Interleukin-2 stimulates a late increase in phosphatidic acid production in the absence of phospholipase D activation

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Abstract The signal transduction pathways involving phospholipid metabolism during T-cell proliferation remain partly undefined. Herein we show that interleukin-2 caused a late (>12 h) rise in the intracellular phosphatidic acid content of CTLL-2 cells which was a consequence of the activation of the enzyme diacylglycerol kinase. No activation of phospholipase D was observed at similar times. Incubation of the cells with a recognized diacylglycerol kinase α isoform inhibitor, R59499, prior to interleukin-2 stimulation was able to block cell cycle entry, diacyglycerol kinase activation and phosphatidic acid accumulation. In contrast, when R59499 was added 3 h after interleukin-2, few or no observable effects on the above three parameters were noticed. These results suggest that the early signaling employed by IL-2 involving the α isoform of diacylglycerol kinase is sufficient to control the late increase in phosphatidic acid and that phosphatidic acid is a mitogenic agent in T-cells.

Key words: Lipid mediator; Second messenger; T-lymphocyte; Interleukin-2

1. Introduction

Interleukin-2 (IL-2) is a cytokine which is responsible for the expansion of T-cell populations. Although it was discovered over two decades ago, its signal transduction pathways are still not fully defined. After ligation to its high affinity heterotrimeric receptor, transmembrane signaling cascades are generated within seconds. A large number of the signals have been partially characterized with the involvement of both protein and lipid kinases [1–8] which associate with the individual chains of the IL-2 receptor at specific sites. These signals integrate with each other to stimulate protooncogene production and to control the activity of the cell cycle protein machinery [9]. Activation of multiple pathways leads to enhanced protein phosphorylation, however the role of phospholipids and in particular phospholipid-derived second messengers remains understudied and unclear. It has been demonstrated that IL-2 does not show characteristics of an agent which delivers a classical transmembrane signal in that phosphatidylinositol turnover, calcium fluxes and cyclic nucleotide generation are not observed [10–12]. Two lines of evidence have emerged in an attempt to understand the role of lipid second messengers for IL-2. Firstly, IL-2 causes the rapid phospholipase C-mediated hydrolysis of glycosylphosphatidylinositol [13,14] and secondly, diacylglycerol (DAG) kinase (DGK) activity is increased [15,16]. Phosphatidic acid (PA) is considered, as is DAG, to be a mitogenic agent as it is able to activate a number of enzymes and to regulate intracellular metabolism [17]. PA arises not only through the activity of DGK, but also via the action of phospholipase D (PLD) which generates PA directly [18,19]. On examining the kinetics of the generation of lipid second messengers after extracellular stimulation, it becomes apparent that in addition to the 'early and transient phospholipid signaling', attributable principally to the hydrolysis of polyphosphoinositides, there exists a second wave of phospholipid hydrolysis whose duration is greater than that observed at short times after receptor stimulation. This second wave of phospholipid hydrolysis which increases the intracellular concentration of lipid second messengers, including DAG and PA, gives rise to the sustained activation of protein kinases thereby driving the cells into mitogenesis and/or differentiation [18–20]. The vast majority of investigators who have examined the IL-2-induced signal transduction have done so using short periods of stimulation and have based their conclusions on such experiments. In the light of the very recent observation that IL-2 causes a sustained increase in the level of intracellular DAG in CTLL-2 cells [21], we have re-examined the 'late phase' of IL-2 signal transduction and demonstrate a strong stimulation of DGK which is responsible for rises in intracellular PA.

2. Materials and methods

2.1. Materials

Human recombinant IL-2 was a generous gift from Hoffman-LaRoche Inc. (Nutley, NJ, USA). [^{32}P]Orthophosphate (carrier free) and [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) were purchased from Amersham (Amersham, UK). Fetal calf and newborn calf sera were bought from Gibco (Paisley, UK) and Bio Whittaker (Walkersville, MD, USA) respectively. Silica gel TLC plates (60 Å, LK6D) were from Whatman (Clifton, NJ, USA). R59949 was purchased from Calbiochem (Nottingham, UK). Authentic phospholipid standards for TLC, 1,2-dioleoyl-*sn*-glycerol, phorbol ester (PMA) and cabbage PLD were from Sigma (Poole, UK). Phosphatidylethanol (PEt) was from Avanti Polar Lipids (Alabaster, AL, USA).

