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Activation of polyphosphoinositide phospholipase C by guanosine 5′-*O*-(3-thio)triphosphate and fluoroaluminate in membranes prepared from a human T cell leukemia line, JURKAT

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Polyphosphoinositide hydrolysis was studied in membranes prepared from a human T cell leukemia line, JURKAT, prelabeled with *myo*-[2-^{3}H]inositol. The formation of inositol bis- and trisphosphates was stimulated in a buffer with 110 nM free Ca^{2+} with a nonhydrolyzable GTP analogue, GTPγS, and NaF plus $AlCl_3$ in a time- and concentration-dependent manner. GTPγS and NaF-$AlCl_3$ had no significant effect on the inositol monophosphate level. $AlCl_3$ enhanced the NaF-stimulated release of inositol polyphosphates. Optimum concentrations of NaF and $AlCl_3$ produced 1.5-fold more inositol polyphosphates than that produced by optimum concentration of GTPγS. OKT3 monoclonal antibody, an antibody against the T-cell receptor complex, did not stimulate the inositol polyphosphate formation by JURKAT membranes even in the presence of GTP, although the antibody at the concentrations used markedly stimulated the hydrolysis of polyphosphoinositides in intact JURKAT cells.

Inositol trisphosphate; Phosphatidylinositol bisphosphate; GTP-binding protein; Guanine nucleotide; Fluoroaluminate; T-cell receptor

1. INTRODUCTION

Stimulation of a human T cell leukemia line, JURKAT, by phytohemagglutinin and concanavalin A initiates rapid hydrolysis of PtdIns(4,5)P_2, formation of inositol phosphates, and labeling of phosphatidic acid with [^{32}P]phosphate [1–3]. An increase in $[Ca^{2+}]_i$ accompanies the hydrolysis of PtdIns(4,5)P_2 [2,4]. At least one receptor, or possibly the only receptor [4,5], participating in the lectin-induced transmembrane signaling is the T-cell antigen receptor composed of the clonotypic α/β chain heterodimer (Ti) and its associated, monomorphic molecules, three CD3 subunits and CD3-ζ subunits [6]. Thus, monoclonal antibodies directed against either Ti or CD3 induce the increase in $[Ca^{2+}]_i$ and the hydrolysis of PtdIns(4,5)P_2 in JURKAT [4,7–9]. These results indicate that the activation of T lymphocytes by antigen presenting cells and target cells induces the hydrolysis of PtdIns(4,5)P_2 and subsequent increase in $[Ca^{2+}]_i$ and activation of protein kinase C.

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Abbreviations: PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; InsP_3, inositol trisphosphate; InsP_2, inositol bisphosphate; InsP_1, inositol monophosphate; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate

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Recent studies in hepatocytes [10], blowfly salivary glands [11], cloned rat pituitary cells (GH_3) [12,13], WRK1 cells [14], and HL-60 cells [15] have successfully demonstrated the receptor-stimulated hydrolysis of PtdIns(4,5)P_2 in cell-free systems. These studies have shown that a guanine nucleotide-binding regulatory protein(s) (G-protein) is involved in the coupling of the various receptors for Ca^{2+}-mobilizing agonists to the hydrolysis of PtdIns(4,5)P_2 and PtdIns4P. Ti-α and β chains and CD3-δ and ϵ chains in the Ti/CD3 complex have been cloned and sequenced [16–18]. Therefore, it is interesting to know how the Ti/CD3 complex participates in the hydrolysis of PtdIns(4,5)P_2. The present study has been undertaken to characterize the polyphosphoinositide phospholipase C in JURKAT membranes with the hope of elucidating the mechanism of activation of phospholipase C by the monoclonal antibody against CD3.

2. MATERIALS AND METHODS

2.1. *Materials*

OKT3 monoclonal antibody was prepared from culture supernatant of the OKT3 hybridoma, obtained from the American Type Culture Collection, by protein A-Sepharose CL-4B (Pharmacia) chromatography. *myo*-[2-^{3}H]Inositol (2.6 Ci/mmol) was prepared by reduction of *scyllo*-inosose (Sigma) with NaB^3H_4 (10.3 Ci/mmol, Amersham), followed by extensive purification by paper chromatography. Commercial sources of chemicals were as follows: GTPγS, Boehringer Mannheim; GTP, Sigma; AG1-X8 (200–400 mesh, formate form), Bio-Rad.

