The protein tyrosine kinase p56lck regulates the serine-base exchange activity in Jurkat T cells

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Abstract Different classes of protein kinase inhibitors for protein kinase C, cAMP-dependent protein kinase or protein tyrosine kinases have been studied for their effect on phospholipid metabolism. The results show that among the compounds studied, only 4′-aminohydroxyflavone (AHF), previously described as a specific inhibitor of the protein tyrosine kinase p56lck, markedly increased phosphatidylserine synthesis in Jurkat T cells. The biosyntheses of phosphatidylcholine and phosphatidylethanolamine were not affected. Also, the synthesis of phospholipids from tritium-labeled fatty acid as precursor was left unchanged by the p56lck inhibitor. The decreased phosphatidylserine synthesis induced when triggering the CD3-TCR complex was impaired by AHF, suggesting that p56lck could be implicated in the regulation of the serine-base exchange enzyme system. Direct evidence for the participation of p56lck in the regulation of the serine-base exchange enzyme system was obtained by using p56lck-deficient Jurkat cells (J.CaM 1.6) in which the basal base exchange activity was markedly increased and on the other hand AHF had no effect. In addition, transfection of J.Cam 1.6 cells with p56lck-cDNA allowed recovery of the AHF activity.

Key words: p56lck; Phosphatidylserine; Phospholipid; Base exchange; Jurkat cell

1. Introduction

The phospholipid base exchange enzyme system that catalyzes incorporation of exogenous choline, ethanolamine and serine into phospholipids involves separate enzymes specific for each polar head group of phospholipids. In the past 5 years, evidence accumulated demonstrating that the phospholipid base exchange activity is regulated upon membrane receptor triggering in different cell lines. In rat liver plasma membrane, phospholipid base exchange activity is regulated by G-proteins and P2y-purinergic receptor [1], in the neuroblastoma cell line LA-N-1, the serine-base exchange activity is modulated by muscarinic receptors [2]. In glioma C6 cells, the serine-base exchange is inhibited by glutamate and acetylcholine [3]. We have previously shown that in T cell lines triggering the T cell receptor complex induces a rapid and sustained decrease of phosphatidylserine (PtdSer) synthesis [4,5]. PtdSer synthesis in mammalian cells occurs only through the serine-base exchange enzyme system. The enzyme is located on the membrane of the endoplasmic reticulum [6,7] and its activity is maintained by the high Ca^{2+} concentration existing in the endoplasmic reticulum in non-activated cells. Upon activation of lymphocytes with CD3 mAb or activation of glioma C6 cells with acetylcholine or glutamate, Ca^{2+} ions are released into the cytoplasm and it is accepted that decreasing the Ca^{2+} concentration in the endoplasmic reticulum results in decreased activity of the serine-base exchange enzyme and reduced PtdSer biosynthesis. This was confirmed by the fact that thapsigargin, a blocker of the endoplasmic reticulum Ca^{2+}-ATPase and Ca^{2+} ionophores induce a strong decrease of PtdSer synthesis [3,5,8]. On these experimental bases, we have repeatedly used the measure of PtdSer synthesis as a tool to follow Jurkat T cell activation [9–11].

Little is known about the eventual regulation of the serine-base exchange activity by protein kinases; a possible involvement of PKC has been suggested since, in HL60 cells, phorbol ester inhibits PtdSer synthesis [12] but this was not the case in Jurkat T cells [4]. The participation of PKA has also been suggested from experiments performed in an acellular system [13].

It is generally accepted that T cell activation starts with the phosphorylation on tyrosine residues of several proteins. Among these proteins several protein tyrosine kinases have been characterized such as $p59^{fyn}$, ZAP-70 and $p56^{lck}$ that play a major role in T cell activation [14–16]. The tyrosine kinase pathway, is linked to the Ca^{2+} pathway through the phosphorylation on tyrosine residue of phospholipase Cγ1. This phospholipase activated by phosphorylation hydrolyses phosphatidylinositol bisphosphate into the two second messengers diacylglycerol, a protein kinase C activator and inositol trisphosphate that liberates Ca^{2+} from the endoplasmic reticulum. On these bases, it was imaginable to link the CD3-induced decrease of PtdSer synthesis to one kinase involved in the tyrosine kinase pathway. p56lck is a central kinase in the activation schemas of T cells. 4-Aminohydroxyflavone (AHF) a specific inhibitor of this kinase has been recently developed [17]. We show here by using AHF and J.Cam 1.6 p56lck-deficient cells, that p56lck participates in the regulation of the serine-base exchange enzyme.

