Implication of three isoforms of PLA_2 in human T-cell proliferation

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Abstract We observed that human (Jurkat) T-cells constitutively expressed the mRNA, encoding for the four isoforms of phospholipase A_2 (PLA_2), i.e. two secretory (type IB and type V), and two cytosolic (type IV, Ca^{2+}-dependent and type VI, Ca^{2+}-independent). In order to assess whether these PLA_2 isoforms are active, we labeled Jurkat T-cells with [^{3}H]arachidonic acid ([^{3}H]AA) and determined its release into the extracellular medium in the presence of phorbol 12-myristate 13-acetate (PMA) and ionomycin. The three PLA_2 isoforms seem functional as aristolochic acid and bromoenol lactone (BEL), the respective inhibitors of type IB/type V and type VI PLA_2s, significantly inhibited the release of free [^{3}H]AA. On the other hand, arachidonyl trifluoromethyl ketone ($AACOCF_3$), an inhibitor of type IV PLA_2, failed to curtail significantly the release of free [^{3}H]AA into the extracellular medium. We assessed the implication of these PLA_2 isoforms in transcription of the interleukin-2 (IL-2) gene, involved in T-cell proliferation. Hence, aristolochic acid and BEL, but not $AACOCF_3$, significantly inhibited the PMA and ionomycin-induced induction of mRNA of IL-2. Similarly, aristolochic acid and BEL, but not $AACOCF_3$, significantly inhibited the PMA and ionomycin-induced secretion of IL-2 in the culture supernatants. Together these results suggest that human Jurkat T-cells possess two secretory and two cytosolic PLA_2 isoforms and only three of them (type IB, type V and type VI) are implicated in T-cell proliferation. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Arachidonic acid; Jurkat T-cell; Phospholipase

1. Introduction

Phospholipase A_2 (PLA_2, EC 3.1.1.4) represents a class of enzymes that catalyze the hydrolysis of the sn-2 fatty acyl bond of phospholipids to give free fatty acids and lysophospholipids [1,2]. These enzymes have been divided into several groups based on molecular weight, amino acid sequence homology, calcium dependence and cellular localization [2,3]. There are essentially two large subgroups within the PLA_2 family: the small and usually secretory PLA_2 ($sPLA_2$) among which are pancreatic type IB and type V, and the intracellular cytosolic PLA_2 including the well characterized 85 kDa cytosolic Ca^{2+}-dependent type IV PLA_2 and the recently cloned Ca^{2+}-independent type VI PLA_2 [4–6]. During the last decade, the involvement of distinct types of PLA_2 in various biological processes has been demonstrated [3,7,8–10]. In particular, these enzymes have been shown to play a direct role in proliferation of many cell types such as fibroblasts, stromal and other cell types [9–12].

T-lymphocytes, antigen-specific cells of the immune system, are generally quiescent and require antigenic stimulation to progress from the G_0 stage of the cell cycle [13]. Resting lymphocytes are activated by either aggregation of T-cell receptor (TCR) with an antigen or mitogen stimulation such as phorbol 12-myristate 13-acetate (PMA) and ionomycin [14]. The early signaling events, following T-cell activation, include the activation of protein tyrosine kinases and the phospholipase C pathway to generate intracellular second messengers, leading to cell proliferation and differentiation [15]. However, the signaling mechanisms of T-cell activation, especially the implication of potential lipid second messengers as well as specific enzyme systems such as PLA_2, are not fully elucidated.

For several years, the presence and the role of different isoforms of PLA_2 in T-cell activation and proliferation have been controversial [16,17]. Though a Ca^{2+}-dependent type IV PLA_2 has been shown to be involved in T-cell activation, neither mRNA nor protein for this PLA_2 could be detected in human T-cells [18]. Hence, the presence of type VI PLA_2 in peripheral blood B- and T-cells and Jurkat T-cells has been shown [18]. This PLA_2 isoform plays a key role in the control of cell growth [18]. However, the mechanism of action of this PLA_2 isoform remains unknown.