2.2. *Cell culture and membrane preparation*

JURKAT-FHCRC cells were cultured in Iscove's modified Dulbecco's medium (Gibco) supplemented with 5% heat-inactivated (56°C for 30 min) fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin [19]. The cells were labeled for 2 days in the same medium with *myo*-[2-^{3}H]-inositol, which was added at 10 μCi/ml unless otherwise stated. The labeled cells were washed twice with Tris-buffered saline without Ca^{2+} and Mg^{2+}, and suspended at a concentration of 2×10^7 cells/ml in a homogenization medium composed of 10 mM Hepes at pH 7.4, 110 mM KCl, 1 mM EGTA, 10 mM LiCl, 1 mM ATP, and 5 mM $MgCl_2$. Cells were disrupted at 4°C by N_2 cavitation with Yeda press treatment. N_2 pressure was applied for 20 min at 800 lb/inch^2 with constant stirring. The homogenate was centrifuged at $670 \times g$ for 10 min at 4°C. The pellet was discarded and the supernatant was subjected to a second centrifugation at $2 \times 10^5 \times g$ for 30 min at 4°C. The final pellet was resuspended at a concentration of 1–2 mg protein/ml in the homogenization medium and used immediately. This preparation is designated as JURKAT membranes. Protein was measured by a modification of the method of Lowry et al. [20].

2.3. *Assay for the formation of inositol phosphates by JURKAT membranes and lipid analysis*

Membranes from $8–17 \times 10^5$ JURKAT cells in 50 μl of the homogenization medium were mixed with 150 μl of an incubation medium (10 mM Hepes at pH 7.4, 1 mM EGTA, 10 mM LiCl, 5 mM $MgCl_2$, and 0.8 mM $CaCl_2$) which contained guanine nucleotides, NaF, $AlCl_3$, or OKT3 monoclonal antibody as indicated. A fluorimetric determination by the use of quin2 indicates that the mixture contained 110 nM free Ca^{2+}. The mixture was incubated at 37°C for 5 min or the indicated time. Reactions were terminated by addition of 0.75 ml chloroform/methanol (1:2, v/v). After phase separation by adding 0.25 ml each of chloroform and water, the upper phase was used to determine InsP_1, InsP_2, and InsP_3 by chromatography on AG1-X8 (formate form) columns [2,21,22]. When lipids were analyzed, insoluble residues on the first extraction were re-extracted with 1 ml chloroform/methanol/conc. HCl (200:100:1, v/v). The second extract was partitioned into two phases by adding 0.2 ml of 0.1 N HCl. The first and second lower phases were combined and used to determine PtdIns, PtdIns4P, and PtdIns(4,5)P_2 by thin-layer chromatography on precoated silica gel plates (LK5D; Whatman), which had been impregnated with 1% potassium oxalate/2 mM EDTA [2]. Chloroform/methanol/4 M aqueous ammonia (9:7:2, v/v) was used as developing solvent.

3. RESULTS AND DISCUSSION

A polyphosphoinositide phospholipase C controlled by a G-protein can be most easily demonstrated by activating the G-protein with either GTPγS, a nonhydrolyzable GTP analogue, or NaF plus $AlCl_3$ [10,12,23,24]. It is known that Al^{3+} in the presence of F^- modulates the activity of G-proteins, N_s, N_i, and transducin. The active principle is thought to be AlF_4^- [23,25], which activates the α-subunits GDP of these proteins by mimicking the role of the γ-phosphate of GTP in its binding site [26].

