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SIMILAR PHYSICAL AND KINETIC PROPERTIES OF RAT BRAIN SYNAPTIC MEMBRANE AND CYTOSOL PHOSPHOINOSITIDE PHOSPHOLIPASES C[1]

Osamu Kozawa, Masahiko Hoshijima, Tetsuji Tanimoto, Toshihiko Ohmori, and Yoshimi Takai[2]

Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan

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SUMMARY: Phosphoinositide phospholipase C (PLC) was extracted from the synaptic membrane fraction of rat brain by 1% sodium deoxycholate. The molecular weight and sedimentation coefficient of the membrane PLC were about 160,000 and 6.7 as estimated by gel filtration and sucrose density gradient centrifugation, respectively. These values of the membrane PLC were identical with those of the cytosol PLC of the same tissue. Moreover, the membrane PLC showed the substrate specificity for phosphatidylinositol, phosphatidylinositol-4-monophosphate and phosphatidylinositol-4,5-bisphosphate and the sensitivity to Ca^{2+}, sodium deoxycholate and N-ethylmaleimide similar to those of the cytosol PLC. These results indicate that the rat brain synaptic membrane PLC is indistinguishable from the cytosol PLC in physical and kinetic properties.

PLC[3] catalyzes the hydrolysis of phosphoinositides including PI, PIP and PIP_2 to produce diacylglycerol and the respective inositol phosphates, IP_1, IP_2 and IP_3, among which diacylglycerol and IP_3 serve as messengers for protein kinase C activation and intracellular Ca^{2+} mobilization, respectively (for reviews, see

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[2] To whom all correspondence should be addressed.

[3] Abbreviations used are: PLC, phosphoinositide phospholipase C; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-monophosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; EGTA, [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; DCA, sodium deoxycholate; Chaps, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid.

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Refs. 1,2). Although the regulatory mechanism of PLC by receptors has long been unknown, recent studies using the plasma membranes isolated from several cell types have suggested that this enzyme is regulated by receptors through a GTP-binding protein(s) (3-8). In these experiments, isolated plasma membranes respond to the specific agonist to produce the inositol phosphates in a GTP-dependent manner, indicating that these plasma membranes contain the PLC linked to the GTP-binding protein.

PLC has been purified to near homogeneity from the cytosol fraction of several tissues and the cytosol PLC has been well characterized (9-13). PLC has also been found in the membrane fraction of several tissues (14-20), but the membrane PLC has not been well characterized.

The present studies were undertaken to characterize the rat brain synaptic membrane PLC in comparison with the cytosol PLC of the same tissue. This paper describes that the PLC which is extracted and partially purified from the synaptic membranes shows the physical and kinetic properties similar to those of the cytosol PLC.

EXPERIMENTAL PROCEDURES

Materials——Male Sprague-Dawley rats maintained ad libitum on CLEA laboratory chows were used. PI was from Serdary Research Laboratories. PIP and PIP_2 were from Sigma. [Inositol-2-^{3}H]PI (16.3 Ci/mmol) and [inositol-2-^{3}H]PIP (1.0 Ci/mmol) were from Amersham. [Inositol-2-^{3}H]PIP_2 (2.0 Ci/mmol) was from New England Nuclear. Other materials and chemicals were from commercial sources.

Preparation of a crude mitochondrial (P2) fraction and its subfractionation——All manipulations were carried out at 0-4°C. Five brains (5 g wet weight) were homogenized with a Potter-Elvehjem Teflon-glass homogenizer in 33 ml of Buffer A (20 mM Tris/HCl at pH 7.5, 0.32 M sucrose, 2 mM EDTA and 10 mM EGTA). The homogenate was diluted by the addition of 17 ml of Buffer A, and then centrifuged for 10 min at 1,000 x g. The supernatant was centrifuged for 15 min at 12,500 x g. The pellet was washed by resuspending in 50 ml of Buffer A and centrifuged for 15 min at 12,500 x g. The pellet was suspended in 15 ml of Buffer A and employed as a crude mitochondrial (P2) fraction. The P2 fraction was further fractionated into myelin, synaptosomes and mitochondria as described previously (21).

