The Pro-451 to Leu polymorphism within the C-terminal tail of P2X7 receptor impairs cell death but not phospholipase D activation in murine thymocytes

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Running title: P2X7R-induced cell death is independent of phospholipase D activity
Abstract

The P2X family of ATP receptors (P2XR) are ligand-gated channels that have been proposed to regulate cell death of immature thymocytes. However, the nature of the P2XR subtype involved has been controversial until recently. In agreement with previous studies, we found that extracellular ATP (ATPe) induces a caspase-dependent apoptosis of Balb/c thymocytes, as observed by DNA fragmentation. Additionally, ATPe induces a predominant caspase-independent thymocytes lysis characterized by plasma membrane disruption. Both responses to ATPe can be induced by a potent P2X7R agonist, benzoylbenzoyl-ATP (BzATP) while P2X7R antagonists, oxidizedATP (oATP) and pyridoxal-phosphate-6-azophenyl-2’,4’-disulfonic acid (PPADS) inhibited the effect of ATPe. These results are further supported by observations where disruption of the P2X7R gene (P2X7R−/− mice) completely abolishes thymocytes death induced by ATPe. Interestingly, the natural P451L mutation in the C-terminal tail of P2X7R present in C57BL/6 mice, which impairs ATPe-dependent pore formation in T lymphocytes, significantly reduces thymocytes death triggered by ATPe. Furthermore, we found that P2X7R from BW5147 thymoma cells also harbours this point mutation, accounting for their insensitivity to ATPe-induced cell death. Concentrations of ATPe effective in inducing cell death, also increase phosphatidylcholine-hydrolyzing phospholipase D (PC-PLD) activity in Balb/c thymocytes through the stimulation of P2X7R. However, in contrast to ATPe-induced cell death, PC-PLD activation is totally Ca2+-dependent. Moreover, the stimulation of PC-PLD by ATPe is not affected by the P451L mutation present in C57BL/6 thymocytes and BW5147
cells, suggesting that cell death and PC-PLD activity are regulated through distinct domains of the P2X7R. Finally, the inhibition of ATPe-induced PC-PLD stimulation does not affect thymocytes death. Altogether, these data suggest that P2X7R-induced thymocytes death is independent of the stimulation of PC-PLD activity.
Introduction

Extracellular ATP (ATPε) interacts with P2 purinergic receptors which are widely distributed in different cell types. Two distinct subfamilies of P2 receptors have been described, namely the G-protein coupled seven-transmembrane P2Y receptors and the ligand-gated ionotropic P2X receptors (P2XR). Among the P2XR, the P2X7R subclass is notably expressed in hemopoietic tissue (1). Activation of the P2X7R by ATPε opens ion channels which mediate fast permeability changes to monovalent and divalent cations (Na+, K+ and Ca^{2+}). Upon prolonged activation, P2X7R induces the formation of nonselective membrane pores permeable to molecules up to 800 Daltons (2). At present, the mechanisms underlying the opening of the cation channel and its transition or coupling to a pore are still unknown. It has been proposed that P2X7R is able to undergo a progressive increase in size, possibly by recruitment of additional receptor subunits (2). However, Schilling et al. (3) propose another attractive hypothesis, where P2X7R might interact with a distinct cytolytic pore allowing the entry of large molecules into the cell. Moreover, formation of the non selective pore has been shown recently to be dissociated from large scale changes in P2X7R density (4), but is dependent on the cytoplasmic C-terminal part of the P2X7R (5, 6, 7).

In blood cells, P2X7R is known to mediate physiological processes such as the release of cytokines and the generation of reactive oxygen species (2). A role for P2X7R has also been proposed in ATPε-triggered cell death of several cell types, including myeloid cells (8), macrophages (9, 10) dendritic cells (11) and thymocytes (12, 13). The function of P2X7R in the
latter is still controversial, because previous reports have proposed an additional role of P2X1R in thymocyte death (14, 15). In addition, while caspases have been implicated in ATPe-triggered cell death in some cell-types (8, 10, 16), the molecular mechanisms leading to ATPe-induced cell death are not yet fully understood.

Stimulation of P2X7R by ATPe triggers several intracellular signaling events (16, 17, 18) among which is the activation of phospholipase D (PLD), an enzyme which mainly hydrolyzes phosphatidylcholine (PC) into phosphatidic acid (PA) and choline. ATPe has been shown to trigger PLD activation in various cell types such as macrophages (19), lymphocytes (20), astrocytes (21) and PC12 pheochromocytoma (22). In mammals, two types of PLDs have been described, those which are dependent of phosphatidylinositol 4,5-bisphosphate for activity and those which are activated by oleate. Among the first type of PLDs, PLD1 and PLD2 isoforms have been cloned and characterized, (23, 24). The mechanisms of regulation of PLDs are highly dependent on the cell-type studied (25, 26). PLDs are involved in a variety of physiological processes such as vesicular trafficking and secretion, superoxide generation, bacterial killing, proliferation, differentiation and possibly apoptosis (25, 27). Indeed, PLD activity increases when apoptosis is induced by Tumour Necrosis Factor α (TNFα) or anti-Fas/Apo1 monoclonal antibody in HL60 cells and in murine B cell lymphoma A20 cells (28). Oleate-stimulated PLD activity is also associated with apoptosis induced by actinomycin D, TNFα or hydrogen peroxide (H₂O₂) in Jurkat T cells (29). However, PLD activity has also been shown to be involved in anti-apoptotic responses. Thus, PLD2 plays a suppressive role in H₂O₂-induced apoptosis (30)
and in hypoxia-induced cell death (31).