Fig.1 shows the time course of formation of [^{3}H]inositol phosphates by JURKAT membranes, prepared from *myo*-[2-^{3}H]inositol-labeled cells, in a buffer with 110 nM free Ca^{2+}. The formation of InsP_2 and InsP_3 was stimulated by GTPγS (100 μM) and, to a greater extent, NaF (5 mM) plus $AlCl_3$ (10 μM). The accumulation of InsP_2 was more prominent than that of InsP_3. GTPγS and NaF-$AlCl_3$ had no significant effect on the InsP_1 level. The relative proportions of [^{3}H]PtdIns, [^{3}H]PtdIns4P, and [^{3}H]PtdIns(4,5)P_2 in JURKAT membranes were 95.8 ± 4.9, 2.3 ± 0.2, and 1.9 ± 0.0% of total inositol lipids, respectively (means ± SE of 3 determinations). The highest levels of accumulated InsP_2 and InsP_3 in fig.1 correspond to 62.5% of PtdIns4P and 47.1% of PtdIns(4,5)P_2 present in JURKAT membranes at time 0. Small and transient changes in PtdIns4P and PtdIns(4,5)P_2 contents were observed during 15 min incubation after GTPγS and NaF-$AlCl_3$ addition, suggesting the continual replenishment of PtdIns4P and PtdIns(4,5)P_2 by phosphorylating reactions in the membranes.

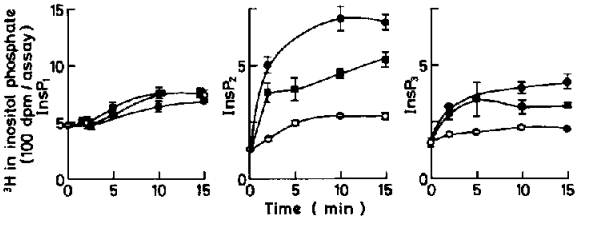

Fig.1. Time course of GTPγS- and AlF_4^--induced formation of inositol phosphates by JURKAT membranes. Membranes (97 μg protein/assay) from JURKAT cells labeled with 1 μCi/ml of *myo*-[2-^{3}H]inositol were incubated at 37°C for the indicated time in the absence (○) or presence of 100 μM GTPγS (■) or 5 mM NaF plus 10 μM $AlCl_3$ (●). The formation of inositol phosphates (InsP_1, InsP_2, and InsP_3) was analyzed. Values are means ± SE of 3 determinations.

Fig.2 shows the effect of NaF concentration on InsP_2 and InsP_3 formation by JURKAT membranes. Increasing the NaF concentration enhanced the accumulation of InsP_2 and that of InsP_3 with different dose-response curves. However, plotting the sum of InsP_2 and InsP_3 vs NaF concentration gives the usual dose-response curve (fig.2), which may suggest that at different NaF concentrations there is either a difference in the rates of InsP_3 hydrolysis to InsP_2 or a difference in the preference by the polyphosphoinositide phospholipase C for PtdIns4P and PtdIns(4,5)P_2. Whichever the reason is, the results shown in figs 1 and 2 indicate that the activation of the polyphosphoinositide phospholipase C in JURKAT membranes can be most appropriately

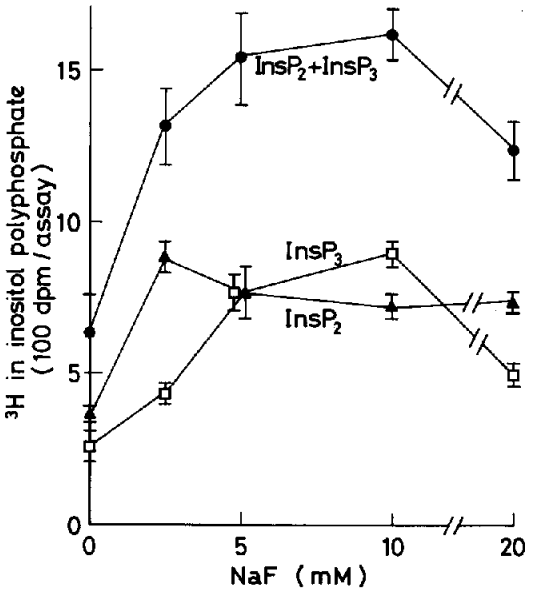

Fig.2. Effect of NaF concentration on inositol polyphosphate formation by JURKAT membranes. *myo*-[2-^{3}H]Inositol-prelabeled JURKAT membranes (57 μg protein/assay) were incubated at 37°C for 5 min in the presence of the indicated concentration of NaF. The formation of inositol phosphates (InsP_1, InsP_2, and InsP_3) was assayed. (□) InsP_3, (▲) InsP_2, (●) InsP_3 and InsP_2 combined. Values are means ± SE of 4 determinations.

expressed by the sum of InsP_2 and InsP_3 formed during the incubation.