2.2. Cell culture and cell cycle analysis

CTLL-2 (clone G7) cells were maintained according to Flores et al. [16]. KIT225 cells were cultured likewise. NIH3T3 cells were grown in petri dishes in DMEM containing 10% v/v newborn calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin buffered to pH 7.2 with 10 mM HEPES. All cell types were passaged twice weekly before they became confluent. Cell cycle analyses were performed as previously described [16].

2.3. Non-radioactive cell treatments

CTLL-2 cells were washed twice in incomplete medium (RPMI supplemented with 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin buffered to pH 7.2 with 10 mM HEPES) before reincubating during a starving period of 8 h. At this point cell treatments were initiated. Cells were incubated at a density of 5×10^5/ml in the absence or presence of 50 U/ml IL-2 for

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various times after which the cells were pelleted, washed twice using ice-cold PBS and stored at −80°C. In the case of experiments using the DGK inhibitor, R59949, cells were either pre-incubated (30 min) with the compound before stimulation with IL-2 or the compound was added 3 h after IL-2. In all cases R59949 was used at 1 μM without observable toxic effects.

2.4. In vivo metabolic labeling

CTLL-2 cells were prepared as above and metabolically labeled to equilibrium during the starvation period using orthophosphate (50 μCi/ml) in phosphate-free incomplete medium followed by treatment with or without R59949 and IL-2 as indicated above. At the end of the incubations, cells were pelleted, washed twice in ice-cold PBS and stored at −20°C. Additionally, in some experiments 1% (v/v) ethanol was included in the cell incubations during the last 30 min of the IL-2 stimulation period. NIH3T3 cells (grown in six-well plates), KIT225 and CTLL-2 cells were metabolically labeled as indicated above. After stimulation with combinations of ethanol, IL-2 and PMA (100 nM) during a period of 30 min, the cells were rapidly washed with ice-cold PBS, collected by centrifugation or trypsinization and stored at −20°C.

2.5. Biochemical and enzymatic assays

DGK activity was determined using the method described by Flores et al. [16]. Phospholipids were extracted by the method of Bligh and Dyer [22] and subjected to TLC using a solvent system consisting of chloroform/methanol/glacial acetic acid (9:1:1, v/v/v) for the separation of labeled PA. After drying, the TLC plates were exposed to film and the bands corresponding to labeled PA (found by comigration with an authentic PA marker) were quantified using phosphorimaging analysis (Molecular Analyst Software, Bio-Rad). Accumulation of PA in cells after metabolic labeling of CTLL-2 cells was determined by phospholipid extraction of the cell pellets followed by TLC employing the same mobile phase. Autoradiography and quantification of radioactive spots corresponding to PA were performed as described above. The formation of PEt (a recognized marker of PLD activity) in metabolically labeled cells was determined by phospholipid extraction of the cell pellets followed by TLC employing the solvent system chloroform/methanol/glacial acetic acid (65:15:2, v/v/v) with authentic PEt as the TLC standard. PLD activity was also assessed in a crude membrane fraction using a fluorescent assay previously described [23]. As a positive control the fluorescent substrate (phosphatidylcholine) was incubated with 25 U of cabbage PLD in the presence or absence of butanol. Fluorescent phospholipids were extracted by the method of Bligh and Dyer [22] and subjected to TLC using a solvent system consisting of chloroform/methanol/glacial acetic acid (65:15:2, v/v/v). After drying, the TLC plates were illuminated with ultraviolet light and photographed. Fluorescent PA and fluorescent phosphatidylbutanol were used as marker phospholipids. The method of Bartlett [24] was used for the assay of total phosphate.