Taking into account the divergent roles of PLD in apoptosis, we examined whether PLD activation via P2X7R is associated with ATPe triggered cell death of thymocytes. During their development immature thymocytes undergo two selection processes. Positive selection insures that thymocytes can interact with self-Major Histocompatibility Complex molecules via their receptor for antigen (TcR). Thymocytes die by neglect when they fail to undergo positive selection. Negative selection eliminates thymocytes bearing a TcR with too high an avidity for self-MHC molecules. Thus a large proportion of thymocytes fails to develop and dies by apoptosis (32). Interestingly, in addition to antigen stimulation through the TcR and the glucocorticoid receptor, a third pathway involving ATPe has been proposed to regulate apoptosis of immature thymocytes (14, 33). It has been suggested that intrathymic elimination of self-reactive thymocytes is mediated by P2X1R (14) while death by neglect may be due to one or more PPADS-sensitive P2X1R, 2R and 7R (33). In dexamethasone-induced apoptosis, it was also shown that P2X1R were upregulated and antagonists to these purinoreceptors prevented cell death (14). However, the role of P2X1R in negative selection has been challenged recently (34).

In the present work, we have examined the consequences of P2X7R stimulation by ATPe in thymocytes by taking advantage of the P2X7R knock-out mice (P2X7R−/−) (35). We showed that ATPe stimulation of P2X7R triggers phosphatidylcholine-hydrolyzing PLD (PC-PLD) activity and cell death of mouse thymocytes. We also found that in contrast to cell death, PC-PLD activation is calcium-dependent and not modified by the P451L mutation present in the C-
terminal cytoplasmic tail of P2X7R of some strains of mice. Altogether, our results strongly suggest that PC-PLD is not directly involved in the pathways leading to ATPe induced cell death of thymocytes.
Materials and methods

Materials

Tissue culture media were from Gibco, Life Technologies SARL. [³H]myristic acid (49 Ci/mmol, 1.81 TBq/mmol - 1 mCi/ml, 37 MBq/ml) was purchased from NEN Life Science Products. ATP, UTP, 2’,3’-O-(benzoyl-4-benzoyl)-ATP (BzATP), oxidized ATP (oATP), pyridoxal-phosphate-6-azophenyl-2’,4’-disulfonic acid (PPADS), 2,3-diphosphoglycerate (2,3 DPG), ionomycin and lactate dehydrogenase (LDH) detection kit were from Sigma Chemicals Co. All solvents were from Merck Eurolab. Annexin-V-fluos was from Boehringer-Mannheim, “In situ cell death detection kit, fluorescein” was from Roche and U73122 from Calbiochem.

Thymocytes were prepared from thymus of 4-6 week-old male Balb/c, BDF1 (C57BL/6 x DBA/2 F1) or C57BL/6 mice (Charles River) by standard procedures. Their viability was always above 98%, as tested by trypan blue exclusion. Thymocytes (about 200-250 millions/mouse) were suspended before the experiments in RPMI medium supplemented with 1 mg/ml lipid-free BSA. Fetal Bovine Serum (FBS) was not used because it may contain components able to stimulate thymocytes. P2X7R⁻/⁻ BDF1 mice were previously described (35). BW5147 cells, a murine thymoma cell line derived from the AKR mouse strain were grown in RPMI medium supplemented with 10% FBS and 1% antibiotics.

Measurement of PC-PLD activity

For determination of PC-PLD activity, we measured the accumulation of
phosphatidylbutanol (PBut) (24). Murine thymocytes (8.5 x 10⁶) suspended in 2.5 ml of RPMI containing 1 mg/ml lipid-free BSA, were labeled with 12.5 µCi of [³H]myristic acid at 37°C for 5h. BW5147 cells (8.5 x 10⁵) suspended in 1 ml of RPMI containing 1 mg/ml lipid-free BSA, were labeled with 6.4 µCi of [³H]myristic acid at 37°C for 24 h. Analysis of [³H]myristic acid-labeled lipids, performed as previously described (37), revealed that PC represents 86 ± 4 % and 70 ± 5% of the labeled phospholipids in Balb/c thymocytes and BW5147 cells respectively. After labeling, the thymocytes and BW5147 cells were washed with RPMI and preincubated at 37°C for 15 min, in the presence of 0.3% butanol-1 in 2 ml of RPMI supplemented with 1 mg/ml lipid-free BSA and 0.5 mM CaCl₂ (CaCl₂ final concentration : 0.925 mM). ATPe or other agents were then added for 45 min incubation. At the end of the experiment, cellular lipids were extracted and separated by thin layer chromatography as described previously (37). The amounts of [³H]PBut formed were expressed as a percentage of the amount of total radiolabeled lipids.