To determine whether one or several PLA_2s are involved in T-cell proliferation, we examined the presence of four different isoforms of PLA_2, i.e. type IB, type V, type IV and type VI, in Jurkat T-cells. In this investigation, we confirmed the expression of type VI PLA_2 and identified the presence of two secreted PLA_2s (type IB and type V) in Jurkat T-cells where these isoforms seem to be implicated in cell proliferation. Moreover, our results revealed, for the first time, that one of the targets of these enzymes is the interleukin-2 (IL-2) gene whose mitogen-induced mRNA expression is down-regulated by the inhibitors of the three PLA_2 isoforms.

2. Materials and methods

2.1. Chemicals

Tissue culture medium (RPMI 1640), L-glutamine, penicillin–streptomycin, HEPES and fetal calf serum (FCS) were obtained from BioWhitaker (Verviers, Belgium). The SuperScript II Reverse Transcriptase, Platinum *Taq* DNA Polymerase, random primers, oligo(dT)18 and the oligonucleotides used as primers in the reverse transcription-polymerase chain reaction (RT-PCR) analysis were purchased from

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Abbreviations: AA, arachidonic acid (20:4 n-6); $AACOCF_3$, arachidonyl trifluoromethyl ketone; BSA, bovine serum albumin; BEL, bromoenol lactone; PMA, phorbol 12-myristate 13-acetate; PLA_2, phospholipase A_2; TCR, T-cell receptor

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Invitrogene, Life Technologies (Cergy Pontoise, France). Agarose was from Promega (Charbonnière, France). Phospholipase A_2 inhibitors, arachidonyl trifluoromethyl ketone ($AACOCF_3$) and bromoenol lactone (BEL), were from Cayman Chemical (Ann Arbor, USA). Aristolochic acid was from Sigma-Aldrich (Saint Quentin Fallavier, France). All other chemicals were of analytical grade. The kit (Bender MedSystem™) for the determination of IL-2 concentrations was purchased from Prolabo (Strasbourg, France).

2.2. Cell culture

For stock culture, Jurkat T-cells were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 50 μg/ml penicillin–streptomycin and 20 mM HEPES at 37°C in a 95% air–5% CO_2 humidified atmosphere. The medium was changed every 48 h.

For RT-PCR analysis, cells ($1.5–2\times10^6$) were seeded in six-well plates and incubated overnight in RPMI 1640 serum-free medium in order to synchronize them. The cells were then treated or not for 15 min with different PLA_2 inhibitors before stimulation for 2 h with 200 nM PMA and 500 nM ionomycin in RPMI 1640 serum-free medium. At the end of the experiment, the cells were centrifuged ($1500\times g\times10$ min) and the corresponding cell pellet was stored at −80°C until RNA extraction.

2.3. RNA isolation and semi-quantitative RT-PCR analysis

Total RNA from cultured Jurkat T-cells was purified using Trizol reagent (Invitrogene Life Technologies) according to the manufacturer's instructions.

Oligonucleotide primer pairs, used for mRNA analysis by RT-PCR, were based on the sequences of the human genes (Table 1). 0.5 μg of total RNA was reverse-transcribed at 42°C for 1 h. The reaction mixture (10 μl) containing random hexamer primers (20 pmol), oligo(dT)18 primers (10 pmol), 1× reaction buffer, deoxynucleoside triphosphate (dNTP, 0.5 mM), SuperScript II Reverse Transcriptase (100 U) was diluted to 50 μl with diethyl pyrocarbonate-treated water at the end of the RT reaction. The cDNA was either used immediately for PCR or stored at −20°C until use. For PCR amplification, a mixture containing specific oligonucleotide primers (20 pmol), dNTP (0.2 mM), $MgCl_2$ (12.5 mM) and Platinum *Taq* DNA Polymerase (0.8 U) was added to 2–10 μl cDNA (corresponding to 20–100 ng cDNA). The total volume was increased to 40 μl with 1× PCR buffer. For PCR amplification of the gene products, two sets of amplification cycles were utilized. In the first five cycles, annealing temperature (4°C+annealing temperature of the primer) for 5 min was followed by denaturation temperature (92°C) for 1 min. In the second set of amplification, for 20–30 cycles depending on each PCR-amplified product, annealing temperature of the primer for 1 min was followed by extension temperature (72°C) for 1 min and another denaturation temperature (92°C) for 1 min. The conditions were such that amplification of the product was in the exponential phase, and the assay was linear with respect to the amount of input cDNA. Human β-actin mRNA primers were used as internal control to normalize the data. Reaction products were electrophoresed on a 1% agarose gel impregnated with ethidium bromide. The RNA pattern was visualized by UV transillumination.

