Anandamide stimulates phospholipase D activity in PC12 cells but not in NIH 3T3 fibroblasts

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Abstract The endogenous cannabinoid arachidonoylethanolamide was previously reported to have no effects on the phospholipase D activity in Chinese hamster ovary cells expressing the human brain-specific cannabinoid receptor, while in mouse peritoneal cells, Δ^9-tetrahydrocannabinol stimulated this enzyme. In this work, arachidonoylethanolamide (0.1–1 μM) was found to stimulate the phospholipase D-mediated phospholipid hydrolysis in rat adrenal pheochromocytoma PC12 cells, but not in mouse NIH 3T3 fibroblasts. The phospholipase D-activating effects of arachidonoylethanolamide were comparable to those elicited by phorbol ester and nerve growth factor, while arachidonic acid (1 μM) had no effects. The results show that, depending on the cell type, arachidonoylethanolamide can be an activator of the phospholipase D system.

Key words: Anandamide; PC12 cell; Phospholipase D; Phorbol ester; Nerve growth factor

1. Introduction

The arachidonic acid derivative arachidonoylethanolamide (anandamide), formed by phosphodiesterase-mediated cleavage of *N*-arachidonoyl-phosphatidylethanolamine [1,2], binds to specific CB1 and CB2 cannabinoid receptors expressed primarily in the nervous system and the periphery, respectively [3–7]. Since anandamide also occurs in the human brain and various peripheral tissues [8], and it can elicit typical cannabimimetic responses [9,10], it is generally considered to be an endogenous cannabinoid. For this reason, it is of great interest to identify the signal transduction mechanisms which are regulated by anandamide. Previous studies identified the adenylyl cyclase system [11–14], calcium channels [15,16], the intercellular calcium signalling system [17] and mitogen-activated protein kinases [18] as targets of anandamide actions.

Phospholipase D (PLD)-mediated hydrolysis of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) produces phosphatidic acid [19,20], which has the potential to act both as a mitogen (reviewed in [19]) and a regulator of intracellular (Golgi) protein trafficking [21,22]. Phosphatidic acid can also be degraded to 1,2-diacylglycerol, an important endogenous activator of the protein kinase C system, which in turn plays a role in the cell growth regulation [23]. Because PLD can influence a variety of different cellular processes, including the formation of anandamide itself, we tested the possible effects of anandamide on PLD-mediated PtdCho and PtdEtn hydrolysis in the rat pheochromocytoma PC12 cells and NIH 3T3 cells. PC12 cells were chosen because they are widely used models to study the neuronal differentiation, they also produce anandamide [24] and express a well-characterized PLD system which responds to nerve growth factor (NGF) and phorbol 12-myristate 13-acetate (PMA) [25–27]. On the other hand, while NIH 3T3 fibroblasts also express the PtdCho- and PtdEtn-hydrolyzing PLD systems [28–30], they are not known to produce anandamide or express cannabinoid receptors.

So far, very few observations have been reported dealing with the possible regulation of PLD by cannabinoids. Thus, in an earlier report [5], anandamide was found to stimulate the arachidonic acid release but not the PLD activity in Chinese hamster ovary cells expressing the human brain-specific cannabinoid receptor. However, in mouse peritoneal cells, Δ^9-tetrahydrocannabinol stimulated the PLD-mediated formation of phosphatidylethanol (PtdEtOH) [31]. Here we show that in PC12 cells, but not in NIH 3T3 cells, relatively low concentrations of anandamide can stimulate the PLD-mediated hydrolysis of both PtdCho and PtdEtn.

2. Materials and methods

2.1. Materials

Anandamide and arachidonic acid were bought from Matreya. PMA, *N*-oleoylethanolamide, arachidonic acid ethyl ester (AAEE) and Dowex-50W (H^+ form) were purchased from Sigma. NGF was from Boehringer Mannheim, PtdEtOH was bought from Avanti Polar Lipids. 2-[^{14}C]ethanolamine (50 mCi/mmol), 1-[^{14}C]palmitic acid (60 mCi/mmol) and mrthyl-[^{14}C]choline chloride (50 mCi/mmol) were purchased from Amersham. Tissue culture reagents, including fetal calf serum, were from Gibco BRL.