Calculated concentrations of ATP⁴⁻ species

In the medium routinely used, Ca²⁺ and Mg²⁺ concentrations were 0.925 and 0.406 mM, respectively. The concentrations of ATP⁴⁻, ATP⁴⁻-Ca²⁺, ATP⁴⁻-Mg²⁺, free Ca²⁺ were calculated using the equilibrium values reported in “Data for Biochemical Research” (edited by Dawson et al., Oxford University Press, 1969).
Measurement of LDH release

Cell lysis was determined by measuring the release of lactate dehydrogenase (LDH). Murine thymocytes (5 x 10^6) and BW5147 cells (8 x 10^5) were incubated in 0.5 ml of RPMI supplemented with 1 mg/ml lipid-free BSA and 0.5 mM CaCl_2 (CaCl_2 final concentration: 0.925 mM). ATPe or other agonists were added and at the end of the incubations, the cells were pelleted for 10 min at 200 x g and the LDH activity released from dead cells was measured in the media according to the manufacturer’s instructions. Total LDH activity was measured by lysing thymocytes and BW5147 cells with 0.1% Triton X-100. The percentage of LDH released represents the fraction of LDH activity found in the supernatants with respect to the overall enzyme activity. Student t-test was used for statistical analysis; p < 0.05 was accepted as a significant difference.

Flow cytometric analysis of Phosphatidylserine externalization

The analysis of this phospholipid on the outer leaflet of apoptotic thymocyte-membranes was performed by using FITC-labeled Annexin-V, which binds to phosphatidylserine (PS), and propidium iodide which allows the differentiation from lytic cells. Thymocytes (5 x 10^5) were incubated in 0.5 ml of RPMI supplemented with 1 mg/ml lipid-free BSA and 0.5 mM CaCl_2 (CaCl_2 final concentration: 0.925 mM) in the absence or the presence of ATPe. At the end of the incubation time, cells were pelleted for 10 min at 200 x g, washed with 1 mg/ml lipid-free BSA in PBS and then incubated for 15 min at 37°C with 0.5 μg/ml propidium iodide and annexin V
diluted in a buffer containing 5 mM CaCl₂, 140 mM NaCl and 10 mM Hepes pH 7.4. The cells were then immediately analyzed by flow cytometry. Values of externalized PS correspond to the percentage of cells found in the quadrant annexin V-positive/PI-negative cells.

Flow cytometric analysis of DNA-fragmentation

DNA fragmentation of thymocytes was followed by flow cytometry using the sensitive TUNEL method. Murine thymocytes (4-5 x 10⁶) or BW5147 cells (1.2 x 10⁶) were incubated in 1 ml of RPMI supplemented with 1 mg/ml lipid-free BSA and 0.5mM CaCl₂ (CaCl₂ final concentration: 0.925 mM) in the absence or the presence of ATPe. At the end of the incubation time, the cells were pelleted for 10 min at 200 x g, fixed with 1% paraformaldehyde and permeabilized in 0.1% Triton X-100. Cells were then labeled with fluorescein dUTP at strand breaks by terminal deoxynucleotidyl transferase and analyzed by flow cytometry.
Results

ATPe induces thymocytes lysis

It is well established that in lytic cells, plasma membrane disruption leads to the release of cytosolic proteins as lactate dehydrogenase (LDH). To check that LDH release discriminates lytic from apoptotic cells, we verified that dexamethasone, a well established apoptotic agent for thymocytes (38), does not induce LDH release. Indeed after 2 h of incubation, LDH released represents 5.0 ± 0.8 and 4.9 ± 0.7 % of total LDH in untreated and 0.1 μM dexamethasone-treated Balb/c thymocytes, respectively. In contrast, 1 mM ATPe (74.5 μM ATP4-) leads to a time-dependent release of LDH, reaching a maximum of about 50% of total LDH after 5 h (Fig. 1A). Increasing the ATPe concentration from 0.1 to 3 mM enhanced LDH release by about 6-fold (Fig. 1B) with a maximal effect at 1 mM ATPe. Cell lysis measured through the index of LDH release is in agreement with the number of trypan blue-positive cells (data not shown). In order to determine whether LDH released originates from cells of the T cell lineage, macrophages, B lymphocytes and dendritic cells were removed from thymocytes suspension by immunomagnetic depletion with monoclonal anti-mouse MHC class II antibodies. We found that ATPe-triggered LDH release in purified thymocytes is identical to that of the unfractionated preparation of thymocytes (data not shown).

To examine whether cell lysis is due to a sustained stimulation with ATPe, we performed experiments in which the medium containing 1 mM ATPe was removed after a 30 min-incubation and then replaced by fresh medium for a further 4 h 30 min. In those conditions, the
amount of LDH released in response to ATPe is reduced by about 40% (Fig. 1C), indicating that prolonged purinergic stimulation is required for a cytolytic effect. ATPe-triggered cell lysis has been shown in some cases to be a caspase-mediated process (10). However, a pan-caspase inhibitor, z-VAD (50 µM), is unable to inhibit LDH released after 2 h of incubation of Balb/c thymocytes with 1 mM ATPe (Fig. 1D), while it completely abrogates dexamethasone-induced apoptosis of thymocytes (data not shown and 38). This result indicates that ATPe-triggered LDH release is a caspase–independent process in thymocytes.