2. Materials and methods

2.1. Cells

The human T cell line Jurkat kindly supplied by Dr. A.M. Schmitt-Verhulst (Centre d'Immunologie, Marseille-Luminy, France) was cloned by limiting dilution. Clone D (JD) was selected on the basis of its interleukin 2 (IL-2) production when activated with phytohemagglutinin and the phorbol ester PMA. The Jurkat clone E6-1 was

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Abbreviations: PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; AHF, 4′-aminohydroxyflavone.

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purchased from ATCC (ATCC TIB 152). The Jurkat mutant defective in $p56^{lck}$ kinase, J.CaM1.6, derived from JE6-1 were also purchased from ATCC (ATCC CRL-2063). Cells were cultured in RPMI 1640 (Gibco, Cergy-Pontoise, France) supplemented with 5% fetal calf serum (Biowhitaker, Fontenay, France), 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM pyruvate.

2.2. Monoclonal antibodies

The CD3 monoclonal antibody, X35, was purified from ascites gifted by Dr. D. Bourel (CTS Lille, France).

2.3. Phospholipid synthesis in Jurkat cells

Jurkat cells (2×10^6) were maintained in 0.5 ml of a buffer (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 2.5 mM glucose, 20 mM HEPES, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ at 37°C in the presence of either 4 μCi of [^{3}H]serine, [^{3}H]choline or [^{3}H]ethanolamine or 2 μCi [^{3}H]arachidonic acid and effectors (see concentrations in the figure legends). After an incubation period varying from 0 to 2 h the cells were rapidly sedimented in an Eppendorf centrifuge, the supernatants were discarded, and cell phospholipids were extracted using chloroform/methanol according to Bligh and Dyer [18]. This two-step extraction procedure allowed the determination of ^{3}H-labeled products incorporated into the cells by measuring a 25 ml sample of the chloroform/methanol extract. Then the addition of chloroform and water allowed the separation of the organic and aqueous phases. The lipid extracts (organic phases) were analysed by thin-layer chromatography on LK6D chromatography plates (Wathman) in a solvent system composed of chloroform/methanol/acetic acid/water (75:45:12:3). Authentic phospholipid standards (Sigma) were run in parallel and detected with iodide vapor. Radioactivity in lipid spots was determined by using an automatic linear radiochromatography analyser, Tracemaster 20 (Berthold) equipped with an 8 mm window and the integration software supplied by the manufacturer.

2.4. Transfection of J.CaM 1.6 cells

J.CaM 1.6 cells were transfected via electroporation by using p56lck cDNA included in the mammalian expression vector pEF-BOS [19,20] The vector was a generous gift from Dr. A.C. Carrera (Centro Nacional Biotecnologia, Universidad Autonoma Campus de Cantoblanco, Madrid, Spain). p56lck positive cells were cloned by limiting dilutions. The presence of p56lck was verified by Western blots of cells extracts with anti-p56lck mAbs. Anti-lck mAbs were purchased from UpstateBiotechnology (Lake Placid, USA).

3. Results and discussion

3.1. Effect of AHF on phospholipid synthesis

We first studied PtdSer synthesis in a Jurkat T cell clone (JD), in the presence of different concentrations of AHF, a compound designed as a specific p56lck inhibitor [17]. The results (Fig. 1A) show that this compound strongly increased PtdSer synthesis with an $EC_{50} = 20$ μM. Kinetic analysis showed that PtdSer synthesis was increased very early, <15 min after AHF treatment (Fig. 1B). The total incorporation of [^{3}H]serine by cells remained unchanged, indicating that AHF does not modify the transport of the amino acid through the membrane (not shown). The action of AHF appeared highly specific for PtdSer since the biosynthesis of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) monitored by measuring the amount of [^{3}H]choline and [^{3}H]ethanolamine incorporated into phospholipids was not affected by the drug (Table 1). Moreover, the incorporation of [^{3}H]palmitic acid into phospholipids (Table 1) and the formation of PtdEtn from decarboxylation of PtdSer were not affected by AHF (Fig. 1C). The elevation in PtdEtn shown in Fig. 1C parallels the increase in PtdSer synthesis, indicating that the decarboxylation pathway remains active during AHF treatment.

