible under conditions in which phospholipase C-mediated $PIP_2$ breakdown continues.

In several recent studies, enhanced breakdown of $PIP_2$ was observed after addition of PA to various cell types (25–27). It is not clear whether all of the reported effects can be ascribed to the action of PA. Jalink *et al.* (28) demonstrated that lyso-PA present in commercially available preparations of PA accounted for the effects originally observed in A 431 cells. This, however, has not been confirmed in other systems (26, 27). It has been proposed that the effects of exogenously added PA on cellular phosphoinositide turnover are due to activation of phosphoinositide-specific phospholipase C, either directly or indirectly (25–27). Direct stimulation of this enzyme by PA has been shown in *in vitro* studies (8, 9). In view of our findings, however, the observed enhancement of phosphoinositide turnover by addition of exogenous PA to intact cells may not solely be due to activation of phospholipase C but to the stimulation of PIP kinase activity as well. The latter activation may result in a faster replenishment of the $PIP_2$ pool accessible for enzymatic breakdown. Hence, PA may evoke a synergistic activation of PIP kinase and $PIP_2$-specific phospholipase C, providing a powerful positive feedback signal for the amplification of cellular responses after hormonal activation.

Future investigations will concentrate on the kinetic mechanisms by which PA activates PIP kinase, and on the ability of other PA-analogs, including PA-isomers, to substitute for PA in the activation process.

## REFERENCES

1. Berridge, M. J., and Irvine, R. F. (1984) *Nature* **312,** 315–321
2. Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56,** 159–193
3. Rana, R. S., and Hokin, L. E. (1990) *Physiol. Rev.* **70,** 115–164
4. Carpenter, C. L., and Cantley, L. C. (1990) *Biochemistry* **29,** 11147–11156
5. Farago, A., and Nishizuka, Y. (1990) *FEBS Lett.* **268,** 350–354
6. Lee, M.-H., and Bell, R. M. (1991) *Biochemistry* **30,** 1041–1049
7. Hannun, J. A., Loomis, C. R., Merril, A. H., and Bell, R. M. (1986) *J. Biol. Chem.* **261,** 12604–12609
8. Jackowski, S., and Rock, C. O. (1989) *Arch. Biochem. Biophys.* **268,** 516–524
9. Quian, Z., and Drewes, L. R. (1991) *FASEB J.* **5,** 315–319
10. Kanoh, H., Kondoh, H., and Ono, T. (1983) *J. Biol. Chem.* **258,** 1767–1774
11. Sakane, F., Yamada, K., and Kanoh, H. (1989) *FEBS Lett.* **255,** 409–413
12. Cochet, C., and Chambaz, E. M. (1986) *Biochem. J.* **237,** 25–31
13. Ling, L. E., Schulz, J. T., and Cantley, L. C. (1989) *J. Biol. Chem.* **264,** 5080–5088
14. Moritz, A., De Graan, P. N. E., Ekhart, P. F., Gispen, W. H., and Wirtz, K. W. A. (1990) *J. Neurochem.* **54,** 351–354
15. Gadella, T. W. J., Moritz, A., Westerman, J., and Wirtz, K. W. A. (1990) *Biochemistry* **29,** 3389–3395
16. Hendrickson, H. S., and Ballou, C. E. (1964) *J. Biol. Chem.* **239,** 1369–1373
17. Shuto, S., Ueda, S., Imamura, S., Fukukawa, K., Matsuda, A., and Ueda, T. (1987) *Tetrahedron Lett.* **28,** 199–202
18. Toner, M., Viano, G., McLaughlin, A., and McLaughlin, S. (1988) *Biochemistry* **27,** 7435–7443
19. Jolles, J., Zwiers, H., Dekker, A., Wirtz, K. W. A., and Gispen, W. H. (1981) *Biochem. J.* **194,** 283–291
20. Kristjansson, G. I., Zwiers, H., Oestreicher, A. B., and Gispen, W. H. (1982) *J. Neurochem.* **39,** 371–378
21. Bradford, M. M. (1976) *Anal. Biochem.* **76,** 248–254
22. Quian, Z., and Drewes, L. R. (1990) *J. Biol. Chem.* **265,** 3607–3610
23. Michell, R. H. (1975) *Biochim. Biophys. Acta* **415,** 81–147
24. Shukla, S. D., and Halenda, S. P. (1990) *Life Sci.* **48,** 851–866

25. Murayama, T., and Ui, M. (1987) *J. Biol. Chem.* **262,** 5522–5529
26. Kroll, M. H., Zavoico, G. B., and Schafer, A. I. (1989) *J. Cell. Physiol.* **139,** 558–564
27. Knauss, T. C., Jaffer, F. E., and Abboud, H. E. (1990) *J. Biol. Chem.* **265,** 14457–14463
28. Jalink, K., Van Corven, E. J., and Moolenaar, W. H. (1990) *J. Biol. Chem.* **265,** 12232–12239
29. de Chaffoy de Courcelles, D., Roevens, P., Van Belle, H., Kennis, L., Somers, Y., and De Clerck, F. (1989) *J. Biol. Chem.* **264,** 3274–3285