The NaF-induced inositol polyphosphate formation by JURKAT membranes was enhanced by $AlCl_3$ (fig.3). In the presence of 5 mM NaF, maximal enhancement was observed at 50 μM $AlCl_3$, which alone had no effect on the inositol polyphosphate formation. It is highly probable that some Al^{3+} is already present in the incubation mixtures without addition of $AlCl_3$. Therefore, the results shown in fig.3 support the conclusion that AlF_4^- is the active principle responsible for the NaF-induced activation of phospholipase C in JURKAT membranes.

Fig.4 shows the effect of GTPγS concentration on the inositol polyphosphate formation by JURKAT membranes. The half-maximal dose of GTPγS on inositol polyphosphate formation in JURKAT membranes is 5–10 μM. In JURKAT membranes, optimum stimulation with NaF plus $AlCl_3$ always released a larger amount of inositol polyphosphates as compared with that formed by optimum stimulation with GTPγS. This can be found by comparing the results in figs 3 and 4, which were obtained by the use of the same preparation of JURKAT membranes. GTP had only a limited positive effect on inositol polyphosphate formation even at 250 μM (fig.4).

Fig.5 shows the results of a study on the effect of OKT3 monoclonal antibody, an antibody against CD3, on the formation of inositol polyphosphates by JURKAT membranes. OKT3 antibody did not stimulate inositol polyphosphate formation by JURKAT membranes even in the presence of 100 μM GTP or 1–10 μM GTPγS. OKT3 antibody at the concentrations used in this experiment markedly stimulated JURKAT cells to produce inositol phosphates in the presence of Li^+ (fig.5), which is a measure of polyphosphoinositide phospholipase C activation in intact cells [1,2,8,27].

At present, we are not able to find the reason for the lack of stimulation by OKT3 antibody of

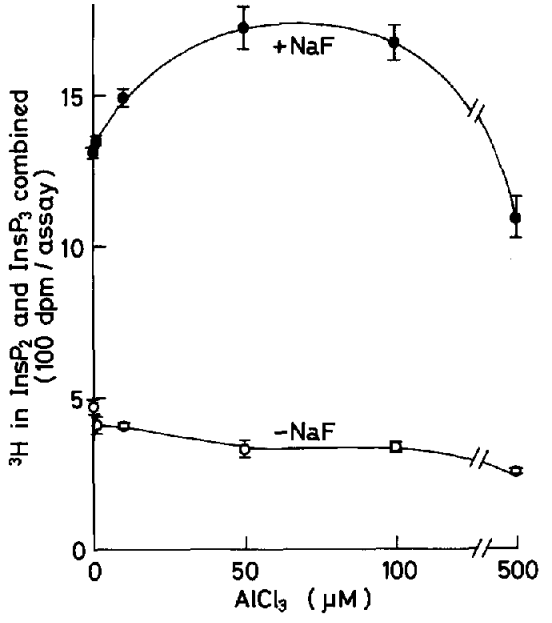

Fig.3. Enhancement by $AlCl_3$ of NaF-induced inositol polyphosphate formation by JURKAT membranes. *myo*-[2-^{3}H]Inositol-prelabeled JURKAT membranes (54 μg protein/assay) were incubated at 37°C for 5 min without (○) or with (●) 5 mM NaF in the presence of the indicated concentration of $AlCl_3$. The formation of inositol phosphates (InsP_1, InsP_2, and InsP_3) was analyzed. Results are expressed as the sum of InsP_2 and InsP_3. Values are means ± SE of 4 determinations.