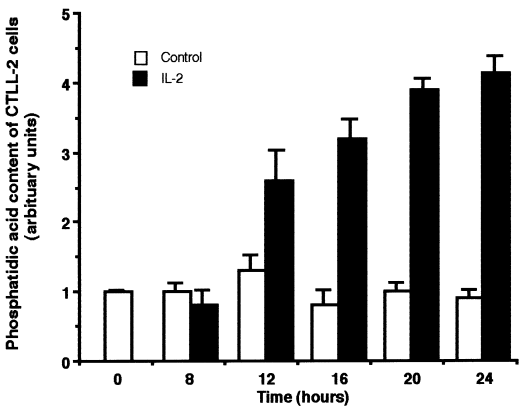

Fig. 1. IL-2 causes a late sustained accumulation of PA. CTLL-2 cells were radiolabeled/arrested and then incubated for various times with or without IL-2. Radiolabeled cellular PA levels were normalized to total phospholipid phosphate (phosphorimaging analysis pixel density of PA/nmol phospholipid phosphate). This figure depicts the combined results of three independent experiments (mean ± S.D.).

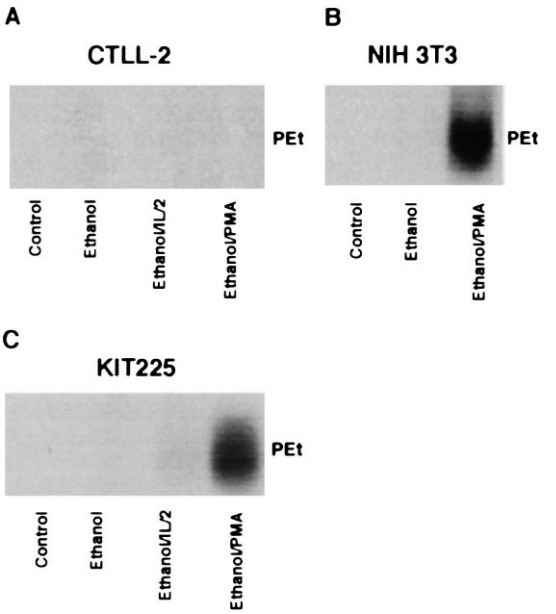

Fig. 2. IL-2 does not acutely stimulate PLD. CTLL-2 (A), NIH3T3 (B) and KIT225 (C) cells were radiolabeled with orthophosphate in phosphate-free medium in the absence of serum and growth factors. Thereafter cells were control treated or treated with ethanol in the presence or absence of IL-2 or PMA for 30 min. After phospholipid extraction, radiolabeled PEt was separated from all other phospholipids by TLC. The results are representative of two further independent experiments.

3. Results

Our first objective was to measure long-term variations in the level of PA in CTLL-2 cells treated with IL-2. Therefore we decided to use orthophosphate radiolabeling as we found that it was readily incorporated into PA and furthermore it would enable us to determine total PA generation. Fig. 1 shows that there was an accumulation of PA in CTLL-2 cells that had been stimulated with IL-2. This accumulation was apparent at 12 h (approximately 3-fold over control) and was sustained until at least 24 h (increasing to approximately 4-fold over control).

As it has been described in other systems that a late sustained accumulation of PA may arise from the action of PLD on phosphatidylcholine [18,19], we decided to determine if IL-2 stimulated PLD at late times after CTLL-2 cell stimulation. We used two techniques. The first involved in vivo metabolic radiolabeling whereas the second method involved an in vitro PLD assay using a fluorescent substrate. At all the times indicated above no activation of PLD was detected in IL-2-stimulated cells (data not shown). To determine if PLD was activated by IL-2 at short time periods (up to 30 min), both PLD assay techniques were employed. Neither IL-2 nor PMA was able to stimulate PLD activity in metabolically radiolabeled CTLL-2 cells (Fig. 2). To confirm this negative result with a positive control, KIT225 and NIH3T3 cells, which had been subjected to the same metabolic radiolabeling procedure as CTLL-2 cells, were stimulated with PMA. A large increase in PLD activity in both cells types was observed after treatment with PMA (Fig. 2).