2.4. Arachidonic acid (AA) release

The experiment on AA incorporation and release was performed as described elsewhere [19]. In brief, Jurkat T-cells were serum-starved for 4 h before labeling with [^{3}H]AA (1.5 μCi/3×10^8 cells) for 2 h in RPMI 1640 serum-free medium supplemented with 0.2% fatty acid-free bovine serum albumin (BSA). At the end of the incubation, cells were washed twice with RPMI 1640 serum-free medium containing 0.2% BSA. The cells were resuspended in 500 μl RPMI 1640 medium supplemented with 0.5% BSA at a final concentration of 12×10^6 cells/ml. Jurkat T-cells were then treated with 5 or 15 μM of PLA_2 inhibitors or vehicle (dimethyl sulfoxide (DMSO), 0.1% final concentration) for 30 min, followed by a 20 min stimulation with PMA (200 nM) and ionomycin (500 nM). Cells were centrifuged ($1250\times g\times3$ min) and 0.4 ml of supernatant was added to 2 ml scintillation cocktail for counting in a liquid scintillation analyzer (Packard 1900 TR).

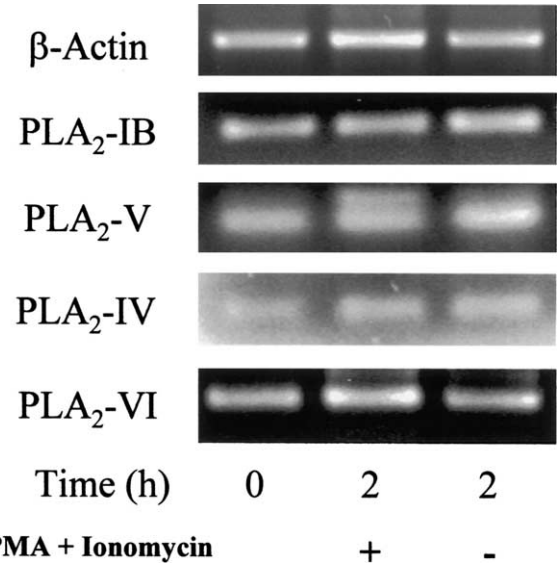

Fig. 1. Constitutive expression of different PLA_2 isoforms in Jurkat T-cells. Quiescent Jurkat T-cells were treated for 2 h with or without PMA (200 nM) and ionomycin (500 nM). Total RNA was isolated and analyzed by RT-PCR using specific primers for human PLA_2-IB, -V, -IV and -VI as described in Section 2. β-Actin mRNA was used as an internal standard. One representative blot of three independent experiments is shown.

2.5. Determination of IL-2 concentrations in culture supernatants

Jurkat T-cells from stock culture were seeded in 25 ml flasks (2×10^5 cells/ml) in RPMI 1640 serum-free medium. After overnight incubation, PLA_2 inhibitors (at 5 or 15 μM) or DMSO vehicle (0.1%, final concentration) were added to cells for 15 min prior to the addition of PMA (200 nM) and ionomycin (500 nM). The cells were cultured for 48 h and, after centrifugation ($200\times g\times15$ min), the culture supernatants were saved for the determination of IL-2 concentrations. IL-2 concentrations were determined by employing enzyme-linked immunosorbent assay (ELISA) as per instructions furnished with the kit (Bender MedSystem™).