Preparation of membrane and soluble fractions from the P2 fraction——The P2 fraction (15 ml) was subjected to osmotic shock with 9 volumes of Buffer B (20 mM Tris/HCl at pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA and 10 mM 2-mercaptoethanol), homogenized, stood for 30 min and centrifuged for 30 min at 20,000 x g. The pellet was resuspended in 15 ml of Buffer B and used as a P2 membrane fraction. The supernatant was used as a P2 soluble fraction.

Extraction of PLC from the P2 membrane fraction with DCA—— PLC was extracted from the P2 membrane fraction (15 ml) by 1% DCA. The suspension was stood for 30 min and then centrifuged for 60 min at 100,000 x g. The supernatant was directly employed.

Preparation of a cytosol fraction from rat brain——Four brains (4 g wet weight) were homogenized in 15 ml of Buffer A as described above. The homogenate was centrifuged for 60 min at 100,000 x g. The supernatant (15 ml) was employed as a cytosol fraction after DCA was added to give a final concentration of 1%.

Assay for PLC——PLC was assayed by measuring the formation of the inositol phosphates from the respective radioactive phosphoinositides as described (12). The standard reaction mixture (0.2 ml) contained 50 mM Hepes at pH 7.0, 1 mM $CaCl_2$, 100 mM NaCl, 0.5 mg/ml of bovine serum albumin, 0.05% DCA, 250 μM [^{3}H]PI, [^{3}H]PIP or [^{3}H]PIP_2 (92,000 dpm) and an enzyme preparation. Free Ca^{2+} concentrations below 1 mM were controlled by Ca^{2+}-EGTA buffer (22). The incubation was carried out for 10 min at 37°C.

Determinations——Protein was determined by the method of Lowry et al. (23) with bovine serum albumin as a reference protein. The Mr of protein was estimated by gel filtration by the method of Andrews (24) using hemoglobin (Mr=64,000), γ-globlins (Mr=160,000) and catalase (Mr=230,000) as reference proteins, and also with an S value by the equation of Martin and Ames (25) using hemoglobin (4.4S) as a reference protein. The S value was determined in the presence of 0.6% Chaps as described earlier (26).

RESULTS

The PLC activity assayed with PI as a substrate was found not only in the cytosol fraction but also in the P2 fraction (Table I). When the P2 fraction was subfractionated into myelin, synaptosomes and mitochondria, most of the PLC activity was found in synaptosomes. When the P2 fraction was fractionated into the membrane and soluble fractions, the enzymatic activity was found in both fractions. The specific activities of PLC in both fractions were roughly the same, but the total activity in the P2 membrane fraction was 3-fold larger than that in the P2 soluble fraction. The P2 membrane PLC could be solubilized by 1% DCA, but could not be solubilized by washing with 0.15 M or 2 M KCl. Sonication of the P2 membrane fraction did not release the enzyme.

Table I

Subcellular distribution of PLC in rat brain

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)
Cytosol fraction	22	63	2.9
P2 fraction	8.4	12	1.4
Starting material (P2 fraction)	8.4	12	1.4
Myelin	1.1	0.45	0.41
Synaptosomes	4.0	7.2	1.8
Mitochondria	1.3	0.90	0.69
Starting material (P2 fraction)	8.4	12	1.4
P2 soluble fraction	2.0	2.9	1.5
P2 membrane fraction	6.6	9.5	1.4

The cytosol and P2 fractions were prepared from 1 g wet weight of rat brain. The enzymatic activity was assayed with [^{3}H]PI as a substrate under the standard conditions except that 20 mM sodium acetate at pH 5.5 was used instead of 50 mM Hepes at pH 7.0 and that 0.05% DCA was removed. One unit of PLC was defined as the amount of enzyme which generated 1 nmol of inositol phosphate from PI per min.

These results indicate that PLC is present not only in the cytosol fraction but also in the synaptic membrane fraction in rat brain.

The PLC solubilized from the P2 membrane fraction was subjected to a Sephacryl S-300 column and the enzyme was eluted under the conditions specified in the legend to Fig. 1. When each fraction was assayed for the PLC activity with PI as a substrate, two peaks appeared (Fig. 1A). The first minor peak appeared in the void volume and seemed to be an aggregated form of the second major peak[4]. The Mr of the major peak was estimated to be about 160,000.