Involvement of P2X7R in ATPe-induced thymocytes lysis

In order to determine the identity of the purinergic receptors involved in ATPe-triggered cell lysis, we first examined the expression of P2XR mRNAs in murine thymocytes. C57BL/6 thymocytes were shown to express P2X1, P2X2, P2X6 and P2X7 purinergic receptors (33). We found that P2X1, P2X2 and P2X7 receptors are expressed in Balb/c thymocytes (R. Auger, unpublished data). We then tested the effects of different purinergic receptor agonists or antagonists. As indicated in Fig. 2A, 250 µM BzATP, a potent P2X7R agonist triggers cell lysis whereas 1 mM UTP, a P2Y agonist, has no effect, suggesting that P2X7R may be implicated in this phenomenon. Thus, we determined the ATPe-triggered cell lysis in the presence of two well known P2X7R antagonists, oATP and PPADS. oATP and PPADS used alone did not affect thymocyte survival significantly. However, they reduced LDH release induced by 1 mM ATPe, to 7 and 15%, respectively (Fig 2A). In addition, ATPe does not trigger LDH release from
P2X7R⁻/⁻ thymocytes whereas about 20% LDH release is observed in P2X7R⁺/+ thymocytes (Fig. 2B). Thus, ATPe-induced cell lysis appears to be controlled by P2X7R.

**ATPε-induced thymocytes lysis is affected by the P451L mutation in the C-terminal tail of P2X7R**

Recently, mutation analyses have shown the importance of the C-terminal tail of P2X7R in pore formation in response to ATPε stimulation (5, 6, 7). To analyze the role of the C-terminal region of P2X7R in ATPε-induced cell lysis, we used thymocytes from C57BL/6 mice which bear a natural mutation (P451L) in the carboxyterminal tail of P2X7R, associated to a drastic reduction of ATPε-dependent pore formation and PS externalization (6). Fig 2C shows that ATPε-triggered lysis in C57BL/6 thymocytes is reduced by almost 45% compared to Balb/c thymocytes. We next used BW5147 thymoma cells, which have also impaired responses to ATPε. Indeed, upon ATPε stimulation, a functional Ca²⁺ channel is formed in these cells while there is no opening of the non-specific pore (39). Fig. 2C shows that BW5147 cells are marginally responsive to ATPε treatment. Since the C-terminal tail of P2X7R has been implicated in cell death induced by ATPε (40), we analyzed the nucleotide sequence encoding the C-terminal tail of P2X7R (residues 239 to 595) in BW5147 cells. Interestingly, we found that, as for the P2X7R of C57BL/6 mice, the BW5147 cells also express a P2X7R mutated at the proline-451 into leucine (data not shown). Altogether, these results demonstrate the importance of the C-terminal cytoplasmic domain of the P2X7R in 1) the control of ATPε-triggered pore
formation (5, 6, 7) and 2) partial control of lysis of thymocytes.

**ATPε-induced apoptosis of thymocytes**

Since the stimulation of P2X7R by ATPε is also involved in apoptosis, we analyzed this process in Balb/c thymocytes. PS externalisation has been shown to be an early step of the apoptotic pathway. In untreated thymocytes, a weak time-dependent PS externalisation is observed, plausibly reflecting a spontaneous death of thymocytes (Fig. 3A and 3B). In contrast, 1 mM ATPε induces a rapid exposure of PS on the outer leaflet of PI negative cells which reaches a maximum of about 15% of total cells after 15 min (Fig. 3A and 3B). This result is different from that obtained with dexamethasone; 0.1 µM dexamethasone increases PS externalization to 9 and 31% of total cells after 1 and 3 h of incubation, respectively.

We next quantified, a late step in the apoptotic pathway: ATPε-triggered DNA fragmentation. Fig. 3C and 3D show that 25 to 30% of ATPε-treated Balb/c thymocytes contain fragmented DNA compared to only 9% of the untreated thymocytes. The percentage of apoptotic cells reaches 46% when the cells are incubated with ATPε for 15 h (Fig. 3D). However, since a spontaneous apoptosis occurs in untreated thymocytes (28%), the range of apoptosis triggered by ATPε is similar to that found after 5 h of incubation (18%). This response is different from the effect of 0.1 µM dexamethasone, which increases DNA fragmentation in thymocytes from 42% after 5 h of incubation to 93% after 15 h (Fig. 3D). However, 50 µM zVAD inhibits apoptosis triggered both by ATPε (Fig. 4A) and dexamethasone (data not shown). We also found that treatment with 0.3 mM ATPε for 30 min followed by 4 h 30 min incubation without ATPε.
induces a percentage of fragmented DNA similar to that obtained after 5 h incubation with ATPe (data not shown). Thus, in contrast to ATPe-dependent cell lysis (Fig. 1C), a 30 min treatment with ATPe is sufficient to trigger its maximal apoptotic signal.

ATPe-triggered DNA fragmentation was also measured in thymocytes from C57BL/6 mice and in BW5147 cells. C57BL/6 thymocytes are less sensitive to ATPe-triggered apoptosis (Fig. 4B), a result comparable to those found by Adriouch et al. for T splenocytes of C57BL/6 animals (6). BW5147 cells, which bear the same mutation in the C-terminal tail of P2X7R as C57BL/6 mice, are unresponsive to ATPe treatment (Fig. 4B). Thus, it appears that signals leading to the apoptotic death of thymocytes are dependent on the C-terminal sequence of the P2X7R as it is the case for cell lysis.