2.2. Cell culture

PC12 cells (American Type Culture Collection) were maintained in 75 cm^2 flasks as loosely attached cultures in RPMI 1640 medium containing 10% heat-inactivated horse serum, 5% fetal calf serum, 50 U/ml of penicillin and 50 μg/ml of streptomycin. Suspended cells were harvested for the experiments after shaking the flasks. NIH 3T3 clone-7 fibroblasts, kindly provided by Dr Douglas R. Lowy (National Cancer Institute, NIH, Bethesda, MD, USA), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, penicillin-streptomycin (50 U/ml and 50 μg/ml, respectively) and glutamine (2 mM).

2.3. Measurement of PtdEtOH formation in PC12 cells

PC12 cells grown in flasks were incubated in complete medium for 48 h with 1-[^{14}C]palmitic acid (0.5 μCi/ml). The cells were harvested, washed and incubated in new flasks for another 2 h in fresh serum-free medium to facilitate esterification of remaining free radiolabelled

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Abbreviations: Anandamide, *N*-arachidonoylethanolamide; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdEtOH, phosphatidylethanol; NGF, nerve growth factor; PMA, phorbol 12-myristate 13-acetate; AAEE, arachidonic acid ethyl ester

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palmitic acid. Washed, suspended cells (~1.3–1.5×10^6 cells/ml) were then incubated in serum-free medium (0.75 ml final volume) for 15 min in the presence of 150 mM ethanol and PLD activators. Incubations were stopped by adding 4 ml chloroform:methanol (1:1) to the tubes. After phase separation, PtdEtOH was separated on potassium oxalate (1%)-impregnated Silica Gel H plates as described earlier [32].

2.4. Determination of the PLD-mediated formation of [^{14}C]choline and [^{14}C]ethanolamine

Loosely attached PC12 cells grown in flasks as well as attached NIH 3T3 fibroblasts grown in 12-well plates in full medium were labelled with [^{14}C]choline (0.75 μCi/ml) or [^{14}C]ethanolamine (0.5 μCi/ml) for 48 h. At the end of the labelling period, the density of PC12 cells was around 1.3–1.5×10^6 cells/ml, while attached fibroblasts were ~90% confluent. Cells were washed and then incubated for 3 h in fresh serum-free medium to complete the incorporation of the remaining radioactive precursors into the corresponding cellular phospholipids. Treatments of newly washed suspended PC12 and attached NIH 3T3 cells were performed in serum-free medium (0.75 ml final volume) for 15 min. In case of PC12 cells, the incubations were terminated as above, while NIH 3T3 cells were scraped into 2 ml ice-cold methanol and rapidly transferred to tubes containing 2 ml chloroform. After phase separation, radiolabelled choline and ethanolamine were separated from other products on Dowex 50W-H$^+$-packed columns as described earlier [33].

3. Results

In [^{14}C]choline- and [^{14}C]ethanolamine-labelled cells, the loss of the corresponding radiolabelled phospholipids upon treatments with PMA, growth factors and numerous other agents usually occurs by a PLD-mediated mechanism [19]. Thus, in the first experiments, PC12 cells prelabelled with these precursors were used to examine the possible effects of anandamide phospholipid hydrolysis. In [^{14}C]choline-labelled PC12 cells, a detectable increase in the formation of [^{14}C]choline from [^{14}C]PtdCho was elicited by 0.1 μM anandamide and a maximal (~2.5-fold) stimulatory effect required about 1 μM anandamide (Fig. 1). In [^{14}C]ethanolamine-labelled PC12 cells, 1 μM anandamide also enhanced the formation of [^{14}C]ethanolamine from [^{14}C]PtdEtn about 1.6-fold (Fig. 1). However, the PtdEtn-hydrolyzing PLD system was clearly less sensitive to anandamide compared to the PtdCho-hydrolyzing PLD. In other experiments, a 15 min incubation period was found to be optimal for the detection of these anandamide effects.