Table 1
Sequences of primers of IL-2 and different PLA_2 isoforms

Gene	Primer sequences	Expected size (bp)
PLA_2-IB	S 5′-CGTGTCGCAGTTCCGCAAAATGAT-3′ AS 5′-GCCGACCCAGAGCACGAGTATGAA-3′	240
PLA_2-V	S 5′-TGGCTTGGTTCCTGGCTTCTTGTAGTG-3′ AS 5′-TTCTCCTCCAGCCGCCCATAGCAG-3′	211
PLA_2-IV	S 5′-CCCAAAGGCACTGAAAATGAAGAT-3′ AS 5′-GGGATACGGCAGGTTAAATGTGAG-3′	347
PLA_2-VI	S 5′-GCAGCGCCACATCATCCCTTCTCC-3′ AS 5′-TGCCCGCCACCCAGTCAAACAG-3′	271
IL-2	S 5′-CACTAATTCTTGCACTTGTCAC-3′ AS 5′-CCTTCTTGGGCATGTAAAACT-3′	186
β-Actin	S 5′-ATGATATCGCCGCGCTCGTCGTC-3′ AS 5′-AGGTCCCGGCCAGCCAGGTCCAG-3′	547

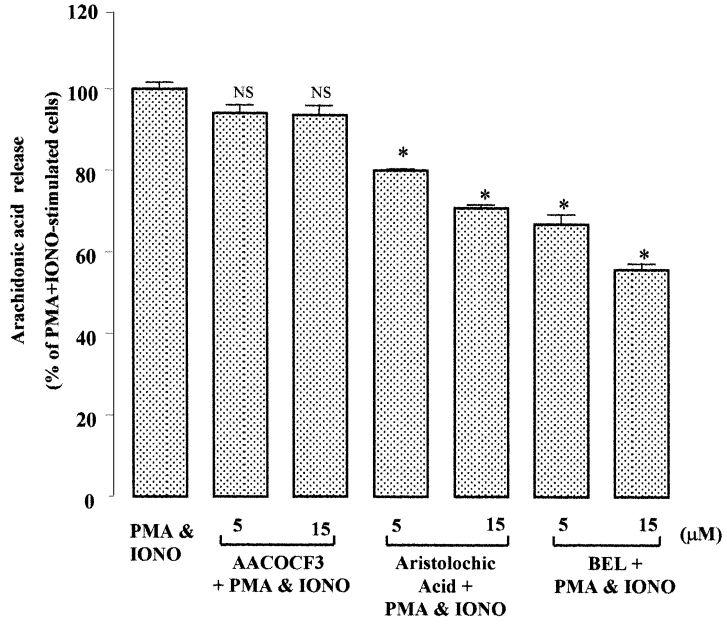

Fig. 2. Effect of PLA_2 inhibitors on [^{3}H]AA release. Serum-starved Jurkat T-cells (3×10^8) were labeled for 2 h with 1.5 μCi [^{3}H]AA. The cells were then treated with 5 or 15 μM $AACOCF_3$, aristolochic acid or BEL or with vehicle (DMSO, 0.1% final concentration) for 30 min, followed by a 20 min stimulation with PMA (200 nM) and ionomycin (500 nM) (PMA and IONO). Cells were harvested as described in Section 2. Results are expressed as mean ± S.E.M. of three independent experiments. Data are expressed as a percentage of PMA- and ionomycin-stimulated cells which was considered to be 100%. Data are significantly different as compared to PMA and IONO group ($*P<0.001$). NS represents insignificant values.

2.6. Statistical analysis

Results are shown as mean ± S.E.M. Statistical analysis of data was carried out using Statistica (version 4.1, Statsoft, Paris, France). The significance of the differences between mean values was determined by analysis of variance one way, followed by a least significant difference test.

3. Results

3.1. PLA_2s of type-IB, -V, -IV and -VI are constitutively expressed in Jurkat T-cells

To shed light on whether the Jurkat T-cells expressed or not PLA_2 mRNA, we designed specific primers directed against four different PLA_2 isoforms (Table 1). Two secreted PLA_2s (type IB and V) as well as two cytosolic PLA_2s (type IV Ca^{2+}-dependent and type VI Ca^{2+}-independent) were selected. As shown in Fig. 1, these four PLA_2 isoforms were constitutively expressed in quiescent Jurkat T-cells. As these PLA_2s were shown to be involved in cell proliferation, we examined the effect of a mitogenic signal on the mRNA expression of these isoforms. Jurkat T-cells were stimulated for 2 h with PMA and ionomycin and the expression of different PLA_2 isoforms was measured by semi-quantitative RT-PCR. The results demonstrated that none of these PLA_2 isoforms were regulated at the mRNA level after the mitogenic stimulation (Fig. 1).