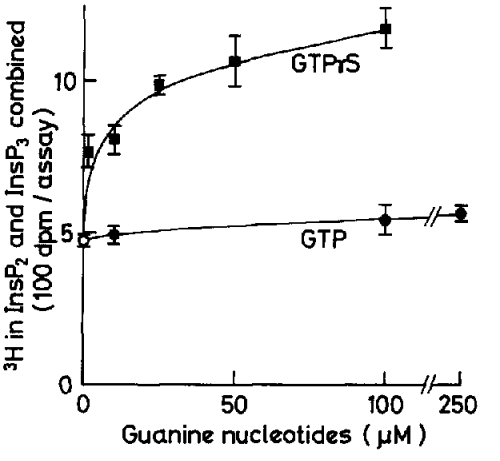

Fig.4. Effects of different concentrations of guanine nucleotides on inositol polyphosphate formation by JURKAT membranes. *myo*-[2-^{3}H]Inositol-prelabeled JURKAT membranes (54 μg protein/assay) were incubated at 37°C for 5 min in the presence of the indicated concentrations of GTP (●) and GTPγS (■). The formation of inositol phosphates (InsP_1, InsP_2, InsP_3) was analyzed. Results are expressed as the sum of InsP_2 and InsP_3. Values are means ± SE of 4 determinations.

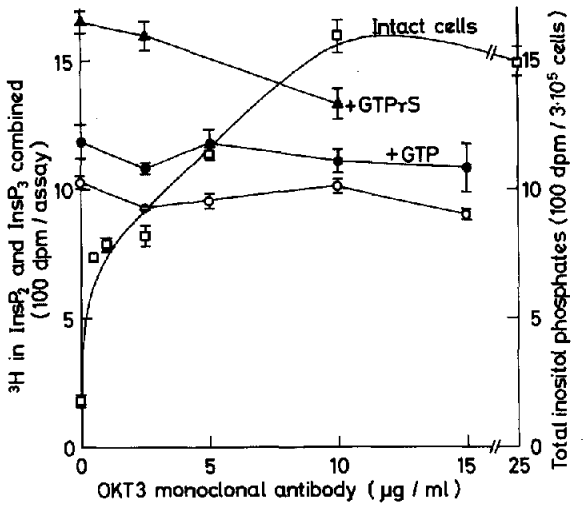

Fig.5. Effects of different concentrations of OKT3 monoclonal antibody on inositol polyphosphate formation by JURKAT membranes and on inositol phosphate production by JURKAT cells. This figure contains results obtained in two different experiments. In one experiment, *myo*-[2-^{3}H]inositol-prelabeled JURKAT membranes (83 μg protein/assay) were incubated at 37°C for 5 min in the presence of the indicated concentration of OKT3 monoclonal antibody without (○) or with either 100 μM GTP (●) or 1 μM GTPγS (▲). The formation of inositol phosphates (InsP_1, InsP_2, InsP_3) was analyzed. Results are expressed as the sum of InsP_2 and InsP_3. Values are means ± SE of 5 determinations. In the other experiment, JURKAT cells (3×10^5 cells/assay) labeled with 2 μCi/ml of *myo*-[2-^{3}H]inositol were incubated at 37°C for 5 min in the presence of the indicated concentration of OKT3 monoclonal antibody in 0.2 ml Dulbecco's phosphate-buffered saline containing 10 mM LiCl. The accumulation of total inositol phosphates (the sum of InsP_1, InsP_2, and InsP_3) was determined as in [1,2,8]. Values (□) are means ± SE of 3 determinations.

polyphosphoinositide phospholipase C in JURKAT membranes. Aggregation of Ti/CD3 complexes is probably an essential step in inducing the activation of the complex following the binding of OKT3 antibody to the complex [28]. In this respect, OKT3 antibody is probably unique among the Ca^{2+}-mobilizing agonists used in demonstrating the receptor-coupled activation of polyphosphoinositide phospholipase C in cell-free systems.

The present results indicate the presence in JURKAT membranes of a G-protein coupled to a polyphosphoinositide phospholipase C. However, it may be argued whether the G-protein is involved in the Ti/CD3 receptor-stimulated polyphosphoinositide hydrolysis in JURKAT cells. Participation of a G-protein has been found in all cell-free systems which show receptor-dependent activation of the phospholipase C [10–15]. A recent study on the mitogen-stimulated activation of ornithine decarboxylase in human T lymphocytes has proved that GTP is essential for the cellular response by the rapid activation of the enzyme [29], which seems to be a secondary response following polyphosphoinositide hydrolysis and a rise in $[Ca^{2+}]_i$ [30].