**PC-PLD activation triggered by ATPe exposure**

We found that in Balb/c thymocytes, mRNAs encoding for the PC-hydrolyzing enzymes PLD1 and PLD2 are expressed (data not shown). Using 1 mM of ATPe, we showed that in [3H]myristic acid-labeled Balb/c thymocytes, the amount of [3H]PBut increases from 0.2% after 15 min incubation and reaches a plateau close to 0.35% after 45 min (Fig. 5A). We found that ATPe triggers similar level of PC-PLD activation in purified thymic T cells (0.32 ± 0.05 % of [3H]PBut) and in unfractionated thymocyte preparation (0.29 ± 0.04 % of [3H]PBut). ATPe induces PC-PLD activation in a dose-dependent manner (Fig. 5B). A maximal stimulation is obtained at 1 mM of ATPe after 45 min incubation and corresponds to a 10 fold stimulation over basal level.
Raising ATPe concentrations from 1 to 3 mM, which leads to a decrease of the free Ca$^{2+}$ concentration from 0.3 to 0.019 mM, reduces drastically PC-PLD activation by ATPe.

**Involvement of P2X7R in ATPe-triggered PC-PLD activation**

In order to determine the type of purinergic receptor coupled to PLD activation, we measured $[^3]$H]PBut formation from $[^3]$H]myristic acid-labeled Balb/c thymocytes in the presence of purinergic receptor agonists or antagonists. Fig. 6A shows that, 250 µM BzATP triggers PC-PLD activation while 1 mM UTP is ineffective. $[^3]$H]PBut accumulation induced by ATPe was reduced by 92% and 75% in the presence of oATP and PPADS, respectively (Fig. 6A). Finally, while PC-PLD is activated by ATPe in thymocytes from P2X7R$^{+/+}$ mice, it is not in thymocytes from P2X7R$^{-/-}$-animals (Fig. 6B). Thus, ATPe-triggered PC-PLD stimulation is mediated through P2X7R activation in thymocytes.

ATPe-triggered PC-PLD activation was also measured in C57BL/6 thymocytes and in BW5147 thymoma cells. Fig 6C shows that the level of ATPe-triggered PC-PLD activation in C57BL/6 thymocytes is identical to that found in Balb/c thymocytes. In C57BL/6 thymocytes, BzATP triggers also PC-PLD activation and a pretreatment with oATP completely abolishes ATPe-triggered PC-PLD activation (data not shown). Fig. 6D shows that ATPe-induced PC-PLD activation is not affected in BW5147 cells as observed in C57BL/6 and Balb/c thymocytes. It thus appears that PC-PLD activation is dependent of P2X7R but is not controlled by the cell ability to form a non-specific pore. Furthermore, PC-PLD activation is not affected by the
P451L mutation.

**Involvement of Ca\(^{2+}\) in ATPe-triggered PC-PLD activation**

Since the fixation of ATPe on P2X7R leads to the formation of channels that mediate fast influx of divalent cations and especially Ca\(^{2+}\), we examined the role of Ca\(^{2+}\) in PC-PLD activation. We found that a calcium ionophore, ionomycin (2 \(\mu\)M), stimulates \(^{3}\text{H}\)PBut accumulation in \(^{3}\text{H}\)myristic acid-labeled Balb/c thymocytes (Fig. 7A), suggesting a role of Ca\(^{2+}\) in PC-PLD activation. Similar results were obtained with thymocytes from C57BL/6 (Fig. 7A), P2X7R\(^{-/-}\) mice (Fig. 7B) and with BW5147 T lymphoma cells (Fig. 7C), indicating that PC-PLD activity by itself is not affected in P2X7R\(^{-/-}\) thymocytes and in cells where P2X7R is mutated in its C-terminal tail. We then tested the effect of extracellular Ca\(^{2+}\) concentration on ATPe-triggered PC-PLD activation in Balb/c thymocytes. Fig. 7D shows that \(^{3}\text{H}\)PBut accumulation increases when the medium is supplemented with CaCl\(_2\) at concentrations ranging from 0.1 to 3 mM. This result is in agreement with the lack of PC-PLD stimulation with 3 mM ATPe, which reduces the free Ca\(^{2+}\) concentration to 0.019 mM (Fig. 5B). Thus, Ca\(^{2+}\) entry through P2X7R is a major component of ATPe-triggered PC-PLD activation in thymocytes.

**Effect of PC-PLD inhibition in ATPe-triggered cell death**
Oleate-dependent PLD activity has been recently associated to rat thymic atrophy (41).

Since in thymocytes, ATPe-induced PC-PLD activation is dependent of extracellular Ca\textsuperscript{2+} concentration, we first tested the effect of Ca\textsuperscript{2+} on LDH release. Fig. 8A shows that when thymocytes were incubated for 2 h with 1 mM ATPe in a Ca\textsuperscript{2+}-free medium, ATPe-triggered LDH release is not reduced but appears even higher than in the presence of 0.925 mM extracellular Ca\textsuperscript{2+} concentration. Thus the ATPe-triggered cell lysis occurs in conditions where PC-PLD is not activated.