3.2. Release of [^{3}H]AA is inhibited by secreted and cytosolic Ca^{2+}-independent PLA_2 inhibitors in Jurkat T-cells

To determine whether these enzymes were active in Jurkat T-cells, we examined the release of [^{3}H]AA after stimulation by PMA and ionomycin in the presence of different PLA_2 inhibitors. The results showed that aristolochic acid, a non-specific inhibitor of type IB/V $sPLA_2$ and BEL, an inhibitor of cytosolic and Ca^{2+}-independent type VI PLA_2, were able to significantly decrease [^{3}H]AA release, induced by PMA and ionomycin (Fig. 2). $AACOCF_3$, an inhibitor of cytosolic Ca^{2+}-dependent type IV PLA_2 exerted no significant effect on [^{3}H]AA release in these cells. In control cells (vehicle control), no significant release of [^{3}H]AA was observed (results not shown).

3.3. IL-2 mRNA expression, induced by PMA and ionomycin, is inhibited by secreted and Ca^{2+}-independent PLA_2 inhibitors

To study the mechanism of action of PLA_2 on T-cell proliferation, we first designed the primers of IL-2 and β-actin mRNA (Table 1) and examined the kinetic of expression of IL-2 mRNA after PMA and ionomycin stimulation; IL-2

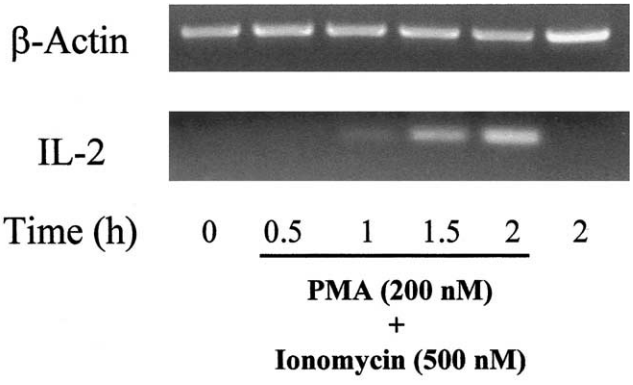

Fig. 3. Kinetics of IL-2 mRNA expression after PMA and ionomycin stimulation. Harvested Jurkat T-cells were cultured for different times in RPMI 1640 serum-free medium with PMA (200 nM) and ionomycin (500 nM). RNA was prepared and subjected to semi-quantitative RT-PCR analysis as described in Section 2. PCR products were visualized by UV transillumination. A representative blot ($n=3$) is shown.