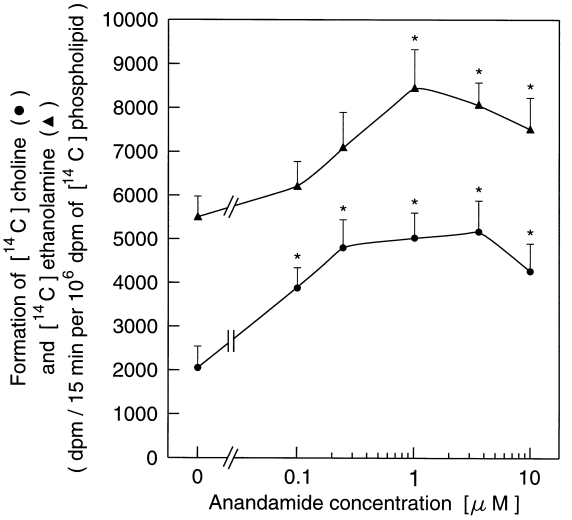

Fig. 1. Stimulatory effects of anandamide on the hydrolysis of PtdCho and PtdEtn in PC12 cells. PC12 cells, prelabelled with either [^{14}C]choline (●) or [^{14}C]ethanolamine (▲) for 48 h, were treated for 15 min with 0.1–10 μM concentrations of anandamide. Newly formed radiolabelled choline and ethanolamine were analyzed as described under Section 2. The average ^{14}C content of PtdCho and PtdEtn was 166 000 and 268 000 dpm/10^6 cells, respectively. Each point represents the mean ± S.E.M. of three experiments each performed in triplicate. *Significantly ($P < 0.05$–0.01) different from the value obtained without anandamide (unpaired t-test).

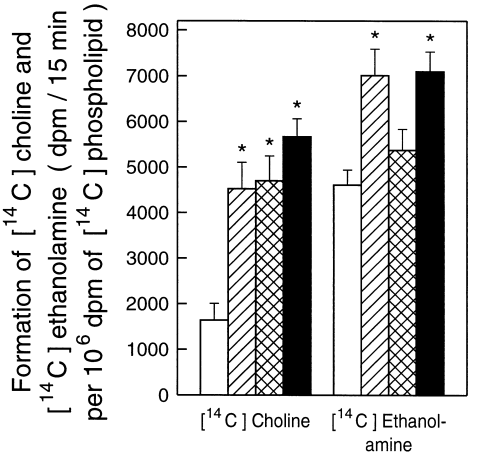

Fig. 2. Comparison of the effects of anandamide, PMA and NGF on the hydrolysis of PtdCho and PtdEtn in PC12 cells. PC12 cells, prelabelled with either [^{14}C]choline or [^{14}C]ethanolamine for 48 h, were incubated for 15 min in the absence (□) or presence of 1 μM anandamide (hatched), 200 ng/ml NGF (cross-hatched) or 100 nM PMA (■). The data are the means ± S.E.M. of three experiments each performed in triplicate. *Significantly ($P < 0.01$) different from the corresponding value obtained in the absence of activators.

In prelabelled PC12 cells, a maximal effective concentration (100 nM) of PMA and 1 μM anandamide induced the hydrolysis of both PtdCho and PtdEtn to similar extents (Fig. 2). However, an optimal concentration (200 ng/ml) of NGF enhanced only PtdCho but not PtdEtn hydrolysis (Fig. 2). Interestingly, neither oleoylethanolamide nor arachidonic acid, each examined at 1 μM concentration, stimulated the PtdCho hydrolysis. In contrast, 1 μM AAEE was about 60–70% as

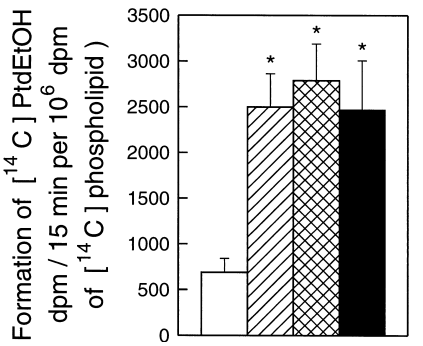

Fig. 3. Comparison of the effects of anandamide, PMA and NGF on the PtdEtOH formation in PC12 cells. [^{14}C]palmitic acid-prelabelled PC12 cells were incubated for 15 min in the presence of 150 mM ethanol in the absence (□) or presence of 1 μM anandamide (hatched), 100 nM PMA (cross-hatched) or 200 ng/ml NGF (■). The average ^{14}C content of total phospholipid was 306 000 dpm/10^6 cells. The data are the means ± S.D. of six incubations in a single experiment. This experiment was repeated once ($n = 4$) with similar results. *Significantly ($P < 0.01$) different from the value obtained without activators.