In order to determine a potential role of PC-PLD in ATP-triggered cell death, we used 2,3-DPG and U73122 which have been shown to indirectly inhibit various agonist-triggered PLD activation in several cellular systems (42, 43). In thymocytes 5 mM 2,3-DPG or 2 µM U73122 reduce by 87 and 100% respectively, the amount of [\textsuperscript{3}H]PBut generated in the presence of 1 mM ATPe (Fig. 8B). When thymocytes were pretreated with 2,3 DPG, U73122 or with 0.3% butanol-1, LDH release induced by 1 mM ATPe was not significantly affected (Fig. 8C).

We finally examined the involvement of PC-PLD activation in the ATPe-dependent apoptotic process. ATPe-triggered DNA fragmentation, a caspase-dependent process is not affected in the presence of 0.3% butanol-1 (Fig. 4A) or the absence of extracellular calcium (data not shown). Taken together these data indicate that PC-PLD activation is not involved in ATPe-induced thymocytes death.
Discussion

It is now established that cell death involves different mechanisms including lysis/necrosis and apoptosis (44, 45). The main biochemical steps leading to apoptosis, PS externalization, caspase activation, DNA fragmentation are now well characterized in many cell types. In contrast, lysis has not been so widely studied from a biochemical point of view (46). Nevertheless, among the morphological features characterizing this latter process, cell swelling and bursting and ultimately release of cytosolic molecules such as $^{51}$Cr-labeled proteins or LDH have been observed (12, 44, 45). In the present study, we found that ATPe triggers PS externalization in plasma membrane of thymocytes from Balb/c mice within 15 min. BzATP also triggers a rapid PS flip in HEK 293 cells expressing the P2X7R, in THP-1 monocytes (47) and in mouse thymocytes (48). However, since translocation of PS induced by P2X7R can be dissociated from apoptosis (47), we analyzed the effect of ATPe on DNA fragmentation, a late event in the apoptotic process. We showed that a transient as well as a long-term exposure to 0.3 mM ATPe triggers DNA fragmentation which reaches a maximal effect following 5 h of incubation. This DNA fragmentation is not dependent on extracellular Ca$^{2+}$ concentration but is fully dependent on caspase activation. Thus, ATPe triggers apoptosis in a smaller number of Balb/c thymocytes compared to the potent apoptotic agent, dexamethasone (Fig. 3D and 38).

Concomitant to the induction of apoptosis, ATPe also triggers lysis of Balb/c thymocytes. Indeed, as soon as 45 min of incubation, 1 mM ATPe induces cell lysis, reaching a maximum after 5 h of incubation. Inhibition of ATPe-dependent lysis by oATP and PPADS suggests a role
of P2X7R in this process. This is in agreement with previous studies suggesting a potent role of P2X7R in thymocytes death (13, 33), even if they were using C57BL/6 and DBA/2 mice in which P2X7R functions are greatly affected, due to a mutation in the C-terminal tail of the receptor (6). Using thymocytes from P2X7R−/− mice, we were able to determine which subclasses of P2XR mediate cell death in murine thymocytes, which has been a subject of debate until now (13, 14). Indeed, the resistance of thymocytes from P2X7R−/− mice to the lytic effect of ATPe demonstrates that P2X7R plays a major role in this phenomenon. It also strongly suggests that P2X1R, which is unable to heterodimerize with P2X7R (49, 50), is not involved in ATPe-induced thymocytes death. As for apoptosis triggered by ATPe, extracellular Ca²⁺ is not required for cell lysis. We also showed that ATPe-triggered lysis is caspase-independent at the difference of the apoptotic response. Moreover, as observed by Apasov et al. (13), protein synthesis is not involved in lysis of thymocytes induced by ATPe (R. Auger unpublished data). Our results on ATPe-induced lysis of thymocytes are in agreement with a recent report showing that high concentrations of ATPe (> 0.5 mM) induce a predominant lytic effect in these cells (15).

If different pathways, leading either to apoptosis or to necrosis/lysis, can occur in a same cell-type (45), the coexistence of two different ATPe-triggered cell deaths in a same cell-type had rarely been mentioned (8, 12). In Balb/c thymocytes, these two phenomena appear to be distinct. Indeed, a transient exposure of thymocytes to 0.3 mM ATPe is sufficient to induce a caspase-dependent apoptosis which is not potentiated when higher ATPe concentrations are
used. In contrast, a caspase-independent cell lysis is detected as soon as 30 to 60-min of treatment with 1 mM ATPe. Moreover, as opposed to apoptosis, cell lysis is reduced when ATPe is applied transiently or when concentrations lower than 1 mM are used. It thus appears that in murine thymocytes, P2X7R activation is able to induce cell death by apoptosis and lysis through independent mechanisms, which remain to be investigated. However, even if both processes are regulated by P2X7R activation, cytolytic effect of ATPe is predominant while apoptosis affects a smaller percentage of thymocytes (5.5 fold vs 2.3 fold increase when 1 mM ATPe was applied for 30 min for LDH release and DNA fragmentation measured 5 h later, respectively; Fig.1C and data not shown).