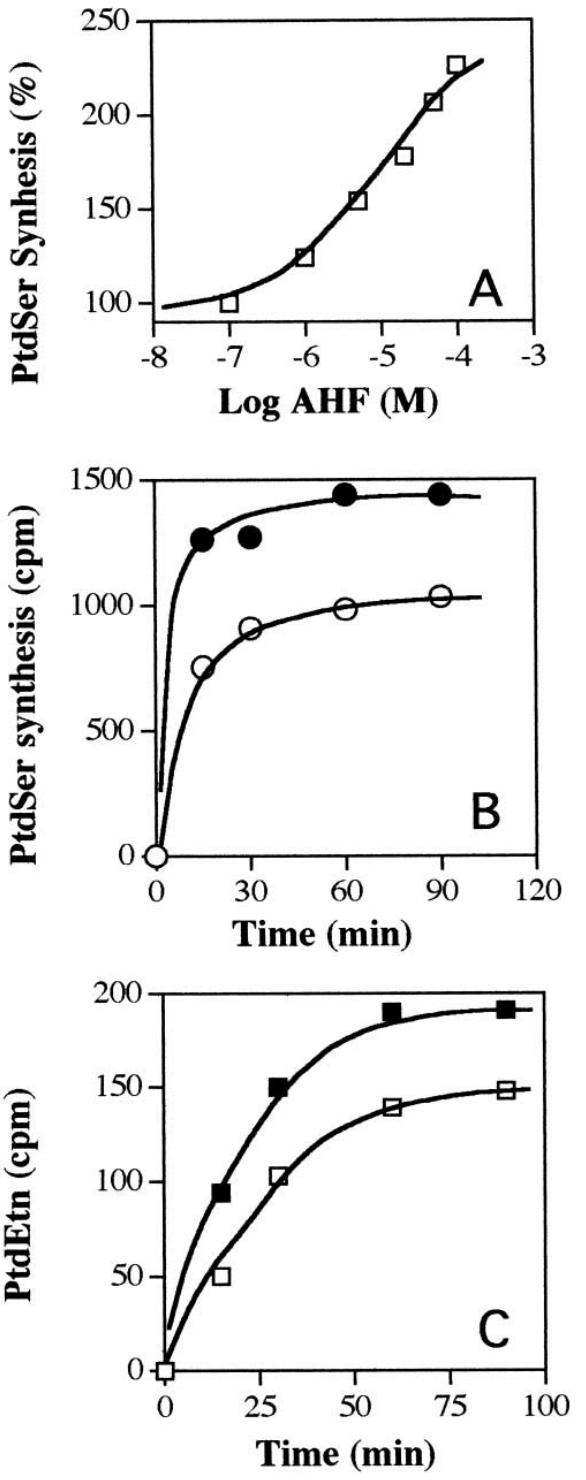

Fig. 1. Effect of AHF on phosphatidylserine synthesis. Jurkat cells, clone D, were incubated at 37°C in the presence of [^{3}H]serine for 120 min in the presence of varying concentrations of AHF. Phospholipids were extracted and analysed by thin-layer chromatography. The radioactivity incorporated into PtdSer was quantified and plotted against AHF concentrations (A). (B) Cells were incubated with [^{3}H]serine without (○) or with 40 μM AHF (●) for different periods of time. (C) Incorporation of [^{3}H]serine into PtdEtn through the PtdSer-decarboxylation pathway in control (□) and 40 μM AHF (■) treated cells. The figure shows an experiment representative of three others.

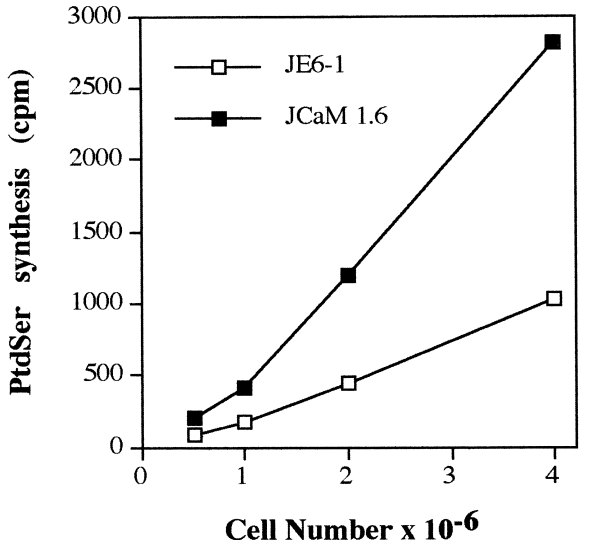

Fig. 2. Jurkat J E6-1 cells (□) synthesize less PtdSer than J.CaM 1.6 (■), a p56lck negative mutant. The incorporation of [^{3}H]serine into PtdSer was plotted as a function of cell concentration. PtdSer synthesis was measured after a 120 min time period.