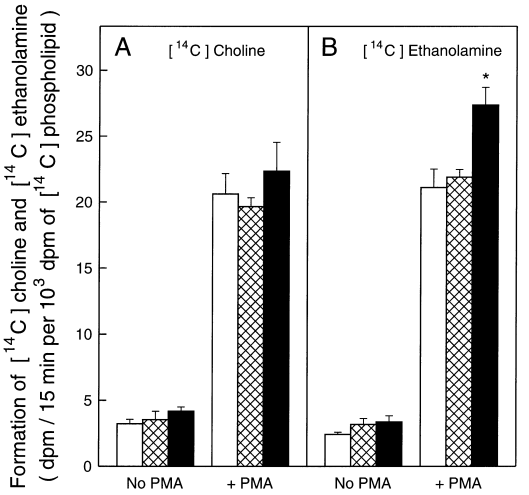

Fig. 4. Anandamide does not stimulate the PLD activity in NIH 3T3 fibroblasts. NIH 3T3 fibroblasts, prelabelled with [^{14}C]choline (A) or [^{14}C]ethanolamine (B) for 48 h, were untreated or treated with 100 nM PMA for 15 min, as indicated, in the absence (□) or presence of 1 μM anandamide (cross-hatched) or 10 μM anandamide (■). The data are the means ± S.D. of six incubations in a single experiment. Similar results were obtained in another experiment ($n = 3$). *Significantly ($P < 0.01$) different from the value obtained with PMA alone.

effective as anandamide in stimulating both PtdCho and PtdEtn hydrolysis (data not shown). It should be added that in PC12 cells the basal rate of ethanolamine formation was about three times greater than the formation of choline. While this may reflect a higher activity state of the PtdEtn-hydrolyzing PLD enzyme, this possibility remains to be determined.

PLD catalyzes the formation of phosphatidylalcohol in the presence of a primary alcohol. Treatment of [^{14}C]palmitic acid-prelabelled PC12 cells with 1 μM anandamide in the presence of 150 mM ethanol resulted in about a 3.6-fold increase in [^{14}C]PtdEtOH formation from prelabelled cellular phospholipids (Fig. 3). PMA and NGF caused similar increases in PtdEtOH formation (Fig. 3).

In NIH 3T3 fibroblasts, numerous agents, including PMA, stimulate PLD-mediated hydrolysis of both PtdCho and PtdEtn [19,28–30]. However, in [^{14}C]choline-labelled NIH 3T3 cells, 1–10 μM concentrations of anandamide were unable to stimulate [^{14}C]PtdCho hydrolysis or to enhance the effect of PMA (Fig. 4A). Similarly, in [^{14}C]ethanolamine-labelled NIH 3T3 cells, anandamide alone had no effect on the hydrolysis of [^{14}C]PtdEtn (Fig. 4B). Interestingly, in these cells, 10 μM anandamide slightly (1.3-fold), but significantly, enhanced the stimulatory effect of PMA on the [^{14}C]PtdEtn hydrolysis (Fig. 4B). However, in other studies, several membrane interacting agents, including sphingosine ([29]), ethanol ([34]) and tamoxifen (Z. Kiss, unpublished observation), also enhanced the effect of PMA on the PtdEtn hydrolysis. Thus, the potentiating effect of 10 μM anandamide on PMA-induced PtdEtn hydrolysis probably occurs by a non-specific mechanism.

4. Discussion

The major finding of this work is that in PC12 cells relatively low concentrations of anandamide were able to stimulate the PLD-mediated hydrolysis of both PtdCho and PtdEtn. The effects of anandamide were not mimicked by oleoylethanolamide or arachidonic acid each used at a relevant (1 μM) concentration. In contrast to PC12 cells, in NIH 3T3 fibroblasts, anandamide did not stimulate the hydrolysis of either of these phospholipids, although the PLD system in these cells responds to many widely different agents, including PMA, growth factors, oxidants, sphingosine and tamoxifen (reviewed in [19]). Overall, these observations suggest that the effects of anandamide on PLD activation are mediated by a specific mechanism(s) not generally present in all cell types.