As the observed IL-2-dependent increase in intracellular PA was not due to stimulated PLD activity, we determined DGK activity in membrane extracts of CTLL-2 cells that had been

treated with IL-2. DGK was clearly activated (approximately 2–3-fold over control) and remained so up to at least 24 h after stimulation (Fig. 3).

DGK enzymes exist as a family of at least eight isoforms whose differences are accounted for within their structure and their sites of expression [25]. Previous work from our laboratory has indicated that IL-2 raises PA levels at early time points after receptor ligation through the specific activation of the α isoform of DGK [16]. The compound R59949 has been shown to be an inhibitor of the α isoform of DGK [25,26]. To investigate if the DGK activity stimulated at later times following IL-2 addition corresponded to the activation of the α isoform we decided to test the effect of R59949 addition either before or after IL-2 stimulation. CTLL-2 cells were either pre-incubated with R59949 before IL-2 stimulation or were treated with the inhibitor 3 h after IL-2 stimulation. As we have previously described [16], pre-incubation of the cells with R59949 before IL-2 stimulation prevented cell cycle entry and led to a build up of cells in G_0/G_1. In contrast, Fig. 4A indicates that those cells which received IL-2 stimulation followed by treatment with R59949 entered into the cell cycle almost identically to those stimulated by IL-2. The proportion of cells committed to DNA synthesis and mitosis in both cases was approximately 3-fold over that observed in unstimulated cells. As pre-treatment, but not post-treatment, of CTLL-2 cells with R59949 was able to prevent IL-2-mediated cell proliferation, we decided to determine the effect of R59949 on PA generation and DGK activation. Fig. 4B indicates that cells that had been treated with R59949 before stimulation with IL-2 failed to increase their PA levels above non-stimulated cells, whereas those cells that had been stimulated with IL-2 3 h before treatment with R59949 accumulated PA to a similar extent to cells which had only been stimulated with IL-2. In addition, the effect of R59949 on long-term IL-2-stimulated DGK activity was investigated. In accordance with the observations that both cell proliferation and PA accumulation at 18 and 24 h in cells stimulated with IL-2 were identical to those in cells treated with R59949 3 h after IL-2 stimulation, no differences in DGK activity in membrane extracts from identically treated CTLL-2 cells were observed (Fig. 5).

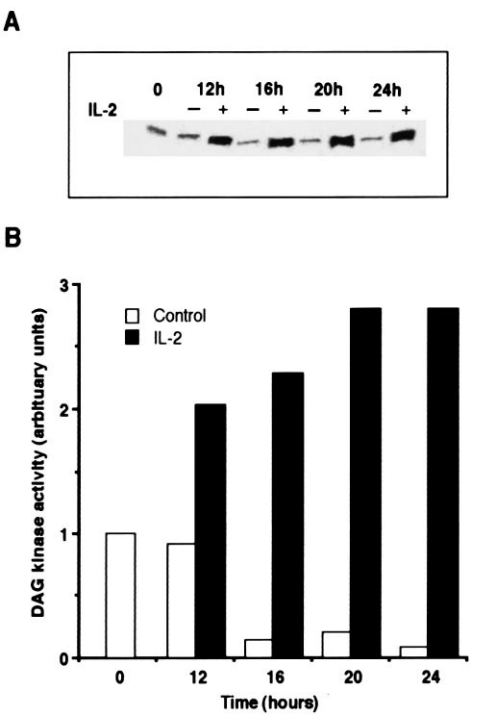

Fig. 3. IL-2 causes a late sustained activation of DGK. CTLL-2 cells were arrested and then incubated for various times with or without IL-2. DGK activity was determined in crude membrane extracts. Shown here is the digitized image of a TLC plate corresponding to the region of PA (A) and its quantification by phosphorimaging (B) which is representative of three experiments with similar results.