The C-terminal cytoplasmic tail of P2X7R, which is 120 amino acids longer than in the other P2XR, is an important modulator of the receptor functions. Indeed, P2X7R contains a domain with homology to the TNF receptor 1 death domain (TNF-DD, residues 436-531) in its C-terminal tail (51). Within this sequence, the E496A mutation found in P2X7R from chronic lymphocytic leukemia patients, confers resistance to ATPe-induced apoptosis of B-lymphocytes (5). Moreover, Wilson et al. (52) showed that the C-terminal domain of P2X7R is implicated in the binding of epithelial membrane proteins, which regulate cell death. In the same line, a recent structure-function analysis of mutated P2X7R has shown that pore formation requires over 95% of the C-terminal tail (7). Taking advantage of a natural P451L mutation in the C-terminal cytoplasmic domain of the P2X7R from C57BL/6 mice, which reduced ATPe-induced pore formation and PS externalization in T cells (6), we found that ATPe-triggered lysis and apoptosis are markedly reduced in C57BL/6 thymocytes. BW5147 cells were described to have impaired
responses to ATPe such as the absence of pore formation (3, 39). Similarly, we found that BW5147 cells are resistant to ATPe-triggered cell lysis and apoptosis, suggesting that pore formation is a prerequisite for the induction of cell death by ATPe. Interestingly, P2X7R from BW5147 cells were also found to bear the same P451L mutation present in the P2X7R of C57BL/6 mice. Taken together these data suggest that the prolyl residue present in the predicted TNF-DD of P2X7R plays an important role in cell death of thymocytes.

ATP e has been described to regulate several intracellular signaling events (16, 17, 18) among which is the stimulation of PLD activity (19, 20, 21, 22, 53). In murine thymocytes, which express PLD1 and PLD2 isoforms, we found that ATPe stimulates PC-PLD activation through P2XR since UTP, a potent agonist of P2Y receptors, is unable to induce PBut formation. Moreover, using oATP and BzATP, potent P2X7R antagonist and agonist respectively, we showed that PC-PLD is activated through the stimulation of this subtype of P2XR. In thymocytes, as observed in peritoneal macrophages by Coutinho-Silva et al. (54), P2X7R appears to be the only P2XR subclass associated with PC-PLD activation since PBut formation was not observed in the ATPe-treated thymocytes of P2X7R−/− mice. ATPe-triggered PC-PLD activation in thymocytes is fully dependent of extracellular Ca2+ concentration. This differs from macrophages, where PLD stimulation by ATPe is dissociated from changes in extracellular Ca2+ concentration (19). We found that protein kinase C, a major physiological regulator of PLD and a potential target of intracellular Ca2+ (25), plays a moderated role in ATPe-stimulated PC-PLD activity (M.-N. Raymond, unpublished data). The downstream signaling pathways linking Ca2+
influx to PC-PLD activation in thymocytes remain to be elucidated.

The role of PLD activity in the regulation of cell death had been controversial up to date (27, 29) since different reports have proposed both anti-apoptotic and pro-apoptotic functions for PLD (29, 30, 41). To explore the role of PLD activation in thymocytes death induced by P2X7R stimulation, we inhibited the formation of PA or the activation of the enzyme. We found that thymocyte lysis and apoptosis are not significantly modified when P2X7R-triggered PLD-dependent PC hydrolysis is blocked. Interestingly, we found that ATPe-triggered PC-PLD activation was unchanged in thymocytes from C57BL/6 mice and BW5147 thymoma cells in which the P2X7R bears a mutation that partially inhibits cell death. Thus, in murine thymocytes, PC-PLD activation is not dependent on the structure of the C-terminal tail of P2X7R and is not involved in ATPe-mediated cell death. These results are in agreement with reports showing that pore formation induced by P2X7R is dependent on the C-terminal tail of the receptor (5, 6, 7) but not on PLD activation (55). In contrast, PC-PLD activation in thymocytes is totally dependent on Ca\(^{2+}\) influx through the P2X7R channel, a process almost independent of the C-terminal tail of the receptor (7). The functions of PC-PLD activation by P2X7R in thymocytes are presently unknown but may include effects on proliferation, differentiation and cell survival during thymocytes development. This remains plausible given the recent evidence showing that Ca\(^{2+}\)-dependent signaling pathways were involved in Jurkat T cell proliferation induced by P2X7R (17).

In summary, our results show that ATPe triggers cell death of murine thymocytes through
activation of the cytolytic P2X7R exclusively. PC-PLD activity is also regulated by this subclass of P2XR. However, PC-PLD activation is regulated by the opening of the cation channel and the consequent entry of extracellular Ca\(^{2+}\), while cell death is dependent on the structure of the C-terminal cytoplasmic tail of the P2X7R. Finally, PC-PLD activation is not involved in cell death of thymocytes induced by P2X7R.
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Abbreviations

P2XR, purinergic 2X receptors; ATPe, extracellular ATP; BzATP, 2’,3’-O-(benzoyl-4-benzoyl)-ATP; oATP, oxidized ATP; PPADS, pyridoxal-phosphate-6-azophenyl-2’,4’-disulfonyl acid; 2,3-DPG, 2,3 diphosphoglycerate; PLD, phospholipase D; PC-PLD, phosphatidylcholine-hydrolyzing PLD; PBut, phosphatidylbutanol; PC, phosphatidylcholine; PA, phosphatidic acid; PS, phosphatidylserine; TNFα, Tumour Necrosis Factor α; TNF-DD, TNF receptor 1 death domain; H2O2, hydrogen peroxide; LDH, lactate dehydrogenase; dex, dexamethasone; FBS, Fetal Bovine Serum; TcR, T cell receptor;
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**Legends to figures**