On the other hand, receptor-mediated effects of anandamide on pre-implantation mouse embryo were observed in the 3.5–14 nM concentration range [35] and 0.1–1 μM concentrations of anandamide were able to enhance the growth of myeloblastic cells by a receptor-independent mechanism [36]. An additional interesting finding (reported here) is that AAEE, which is not known to interact with either the CB1 or CB2 receptor, partially mimicked the effects of anandamide on the PLD activity in PC12 cells. Finally, and perhaps most importantly, in a Chinese hamster ovary cell line highly expressing the brain-specific cannabinoid receptor, anandamide had no effects on the PLD activity [5]. When taken together, these observations do not seem to support the mediatory role of the presently known cannabinoid receptors. However, it should be added that other receptors may exist for anandamide [37] and that the non-receptor-mediated effects of anandamide may be as important as the receptor-mediated ones [17].

Clearly, further work is required to determine the mechanism of anandamide effects on the phospholipid hydrolysis including the involvement of cannabinoid receptors. However, regardless of the mechanism involved, an even more important question concerns the possible physiological relevance of anandamide effects on the PLD activity. In this context, the first important consideration is that the effects of anandamide on the PLD activity required concentrations which may be in the physiological range [8]. Furthermore, since the PtdCho- and PtdEtn-hydrolyzing PLD activities can be activated by many diverse stimuli [19,20], it is conceivable that the activity of the anandamide-synthesizing PLD can also be upregulated by certain physiological factors. Second, PLD is a potentially important regulator of intracellular vesicle-mediated protein trafficking and cell growth [38–42]. Therefore, PLD activation may play a role in the mediation of positive anandamide effects on various growth regulatory signal transduction events such as the activation of mitogen-activated protein kinase [18]. Third, although only two cell lines were analyzed here, the results suggest that anandamide may target PLD activities only in certain tissues. If so, this would provide an additional level of specificity of these anandamide effects.

In summary, here we have reported the novel finding that relatively low concentrations of anandamide stimulate the PLD activity in PC12 but not in NIH 3T3 cells. Although both the mechanism and the physiological significance of these effects remain to be determined, it is likely that stimulation of PLD activity contributes to the physiological actions of anandamide.