A

	18h			24h		
	Sub Go	Go/G1	S+G2/M	Sub Go	Go/G1	S+G2/M
Control	28	57	13	49	29	20
IL-2	8	46	42	10	23	63
R59949 (-30m) / IL-2	12	66	22	14	60	26
IL-2/R59949 (+3h)	10	49	38	9	27	60

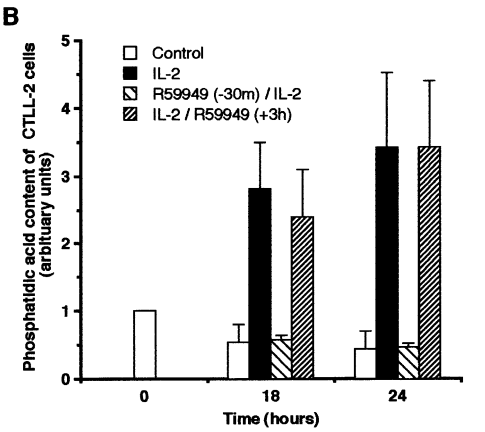

Fig. 4. Effect of the DGKα inhibitor R59949 on IL-2-stimulated cell proliferation and PA accumulation. A: CTLL-2 cells were arrested and then incubated for various times with or without IL-2. R59949 was added to the cells 30 min before or 3 h after IL-2 stimulation. Cell cycle analyses were performed 18 and 24 h after IL-2 stimulation. The percentage distribution of cells within regions of the cell cycle profile is indicated for each cell treatment. Shown here are the results from one experiment representative of two experiments with similar results. B: CTLL-2 cells were arrested, radiolabeled (according to the legend of Fig. 1) and treated as indicated above with combinations of IL-2 and R59949. Intracellular PA was determined at 18 and 24 h after IL-2 stimulation according to the legend of Fig. 1. This figure depicts the combined results of two independent experiments (mean ± S.D.).

4. Discussion

There are several reports in the literature describing that IL-2 does not activate classical mechanisms but instead it provokes the rapid activation of novel phospholipid metabolism [7,8,10–16]. All these events take place within seconds to minutes after the union of IL-2 with its receptor. The rationale behind our study was to investigate the long-term effects of IL-2 on lipid second messenger generation and to fill in the gap in the understanding of the late phase of IL-2 action. Without doubt the roles of lipid second messengers have been extensively studied in several cell types [27]. In the case of lymphocytes, this aspect of cell signaling has been scarcely examined and to the present date there are no data with respect to IL-2-induced long-term increases in PA. With the

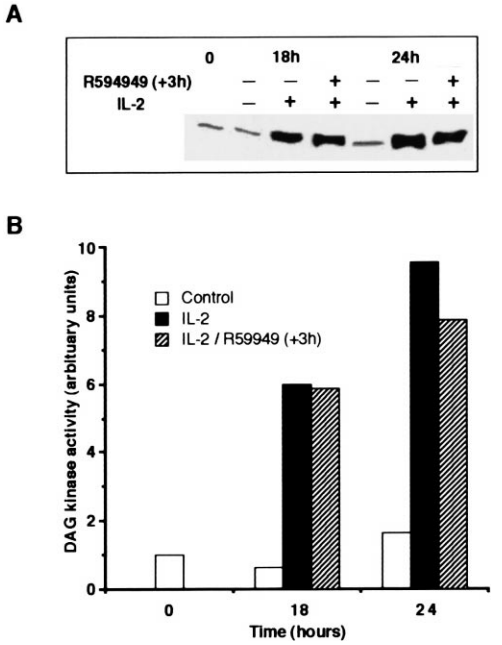

Fig. 5. Effect of the DGKα inhibitor R59949 on IL-2-stimulated DGK activity. After arresting CTLL-2 cells, half were stimulated with IL-2 whereas the other half received identical IL-2 stimulation followed 3 h later by treatment with R59949. DGK activity was determined in crude membrane extracts after 18 and 24 h of IL-2 stimulation. Shown here is the digitized image of a TLC plate corresponding to the region of PA (A) and its quantification by phosphorimaging (B) which is representative of two experiments with similar results.

recent observation that IL-2 causes a delayed and sustained increase in the level of DAG in CTLL-2 cells, which correlates with the induction of S-phase entry, [21] and that numerous growth factors cause the long-term elevation of DAG levels [18–20], we decided to investigate the possibility that in addition to DAG, PA accumulated in the cells as a consequence of IL-2 stimulation. Our results demonstrate that this was indeed the case as a late and sustained increase in PA was observed.