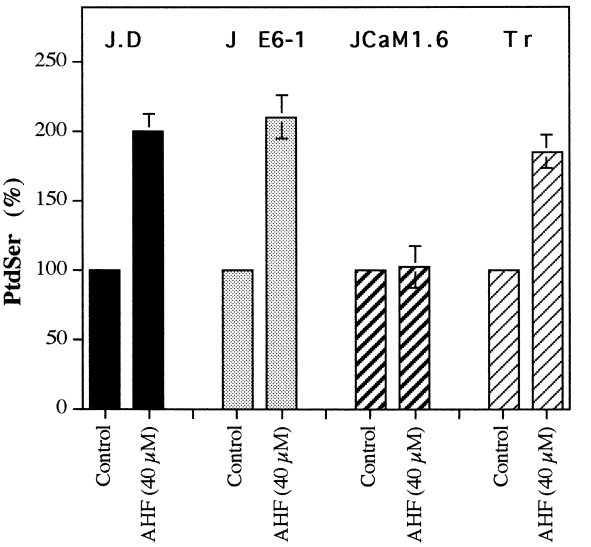

Fig. 3. Effect of AHF in the different Jurkat clones. PtdSer synthesis was monitored in both control and 40 μM AHF treated cells. The incorporation of [^{3}H]serine into PtdSer was measured after an incubation period of 120 min in Jurkat clone D (J.D), J E6-1, J.CaM 1.6 (lacking p56lck) and in a clone of JCAM 1.6 transfected with p56lck cDNA (Tr). Results are expressed as % ± S.D. ($n = 6$) vs. controls (taken as 100%).

3.2. Specificity of AHF

Different protein kinase inhibitors were then used to test the specificity of AHF. Among these inhibitors, H7 and H8 acting on protein kinase A, protein kinase C and protein kinase G according to the concentration used were unable to modify PtdSer synthesis even at 100 μM. Tyrosine protein kinase inhibitors such as Compound 5, lavendustin, genistein, tyrphostin A9, A23 and A49 were also inactive on the serine-base exchange system at concentrations varying between 0.1 and 100 μM. Only staurosporine, a non-specific inhibitor of many tyrosine protein kinases, including p56lck, increased the serine-base exchange activity at concentrations < 1 μM [21].

3.3. Phosphatidylserine synthesis in mutants of Jurkat cells

We next examined serine-base exchange activity in J.CaM 1.6, a mutant Jurkat cell lacking p56lck activity [22] derived from another Jurkat clone, JE6-1. Fig. 2 shows that J.CaM 1.6 incorporated 2-fold more [^{3}H]serine into PtdSer than the parental cell, JE6-1. The effect of AHF (40 μM) on PtdSer synthesis was then compared in JD, JE6-1 and JCaM 1.6. AHF increased similarly in JD and JE6-1 but was inactive in JCaM1.6 (Fig. 3) indicating that in the absence of p56lck activity PtdSer synthesis is upregulated and that AHF interacts specifically with this protein tyrosine kinase. It is interesting to note that under our experimental conditions the PtdSer synthesizing pathway was not saturated by a 2-fold increase since on using other compounds such as oleylamine or stearylamine a 5-fold increase of PtdSer can be observed [21].

3.4. The AHF effect is recovered in p56lck-transfected JCaM 1.6 cells

To determine whether the defect in p56lck was responsible for the changes observed in the activity of the serine-base exchange system, we restored the p56lck activity by transfection with a cDNA clone of p56lck. JCaM 1.6 cells were transfected via electroporation by using p56lck cDNA included in the mammalian expression vector pEF-BOS. Positive clones were isolated, cultured and then cloned by limiting dilution. As shown in Fig. 3, the enhancing effect of AHF on PtdSer synthesis was recovered in p56lck transfectants.

3.5. AHF impairs CD3-induced inhibition of PtdSer synthesis

Triggering the CD3-TCR complex with CD3 mAbs induces a marked decrease of PtdSer synthesis [4,5]. This decrease was previously attributed to the fact that CD3 mAbs empty endoplasmic reticulum Ca^{2+} stores, since a similar decrease of

Table 1
Effect of 4-aminohydroxyflavone (AHF) on phospholipid biosynthesis.