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References

[1] Schmid, H.H.O., Schmid, P.C. and Natarajan, V. (1990) Prog. Lipid Res. 29, 1–43.
[2] Schmid, H.H.O., Schmid, P.C. and Natarajan, V. (1996) Chem. Phys. Lipids 80, 133–142.
[3] Matsuda, L.A., Lolait, S.J., Browstein, M.J., Young, A.C. and Bonner, T.I. (1990) Nature 346, 561–564.
[4] Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. and Mechoulam, R. (1992) Science 258, 1946–1949.
[5] Felder, C.C., Briley, E.M., Axelrod, J., Simpson, J.T., Mackie, K. and Devane, W.A. (1993) Proc. Natl. Acad. Sci. USA 90, 7656–7660.
[6] Munro, S., Thomas, K.L. and Abu-Shaar, M. (1993) Nature 365, 61–65.
[7] Facci, L., Toso, R.D., Romanello, S., Buriani, A., Skaper, S.D. and Leon, A. (1995) Proc. Natl. Acad. Sci. USA 92, 3376–3380.
[8] Felder, C.C., Nielsen, A., Briley, E.M., Palkovits, M., Priller, J., Axelrod, J., Nguyen, D.N., Richardson, J.M., Riggin, R.M., Koppel, G.A., Paul, S.M. and Becker, G.W. (1996) FEBS Lett. 393, 231–235.
[9] Di Marzo, V., De Petrocellis, L., Bisogno, T. and Maurelli, S. (1995) J. Drug Dev. Clin. Pract. 7, 199–219.
[10] Berdishev, E.V., Boichot, E. and Lagente, V. (1996) J. Lipid Med. Cell Signal. 15, 49–67.
[11] Vogel, Z., Barg, J., Levy, R., Saya, D., Heldman, E. and Mechoulam, R. (1993) J. Neurochem. 61, 352–355.
[12] Van der Kloot, W. (1994) Brain Res. 649, 181–184.
[13] Bayewitch, M., Avidor-Reiss, T., Levy, R., Barg, J., Mechoulam, R. and Vogel, Z. (1995) 375, pp. 143–147.
[14] Bonhaus, D.W., Chang, L.K., Kwan, J. and Martin, G.R. (1998) J. Pharmacol. Exp. Ther. 287, 884–888.
[15] Mackie, K., Devane, W.A. and Hille, B. (1993) Mol. Pharmacol. 44, 498–503.
[16] Pan, X.H., Ikeda, S.R. and Lewis, D.L. (1998) Mol. Pharmacol. 54, 1064–1072.
[17] Venance, L., Piomelli, D., Glowinski, J. and Giaume, C. (1995) Nature 376, 590–594.
[18] Wartman, M., Campbell, D., Subramanian, A., Burstein, S.H. and Davis, R.J. (1995) FEBS Lett. 359, 133–136.
[19] Kiss, Z. (1996) Chem. Phys. Lipids 80, 81–102.
[20] Exton, J.H. (1998) Biochim. Biophys. Acta 1436, 105–115.
[21] Ktistakis, N.T., Brown, H.A., Sternweis, P.C. and Roth, M.G. (1995) Proc. Natl. Acad. Sci. USA 92, 4942–4956.
[22] Ktistakis, N.T., Brown, H.A., Waters, M.G., Sternweis, P.C. and Roth, M.G. (1996) J. Cell. Biol. 134, 295–306.
[23] Blobe, G.C., Obeid, L.M. and Hannun, Y.A. (1994) Cancer Metastasis Rev. 13, 411–431.
[24] Bisogno, T., Katayama, K., Melck, D., Ueda, N., De Petrocellis, L., Yamamoto, S. and Di Marzo, V. (1998) Eur. J. Biochem. 254, 634–642.
[25] Altin, J.G. and Bradshaw, R.A. (1990) J. Neurochem. 54, 1666–1676.
[26] Purkiss, J., Murrin, R.A., Owen, P.J. and Boarder, M.R. (1991) J. Neurochem. 57, 1084–1087.
[27] Horwitz, J. and Ricanati, S. (1992) J. Neurochem. 59, 1474–1480.
[28] Kiss, Z. and Anderson, W.B. (1989) J. Biol. Chem. 264, 1483–1487.
[29] Kiss, Z. and Deli, E. (1992) Biochem. J. 288, 853–858.
[30] Kiss, Z. and Anderson, W.H. (1994) Arch. Biochem. Biophys. 311, 430–436.
[31] Burstein, S., Budrow, J., Debatis, M., Hunter, S.A. and Subramanian, A. (1994) Biochem. Pharmacol. 48, 1253–1264.
[32] Kiss, Z. and Tomono, M. (1995) FEBS Lett. 371, 185–187.
[33] Mukherjee, J.J., Chung, T., Ways, D.K. and Kiss, Z. (1996) J. Biol. Chem. 271, 28912–28917.
[34] Kiss, Z., Crilly, K. and Chattopadhyay, J. (1991) Eur. J. Biochem. 197, 785–790.
[35] Paria, B.C., Das, S.K. and Dey, S.K. (1995) Proc. Natl. Acad. Sci. USA 92, 9460–9464.
[36] Derocq, J.-M., Bouaboula, M., Marchand, J., Rinaldi-Carmona, M., Segui, M. and Casellas, P. (1998) FEBS Lett. 425, 419–425.
[37] Ueda, N., Kurahashi, Y., Yamamoto, S. and Tokunaga, T. (1995) J. Biol. Chem. 270, 23823–23827.
[38] Kahn, R.A., Yucel, J.K. and Malhotra, V. (1993) Cell 75, 1045–1048.
[39] Cockcroft, S., Thomas, G.M.H., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N.F., Truong, O. and Hsuan, J.J. (1994) Science 263, 523–526.
[40] Ktistakis, N.T., Brown, H.A., Sternweis, P.C. and Roth, M.G. (1995) Proc. Natl. Acad. Sci. USA 92, 4952–4956.
[41] Liscovitch, M. and Cantley, L.C. (1995) Cell 81, 659–662.
[42] Chen, Y.-G., Siddhanta, A., Austin, C.D., Hammond, S.M., Sung, T.-C., Frohman, M.A., Morris, A.J. and Shields, D. (1997) J. Cell Biol. 138, 495–504.