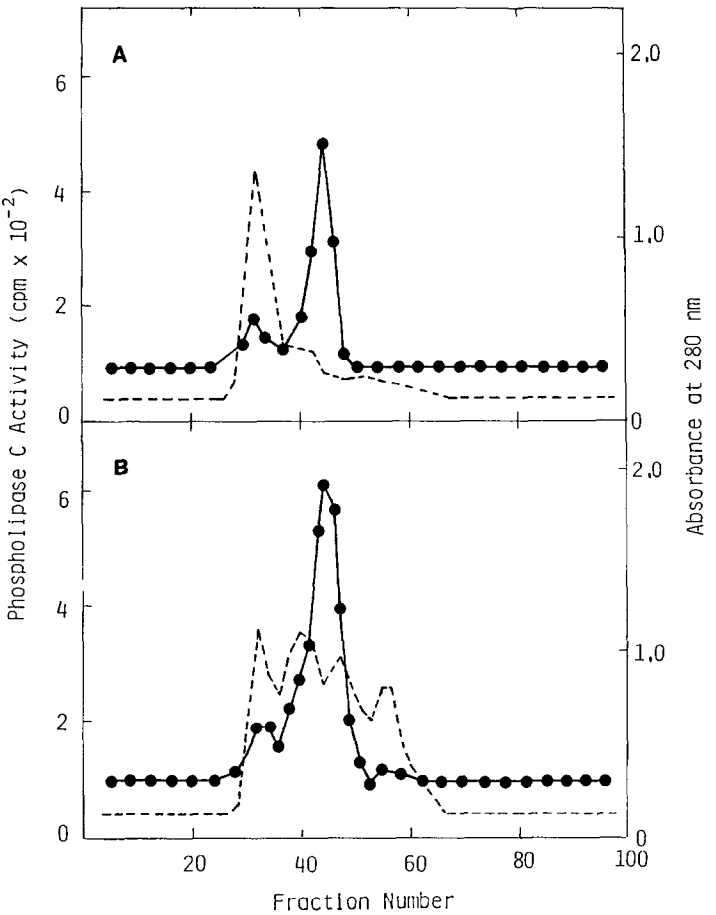

Fig. 1. Sephacryl S-300 column chromatographies of the membrane and cytosol PLCs. The PLC solubilized from the P2 membrane fraction (15 ml, 30 mg of protein) with DCA or the PLC in the cytosol fraction (15 ml, 87 mg of protein) was separately subjected to a Sephacryl S-300 column (2.5 x 102 cm) equilibrated with 20 mM Tris/HCl at pH 7.5 containing 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol and 0.6% Chaps. Fractions of 5 ml each were collected. The PLC activity of each fraction was assayed with [^{3}H]PI as a substrate under the standard conditions except that 20 mM sodium acetate at pH 5.5 was used instead of 50 mM Hepes at pH 7.0 and that 0.05% DCA was removed. (A), the membrane PLC; (B), the cytosol PLC. (●—●), the PLC activity; (----), absorbance at 280 nm.

In a separate experiment, the cytosol fraction was directly subjected to a Sephacryl S-300 column under the same conditions described above. The enzyme appeared in two peaks (Fig. 1B). The first minor peak appeared in the void volume and seemed to be an aggregated form of the second major peak[4]. The Mr of the major peak was estimated to be about 160,000.

[4] The PLC of the first minor peak showed the S value, the substrate specificity for PI, PIP and PIP_2, and the sensitivity to Ca^{2+}, DCA and N-ethylmaleimide similar to those of the PLC of the second major peak.