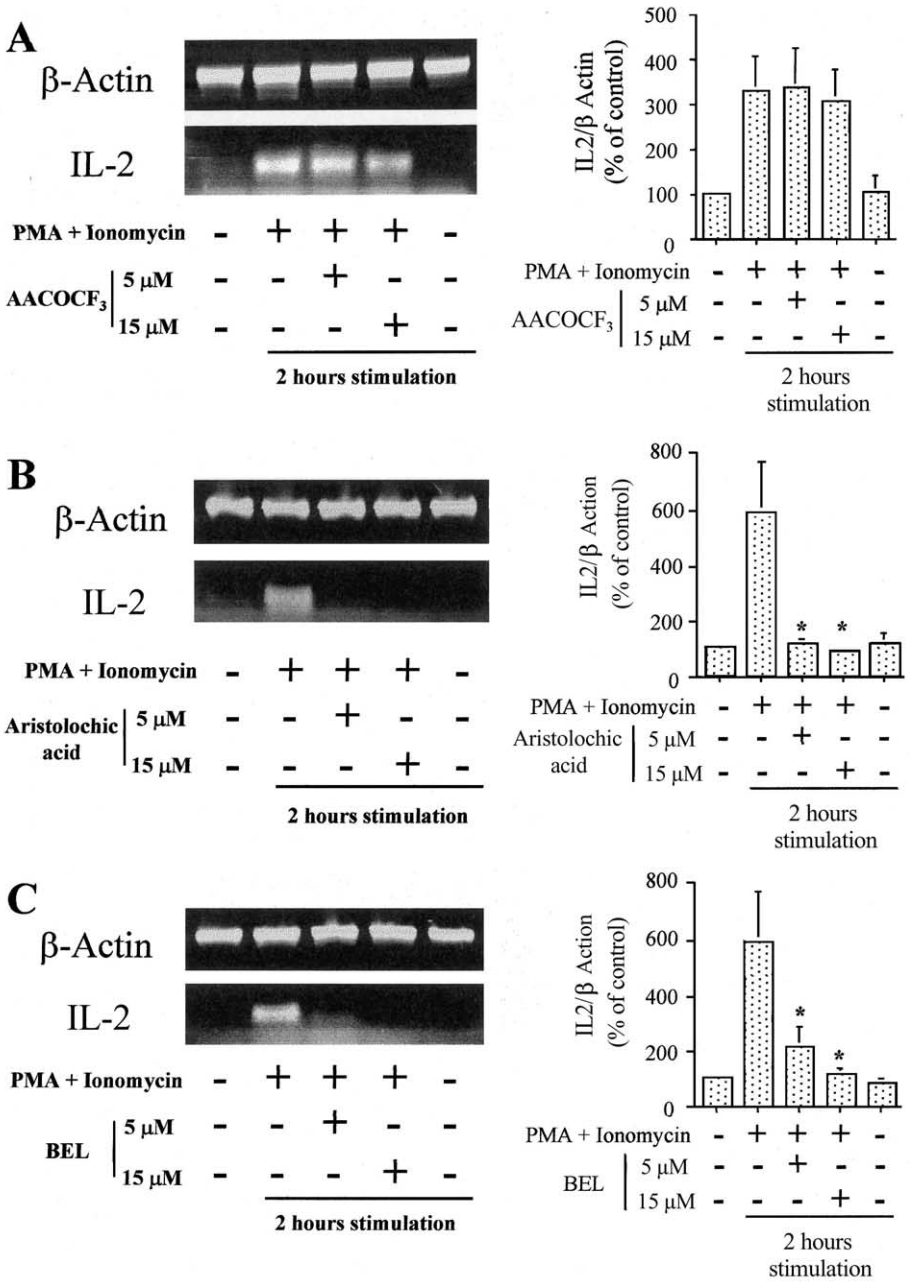

Fig. 4. Effect of different PLA_2 inhibitors on the induction of IL-2 mRNA levels by PMA and ionomycin. Jurkat T-cells were pre-treated for 15 min with (5 or 15 μM) $AACOCF_3$ (A), aristolochic acid (B) and BEL (C) before a 2 h stimulation with PMA (100 nM) and ionomycin (500 nM). Total RNA was prepared and subjected to RT-PCR analysis as described in Section 2. The left panel shows a representative blot ($n = 3$) and the right panel depicts the normalized mRNA levels (mean ± S.E.M.), values are expressed as a percentage of the control which was considered 100%). Data are significantly different as compared to PMA- and ionomycin-stimulated cells ($^*P < 0.001$).

being one of the first genes expressed during the early events of T-cell activation and proliferation. Fig. 3 showed that no IL-2 mRNA was detectable in quiescent Jurkat T-cells; however, the cells started to express IL-2 mRNA after 1 h of treatment with PMA and ionomycin. Maximum IL-2 levels were obtained after 2 h of stimulation. We treated T-cells with either $AACOCF_3$, aristolochic acid or BEL and examined IL-2 mRNA expression after 2 h of PMA and ionomycin treatment (Fig. 4). $AACOCF_3$ failed to curtail the expression of IL-2 mRNA induced by PMA and ionomycin, though aristolochic acid and BEL completely abolished this induction (Fig. 4).

3.4. IL-2 secretion, stimulated by PMA and ionomycin, is inhibited by secreted and Ca^{2+}-independent PLA_2 inhibitors

To confirm the role of these PLA_2s in T-cell proliferation, Jurkat T-cells were stimulated by PMA and ionomycin with $AACOCF_3$, aristolochic acid or BEL. After 48 h of stimulation, culture supernatants were used for the determination of secreted IL-2. We observed that only aristolochic acid and BEL were able to significantly inhibit the secretion of IL-2, induced by PMA and ionomycin (Fig. 5). $AACOCF_3$ exerted mild, but insignificant, effect on IL-2 secretion in the stimulated T-cells (Fig. 5).