PLD is the unique phospholipase which, when in the presence of a primary aliphatic alcohol, such as ethanol, may catalyze a transphosphatidylation reaction in which the phosphatidyl moiety is transferred to an alcohol molecule forming a phosphatidylalcohol (for example PEt). This reaction is considered to be unequivocal evidence for PLD activity [18,19]. In both highly metabolically labeled cells and cell extracts, no PLD activity was observed at the times when the late IL-2-stimulated PA accumulation occurred. In addition, no acute IL-2 stimulation of PLD in CTLL-2 cells was observed which was in disagreement with the observations by Cano and co-workers [28]. Using NIH3T3 fibroblasts as a positive control, PMA, the activator of classical isoforms of PKC, was able to increase PLD activity. This effect was not observed in CTLL-2 cells. As there was no activation of PLD using IL-2 or PMA, it was considered that PLD was not expressed in CTLL-2 cells. Unfortunately, no antibodies are currently available to examine the expression of any of the PLD isoforms so far known. Our lack of PMA-responsive PLD activity in murine CTLL-2 cells in contrast to PMA-responsive PLD activity in human KIT225 cells is supported in part by the finding that in the murine EL4 T-lymphocyte cell line no activation of PLD by PMA is observed [29] whereas in human Jurkat cells PLD activation by PMA and TCR antibody cross linking is seen [29,30], suggesting that, in lymphocytes, PLD activation does take place but its specificity depends on the ligand and/or the origin of the lymphocytes used.

As PLD activity was not stimulated by IL-2, the other route by which IL-2 could have generated PA was through the activation of DGK. Our results demonstrate a late and sustained increase in PA that mirrored the stimulation of DGK activity. The exact role of elevated PA levels in CTLL-2 cells is unknown, but as PA is considered to be a mitogenic agent in many cell types [17–19] similar mitogenic activity may be important in CTLL-2 cells. This late activation of DGK by IL-2 shows some similarity to that seen in other cell types [31,32]. With the clear correlation between the time course of DGK activation and PA accumulation induced by IL-2, it was concluded that this elevated activity was totally responsible for the IL-2-stimulated accumulation of PA.

Flores and co-workers have demonstrated that IL-2 delivers its mitogenic signal in part through the α isoform of DGK [16] which is an isoform both sensitive to R59949 and highly expressed in T-cells [33]. Herein we have built on this finding by investigating if by pharmacological modulation of the early activation of DGKα, the subsequent second wave of PA accumulation is affected. This was indeed the case. However, this was not observed when the R59949 was added to the cells 3 h after IL-2. This would implicate that the 'early signaling' involving DGKα, i.e. that occurring within 3 h of IL-2 stimulation, is able to positively regulate PA production at 10 or more hours later. No inhibition following post 3 h treatment of IL-2-stimulated cells with R59949 rules out that the αDGK isoform is responsible for the long-term generation of PA. The DGK assays performed herein used a crude membrane fraction as antibodies against murine isoforms of DGK still remain unavailable. Therefore it was difficult to identify which of the known DGK isoenzymes could have been activated at late time periods by IL-2. In addition, the possibility of as yet undiscovered novel isoforms of DGK participating during late IL-2-mediated phospholipid metabolism cannot be excluded. Experiments are under way to identify DGK isoforms which are responsible for the late IL-2-stimulated generation of PA.

In summary, this paper has demonstrated for the first time that in CTLL-2 cells IL-2 causes the late generation of not only DAG but also PA. This observation shows general similarity to that seen in other transmembrane signaling systems but the pathways for their generation in CTLL-2 cells by IL-2 are substantially different. The lipid second messengers termed DAG and PA are in reality only generic names for the many subtypes, due to the multiplicity of fatty acids and their corresponding acyl and/or alkyl linkages to glycerol, found within these lipid fractions [27]. This is becoming an important feature of agonist-stimulated signal transduction in that the identification of individual species of DAG and PA that confer microspecificity on the activation and/or inhibition of specific enzymes are the targets for the future design of valid drugs to combat various immunological diseases and undesirable states.

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