Label	Phospholipids	Control	AHF (40 μM)
[^{3}H]Palmitic acid	PtdIns-P/PtdIns-P2	2412 ± 59	2704 ± 211
	PtdCho	3078 ± 339	3246 ± 68
	PtdSer/PtdIns	407 ± 56	400 ± 73
	PtdEtn	3681 ± 223	3543 ± 309
	Neutral lipids	3521 ± 70	3516 ± 83
[^{3}H]Choline	PtdCho	1279 ± 97	1176 ± 60
[^{3}H]Ethanolamine	PtdEtn	4233 ± 686	4558 ± 324

Jurkat cells were labeled with either [^{3}H]palmitic acid, [^{3}H]choline or [^{3}H]ethanolamine for 2 h in the absence (control) or presence of 40 μM AHF. Phospholipids were extracted then separated by thin-layer chromatography and quantified with a radiochromatography scanner. Results are expressed as cpm ± S.D. ($n = 4$).

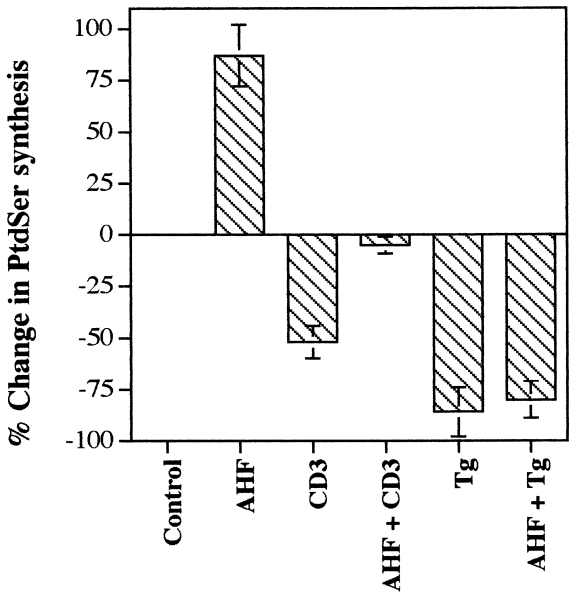

Fig. 4. AHF impairs CD3-induced inhibition of PtdSer synthesis. Jurkat cells were left untreated (control) or treated with either 40 μM AHF, CD3 mAb (2 μg/ml) or thapsigargin (Tg) 10^{-7} M in the presence or absence of AHF. PtdSer synthesis was measured after 120 min. The results are the mean ± S.D. from two experiments performed in triplicate ($n = 6$).

PtdSer synthesis is also observed with the Ca^{2+}-ATPase blocker, thapsigargin. As expected, AHF was capable of blocking the CD3-induced inhibition of PtdSer synthesis. By contrast, the effect of thapsigargin was not reversed by the drug (Fig. 4). Since CD3-induced intracellular signaling involves the activation of protein tyrosine kinases while thapsigargin bypasses these early events, it appeared likely that AHF would block the protein tyrosine kinase p56lck.

Taken together, the results presented indicate that the protein tyrosine kinase, p56lck, probably participates in the regulation of the serine-base exchange enzyme system in Jurkat cells. Previously, we have repeatedly shown that increasing PtdSer synthesis results in decreased interleukin-2 (IL-2) production by activated T cells, suggesting that PtdSer plays a major role in T cell activation. In vivo experiments also supported this conclusion [23]. We have shown that AHF inhibits IL-2 production in Jurkat cells activated by CD3 mAb in the presence of the phorbol ester, TPA. AHF also inhibits the surface expression of CD25, a chain of the IL-2 receptor and the surface expression of CD69, an early activation-induced protein (unpublished results). Besides its role in T cell activation, another important role for PtdSer in the regulation of lymphocytes arises from the discovery that T cell apoptosis is accompanied by the exposure of PtdSer at the cell surface where this phospholipid serves for the recognition by macrophages in a way to eliminate dying cells [24–26]. Also, the recent demonstration [27] that src tyrosine kinases bind to membranes through electrostatic interactions with acidic phospholipids and particularly to PtdSer indicates that this phospholipid might play a role in the membrane targeting of p56lck during T cell activation. Accordingly, the study of the fine regulation of PtdSer biosynthesis appears to be an important step in the understanding of the regulation of T cell functions.

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