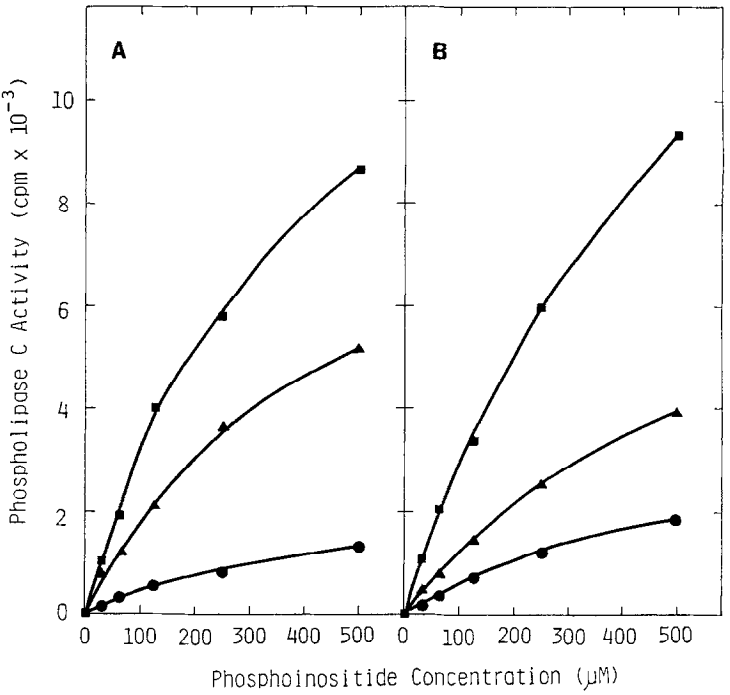

Fig. 2. Dose response curves of PI, PIP and PIP_2 for the membrane and cytosol PLCs. The enzymatic activity was assayed with various doses of either [^{3}H]PI, [^{3}H]PIP or [^{3}H]PIP_2 as a substrate under the standard conditions except that 1 mM Ca^{2+} was used for [^{3}H]PI and [^{3}H]PIP and that 10 μM Ca^{2+} was used for [^{3}H]PIP_2. Fraction 44 (4.5 μg of protein) in Fig. 1A and Fraction 44 (7.6 μg of protein) in Fig. 1B were used as the membrane and cytosol PLCs, respectively. (A), the membrane PLC; (B), the cytosol PLC. (●—●), with [^{3}H]PI; (■—■), with [^{3}H]PIP; (▲—▲), with [^{3}H]PIP_2.

In the next set of experiments, the physical and kinetic properties of the membrane and cytosol PLCs were compared. The S values of both PLCs were identical and were 6.7 as estimated by sucrose density gradient centrifugation (data not shown). This value corresponded to a Mr of about 120,000. Both the membrane and cytosol PLCs hydrolyzed not only PI but also PIP and PIP_2 to produce the respective inositol phosphates. Dose response curves of each phosphoinositide were similar between both PLCs (Fig. 2). Both PLCs displayed maximum reaction velocities in the order PIP> PIP_2>PI. Both the membrane and cytosol PLCs required low concentrations of Ca^{2+} for the hydrolysis of PI, PIP and PIP_2 (Fig. 3). Again, the dose response curves of Ca^{2+} with each phosphoinositide as a substrate were very similar between both