REFERENCES

[1] Sasaki, T. and Hasegawa-Sasaki, H. (1983) Biomed. Res. 4, 281–288.
[2] Sasaki, T. and Hasegawa-Sasaki, H. (1985) Biochem. J. 227, 971–979.
[3] Sasaki, T. and Hasegawa-Sasaki, H. (1985) Biochim. Biophys. Acta 833, 316–322.
[4] Weiss, A., Imboden, J., Shoback, D. and Stobo, J. (1984) Proc. Natl. Acad. Sci. USA 81, 4169–4173.
[5] Weiss, A., Imboden, J., Hardy, K., Manger, B., Terhorst, C. and Stobo, J. (1986) Annu. Rev. Immunol. 4, 593–619.
[6] Weissman, A.M., Samelson, L.E. and Klausner, R.D. (1986) Nature 324, 480–482.
[7] Imboden, J.B., Weiss, A. and Stobo, J.D. (1985) J. Immunol. 134, 663–665.
[8] Imboden, J.B. and Stobo, J.D. (1985) J. Exp. Med. 161, 446–456.
[9] Sasaki, T., Takei, T. and Hasegawa-Sasaki, H. (1987) Microbiol. Immunol., in press.
[10] Uhing, R.J., Prpic, V., Jiang, H. and Exton, J.H. (1986) J. Biol. Chem. 261, 2140–2146.
[11] Litosch, I., Wallis, C. and Fain, J.N. (1985) J. Biol. Chem. 260, 5464–5471.
[12] Lucas, D.O., Bajjalieh, S.M., Kowalchyk, J.A. and Martin, T.F.J. (1985) Biochem. Biophys. Res. Commun. 132, 721–728.
[13] Straub, R.E. and Gershengorn, M.C. (1986) J. Biol. Chem. 261, 2712–2717.
[14] Guillon, G., Balestre, M.-N., Mouillac, B. and Devilliers, G. (1986) FEBS Lett. 196, 155–159.
[15] Kikuchi, A., Kozawa, O., Kaibuchi, K., Katada, T., Ui, M. and Takai, Y. (1986) J. Biol. Chem. 261, 11558–11562.
[16] Robertson, M. (1985) Nature 317, 768–771.

[17] Van den Elsen, P., Shepley, B.-A., Borst, J., Coligan, J.E., Markham, A.F., Orkin, S. and Terhorst, C. (1984) Nature 312, 413–418.
[18] Gold, D.P., Puck, J.M., Pettey, C.L., Cho, M., Coligan, J., Woody, J.N. and Terhorst, C. (1986) Nature 321, 431–434.
[19] Frank, M.B., Watson, J., Mochizuki, D. and Gillis, S. (1981) J. Immunol. 127, 2361–2365.
[20] Markwell, M.A.K., Haas, S.M., Tolbert, N.E. and Bieber, L.L. (1981) Methods Enzymol. 72, 296–303.
[21] Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P. and Irvine, R.F. (1983) Biochem. J. 212, 473–482.
[22] Berridge, M.J. (1983) Biochem. J. 212, 849–858.
[23] Blackmore, P.F., Bocckino, S.B., Waynick, L.E. and Exton, J.H. (1985) J. Biol. Chem. 260, 14477–14483.
[24] Cockcroft, S. and Taylor, J.A. (1987) Biochem. J. 241, 409–414.
[25] Sternweis, P.C. and Gilman, A.G. (1982) Proc. Natl. Acad. Sci. USA 79, 4888–4891.
[26] Bigay, J., Deterre, P., Pfister, C. and Chabre, M. (1985) FEBS Lett. 191, 181–185.
[27] Berridge, M.J., Downes, C.P. and Hanley, M.R. (1982) Biochem. J. 206, 587–595.
[28] Tax, W.J.M., Hermes, F.F.M., Willems, R.W., Capel, P.J.A. and Koene, R.A.P. (1984) J. Immunol. 133, 1185–1189.
[29] Mustelin, T. (1987) FEBS Lett. 213, 199–203.
[30] Mustelin, T., Pösö, H. and Andersson, L.C. (1986) EMBO J. 5, 3287–3290.