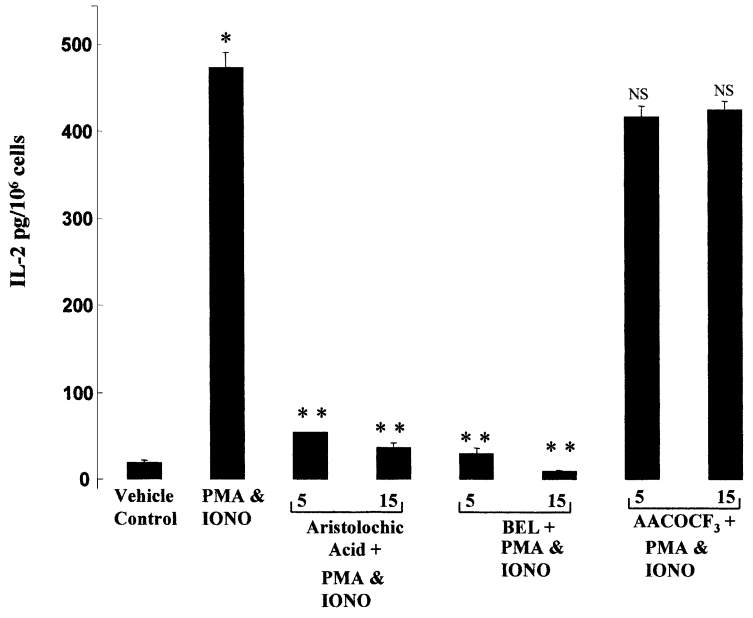

Fig. 5. Effect of different PLA_2 inhibitors on IL-2 secretion in the culture supernatants. Jurkat T-cells (2×10^5 cells/ml) were treated with (5 or 15 μM) $AACOCF_3$, aristolochic acid or BEL or with DMSO vehicle (0.1%) alone for 15 min prior to a stimulation with 200 nM PMA and 500 nM ionomycin (PMA and IONO) in RPMI 1640 serum-free medium. After 48 h, the cells were centrifuged ($200\times g\times15$ min) and the culture supernatants were used for the determination of IL-2 concentrations by ELISA, according to the instructions furnished with the kit (Bender MedSystem®). Data represent mean ± S.E.M. of quadruple independent experiments. Data are significantly different as compared to vehicle control ($^*P<0.001$) and as compared to PMA- and ionomycin-treated groups ($^{**}P<0.001$). NS represents insignificant values as compared to the PMA- and ionomycin-treated groups.

4. Discussion

In the present study, we have observed that Jurkat T-cells constitutively express mRNA encoding for four different PLA_2 isoforms (type-IB, -V, -IV and -VI) among which secreted (type IB and type V) as well as cytosolic Ca^{2+}-independent (type VI) PLA_2s appear to play a critical role in T-cell proliferation.

Though some cell types of the immune system, like murine macrophages and mast cells, have been reported to possess functional $sPLA_2$ type V, the lymphocytes do not seem to possess this $sPLA_2$ isoform [15,20]. However, our study on the expression of mRNA of type VI PLA_2 as well as type V PLA_2 in Jurkat T-cells is consistent with a recent report in which human peripheral blood T- and B-lymphocytes and Jurkat T-cells have been demonstrated to possess these PLA_2 isoforms [18]. We should recall that the existence of mRNA for 85 kDa PLA_2 and its protein has been reported in activated thymocytes and immature B-cells; however, this isoform of PLA_2 has been found to be absent and the expression of its mRNA could not be induced in mature T- and B-cells [18,21]. On the contrary, in the present report, only low, but detectable, mRNA levels of this isoform of PLA_2 (type IV) in Jurkat cells are noticed. Our results are in agreement with the findings of Boilard and Surette [17] who have recently identified type IV PLA_2 in human T-lymphocytes.

In the present report, we demonstrate, for the first time, the expression of mRNA of type IB PLA_2 in Jurkat T-cells. To date, expression of this PLA_2 isoform, which was first discovered in pancreatic juice, then in several cell types and tissues, has never been shown in T-lymphocytes [22,23].