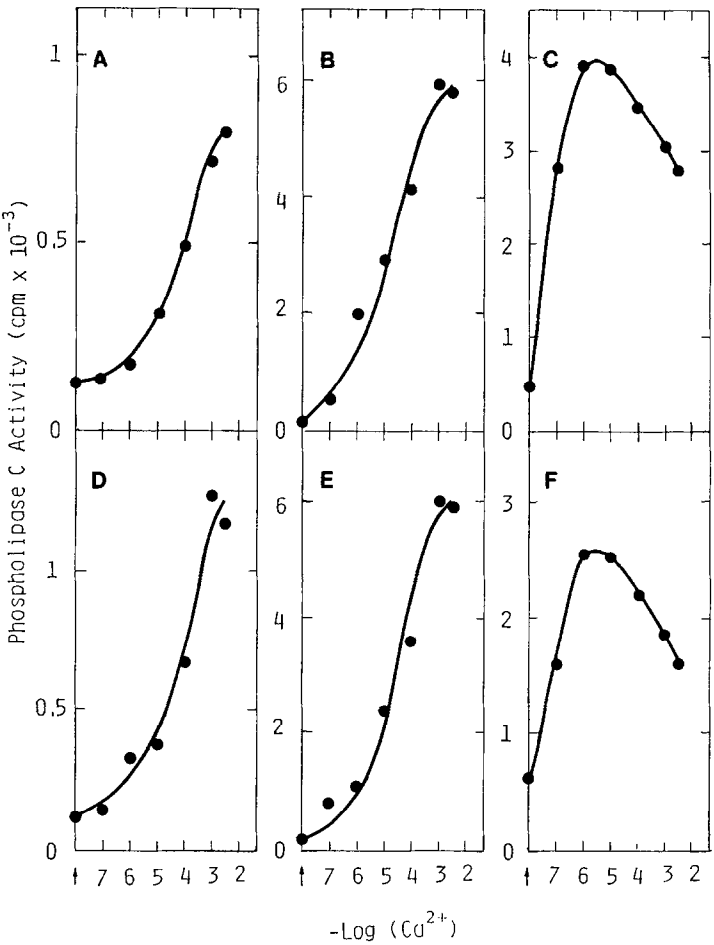

Fig. 3. Dose response curves of Ca^{2+} for the membrane and cytosol PLCs. The enzymatic activity was assayed with 250 μM [^{3}H]PI, [^{3}H]PIP or [^{3}H]PIP_2 as a substrate under the conditions described in the legend to Fig. 2 except that various doses of Ca^{2+} were added as indicated. Where indicated with an arrow, EGTA was added at a final concentration of 5 mM instead of $CaCl_2$. (A-C), the membrane PLC; (D-F), the cytosol PLC. (A,D), with [^{3}H]PI; (B,E), with [^{3}H]PIP; (C,F), with [^{3}H]PIP_2.

PLCs. Both PLCs were the most sensitive to Ca^{2+} with PIP_2 as a substrate. Both the membrane and cytosol PLCs were activated by 0.05% DCA but inhibited by this detergent at the doses higher than 0.2% when assayed with PI as a substrate. Dose response curves of DCA for both PLCs were very similar (data not shown). Both the membrane and cytosol PLCs were inactivated by treatment with 5 mM N-ethylmaleimide and these inhibitions were blocked by the simultaneous presence of 20 mM 2-mercaptoethanol when assayed with PI as a substrate (data not shown).

DISCUSSION

PLC was previously shown to be present in the membrane fractions of rat brain (14,15). However, the rat brain membrane PLC was neither purified nor characterized with PIP and PIP_2 as substrates. The present paper has described for the first time the properties of the PLC partially purified from rat brain synaptic membranes, in comparison with those of the cytosol PLC of the same tissue. The membrane PLC characterized here is not just a contaminant of the cytosol PLC, since the membrane PLC is extractable from the membranes by detergent but not by washing with an isotonic or hypertonic solution or by sonication. The membrane PLC hydrolyzes not only PI but also PIP and PIP_2 to produce the respective inositol phosphates. The membrane PLC requires low concentrations of Ca^{2+} for its enzymatic activity and is the most sensitive to this divalent cation with PIP_2 as a substrate. These kinetic properties of the membrane PLC are very similar to those of the cytosol PLC of the same tissue. Moreover, the membrane PLC shows the Mr similar to that of the cytosol PLC. These results suggest that not only the cytosol PLC but also the membrane PLC could be linked to the GTP-binding protein which serves as a transducer for receptors. It is more likely that the membrane PLC rather than the cytosol PLC is linked to this transducer, since it is well established that the various GTP-binding proteins are present in plasma membranes (for reviews, see Refs, 27,28). Further investigation is still necessary to clarify the regulatory mechanism of PLC by receptors.

Recently, Wang et al. (29) have partially purified PLC from the membrane and cytosol fractions of murine thymocytes, and have shown that the cytosol PLC is less sensitive to Ca^{2+} than the membrane PLC, particularly when PIP_2 is used as a substrate. Moreover, they have described that both the membrane and cytosol

PLCs show the same Mr of 70,000. These results are different from those of the brain PLCs presented here. The exact reason for these differences is not known, but may be due to the tissue specificity.