In order to gain insight into the functional implication of different PLA_2 isoforms, we employed their inhibitors and conducted studies, at first hand, on the release of $[^3H]AA$. We used aristolochic acid, an inhibitor of $sPLA_2$ and $AACOCF_3$, an inhibitor of 85 kDa PLA_2, including type IV [24]. We also employed BEL, a mechanism based inhibitor, used to inhibit the cytosolic Ca^{2+}-independent PLA_2 [25]. We have observed that aristolochic acid and BEL, but not $AACOCF_3$, inhibited significantly PMA- and ionomycin-stimulated release of AA into extracellular environment. In order to ascertain whether PLA_2 inhibitors diminish the transcription of IL-2 gene, we assessed the effects of these inhibitors on mitogenic expression of IL-2 mRNA. As anticipated, aristolochic acid and BEL, but not $AACOCF_3$, inhibited the PMA and ionomycin-induced expression of IL-2 mRNA. Similarly, the two PLA_2 inhibitors, aristolochic acid and BEL, but not $AACOCF_3$, significantly inhibited the PMA- and ionomycin-stimulated secretion of IL-2 in the culture supernatants. In these experiments, $AACOCF_3$ mildly curtailed IL-2 secretion and this inhibitory effect can be explained by the fact that this PLA_2 inhibitor, being present for 48 h in the culture medium, might exert its effect non-specifically on cytosolic Ca^{2+}-independent PLA_2 as suggested by Anderson et al. [26]. These observations altogether suggest that human Jurkat T-cells express the mRNA encoding for two secretory and two cytosolic (Ca^{2+}-independent and Ca^{2+}-dependent) isoforms of PLA_2 and only the three species (two secretory and one cytosolic Ca^{2+}-independent) are implicated in T-cell blastogenesis.

The exact mechanisms of action of PLA_2s in the inhibition of IL-2 secretion, considered as an indicator of T-cell proliferation, is not known. We can speculate that T-cell activation via TCR may be coupled to PLA_2 activation as observed by Bouilard and Surette [17] that anti-CD3 antibodies induced TCR signalling and phosphorylation of a type IV PLA_2 in

human T-cells. Furthermore, the importance of PLA_2 in T-cell proliferation has also been shown by Kudo et al. [27] as these investigators have observed that addition of exogenous PLA_2 to human T-cells can greatly enhance the signal-induced cell activation [27]. Nonetheless, we can speculate that released free AA, as observed in our study, may act in an autocrine manner to accelerate T-cell blastogenesis. Hence, we can envisage per se effects of free AA on cell signalling as Jurkat T-cells do not possess the enzymes that can metabolize the free fatty acids into eicosanoids [28,29]. We have recently shown that free fatty acids modulate calcium signaling and mitogen activated protein kinase activation in Jurkat T-cells [30–32]. Alternatively, free AA, in place of being released to the extracellular environment, may be translocated to the nucleus as it has been observed in a stromal cell line [33]. Nuclear AA may directly affect the transcription of several genes via PPARγ whose implication in T-cell proliferation has been recently documented [34]. PLA_2 may also regulate lymphocyte phospholipid turnover in coordination with lysotransferase enzymes.

Other products of PLA_2-induced hydrolysis of phospholipids are lysophospholipids [1,2]. The lysophospholipids like lyso-phosphatidylcholine have been found to be co-activators of protein kinase C (PKC), implicated in T-cell proliferation [35]. For instance, lyso-phosphatidylcholine per se is toxic for human T-cells but in the presence of endogenous diacylglycerol, this agent stimulates several fold the activation of PKC, implicated in Jurkat T-cell proliferation [32,35]. It is also possible that PLA_2 inhibitors diminish, in part, T-cell proliferation, by diminishing the production of lysophospholipids.

In our study, the use of different PLA_2 inhibitors allowed us to show that three enzymes are functional and play a crucial role in T-cell activation. These results open the door to the synthesis of more potent PLA_2 inhibitors that will bear immunosuppressive properties and can be used in immunotherapy of autoimmune diseases.

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