It has been described that there are multiple Mr forms of PLC in rat brain cytosol (30). In the present studies, both the membrane and cytosol PLCs of rat brain show one Mr of about 160,000 as estimated by gel filtration in the presence of Chaps, and one Mr of about 120,000 as estimated by sucrose density gradient centrifugation in the presence of the same detergent. However, when the membrane and cytosol PLCs are subjected to gel filtration in the absence of this detergent, at least three peaks with Mrs of over 160,000 are detected (data not shown). In the previous study (30), multiple Mr forms of the rat brain cytosol PLC were observed on gel filtration in the absence of any detergent. Therefore, the multiplicity of the rat brain cytosol PLC observed previously may be due just to aggregation of the enzyme, and there may be one Mr form of PLC in both the membrane and cytosol fractions of rat brain. These results of ours are consistent with the earlier observations (10,11) that the PLC purified from the platelet cytosol shows one Mr of about 140,000 as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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REFERENCES

1. Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315-321
2. Takai, Y., Kikkawa, U., Kaibuchi, K. and Nishizuka, Y. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res. 18, 119-158
3. Litosch, I., Wallis, C. and Fain J.N. (1985) J. Biol. Chem. 260, 5464-5471

4. Smith, C.D., Lane, B.C., Kusaka, I., Verghese, M.W. and Snyderman, R. (1985) J. Biol. Chem. 260, 5875-5878
5. Lucas, D.O., Bajjalieh, S.M., Kowalchyk, J.A. and Martin, T.F.J. (1985) Biochem. Biophys. Res. Commun. 132, 721-728
6. Uhing, R.J., Prpic, V., Jiang, H. and Exton, J.H. (1986) J. Biol. Chem. 261, 2140-2146
7. Guillon, G., Balestre, M.-N., Mouillac, B. and Devilliers, G. (1986) FEBS Lett. 196, 155-159
8. Kikuchi, A., Kozawa, O., Kaibuchi, K., Katada, T., Ui, M. and Takai, Y. (1986) J. Biol. Chem. 261, 11558-11562
9. Takenawa, T. and Nagai, Y. (1981) J. Biol. Chem. 256, 6769-6775
10. Hakata, H., Kambayashi, J. and Kōsaki, G. (1982) J. Biochem. 92, 929-935
11. Low, M.G., Carroll, R.C. and Cox, A.C. (1986) Biochem. J. 237, 139-145
12. Wilson D.B., Bross T.E., Hoffmann S.L. and Majerus, P.W. (1984) J. Biol. Chem. 259, 11718-11724
13. Banno, Y., Nakashima, S. and Nozawa, Y. (1986) Biochem. Biophys. Res. Commun. 136, 713-721
14. Durell, J., Garland, J.T. and Friedel, R.O. (1969) Science 165, 862-866
15. Lapetina, E.G. and Michell, R.H. (1973) Biochem. J. 131, 433-442
16. Allan, D. and Michell, R.H. (1978) Biochim. Biophys. Acta 508, 277-286
17. Akhtar, R.A. and Abdel-Latif, A.A. (1978) Biochim. Biophys. Acta 527, 159-170
18. Rothenberg, R.J., Moskowitz, R.W. and Malemud, C.J. (1983) Biochem. Biophys. Res. Commun. 115, 331-338
19. Cockcroft, S., Baldwin, J.M. and Allan, D. (1984) Biochem. J. 221, 477-482
20. Seyfred, M.A. and Wells, W.W. (1984) J. Biol. Chem. 259, 7666-7672
21. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y. (1982). J. Biol. Chem. 257, 13341-13348
22. Bartfai, T. (1979) Adv. Cyclic Nucleotide Res. 10, 219-242
23. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
24. Andrews, P. (1965) Biochem. J. 96, 595-606
25. Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372-1379
26. Takai, Y., Nakaya, S., Inoue, M., Kishimoto, A., Nishiyama, K., Yamamura, H. and Nishizuka, Y. (1976) J. Biol. Chem. 251, 1481-1487
27. Gilman, A.G. (1984) Cell 36, 577-579
28. Ui, M. (1984) Trends Pharmacol. Sci. 5, 277-279
29. Wang, P., Toyoshima, S. and Osawa, T. (1986) J. Biochem. 100, 1015-1022
30. Low, M.G., Carroll, R.C. and Weglicki, W.B. (1984) Biochem. J. 221, 813-820