**Figure 1 : Effect of ATPe on LDH release of Balb/c thymocytes.** A) Time-course of ATPe-induced LDH release. Thymocytes from Balb/c mice were suspended in RPMI medium supplemented with BSA (final concentration : 1 mg/ml) and CaCl$_2$ (final concentration : 0.925 mM). 1 mM ATPe was added for 15 min to 5 h and supernatants were collected for LDH content determination. Data are means ± SEM of 4 independent experiments. B) Dose–dependent effect of ATPe on LDH release. Experiments were performed as described in A) for 3 h. Data are
means ± SEM of 4 independent experiments. C) Effect of a pulse of ATPe on LDH release. Balb/c thymocytes were incubated with 1 mM ATPe for 30 min or 5 h. In the case of the 30 min ATPe-pulse, thymocytes were washed after the pulse and resuspended in a fresh medium without ATPe for a further 4 h 30 min. Data are means ± SEM of 3 independent experiments. D) Effect of a caspase inhibitor on ATPe-triggered LDH release. Experiments were performed as described in A) for 2 h in the absence or the presence of 50 µM z-VAD. Data are means ± SEM of 3 independent experiments.

Figure 2: Implication of P2X7R on ATPe-induced LDH release of murine thymocytes. A) Effect of different purinergic receptor agonists or antagonists on LDH release. Balb/c thymocytes were treated with 1 mM ATPe, 0.25 mM BzATP or 1 mM UTP for 3 h. Supernatants were collected for LDH content determination. When antagonists were tested, thymocytes were preincubated for 2 h with 0.3 mM oATPe and for 15 min with 0.3 mM PPADS. The amounts of LDH released are expressed as a percentage of the amounts of total LDH. Data are means ± SEM of 3 independent experiments. B) Effect of ATPe on LDH release in thymocytes from P2X7R-deficient mice. Thymocytes from wild-type BDF1 (WT) mice or P2X7R-/- mice were stimulated with 1 mM ATPe for 2 h. Supernatants were collected for LDH content determination. Data are means ± SEM of 3 independent experiments. C) Effect of ATPe-triggered LDH release from C57BL/6 thymocytes and BW5147 cells. Cells were incubated with 1 mM ATPe for 5 h and supernatants were collected for LDH content determination. Data are means ± SEM of 3 independent
Figure 3: Phosphatidylserine externalization and DNA fragmentation triggered by ATPe treatment of Balb/c thymocytes. A) Effect of ATPe on PS externalization. Balb/c thymocytes resuspended in RPMI medium supplemented with BSA (final concentration: 1 mg/ml) and CaCl₂ (final concentration: 0.925 mM) were treated with 1 mM ATPe for 2 h. Cells were then labeled by annexin V binding and propidium iodide and analyzed by flow cytometry. B) Time-course of ATPe-induced PS externalization. Experiments were performed as described in A) during 3 h. Experiments were performed three times in duplicate and values represent the mean of a representative experiment. C) Effect of ATPe on DNA fragmentation. Balb/c thymocytes resuspended in RPMI medium supplemented with BSA and CaCl₂, were treated with 0.3 mM of ATPe for 5 h. Cells were then analyzed for fragmented DNA by the TUNEL assay. D) Dose–and time-dependent effects of ATPe on DNA fragmentation. Balb/c thymocytes were treated with various concentrations of ATPe or 0.1 µM dexamethasone for 5 or 15 h. Cells were then analyzed for fragmented DNA by the TUNEL assay. Fragmented DNA represents the percentage of cells recovered in the M2 gates defined in C). Data are means ± SEM of 3 independent experiments.

Figure 4: Role of the C-terminal tail of P2X7R and PLD activation on DNA fragmentation induced by ATPe treatment of murine thymocytes. A) Effect of ATPe on DNA fragmentation in thymocytes from Balb/c and C57BL/6 mice and BW5147 cells. Thymocytes were treated with...
0.3 mM ATPe for 5 h. Cells were then analyzed for fragmented DNA by the TUNEL assay. Data are means ± standard deviation of 2 independent experiments. 

**B** Effect of z-VAD and butanol-1 on ATPe-triggered DNA fragmentation. Balb/c thymocytes were treated with 0.3 mM ATPe in the absence or the presence of 0.3 % butanol-1 or 50 µM z-VAD for 5h. Cells were then analyzed for fragmented DNA. Data are means ± SEM of 3 independent experiments.

**Figure 5 : Effect of ATPe on PC-PLD activation in Balb/c thymocytes.** 

**A** Time-course of ATPe-induced PC-PLD activation. Balb/c thymocytes labeled with [³H]myristic acid were resuspended in RPMI medium supplemented with BSA (final concentration : 1 mg/ml) and CaCl₂ (final concentration : 0.925 mM). Fifteen minutes after addition of 0.3% butanol-1, 1 mM ATPe was added and incubations were continued up to 60 min. The amounts of [³H]PBut formed are expressed as a percentage of the amounts of total radiolabeled lipids. 

**B** Dose–dependent effect of ATPe on PC-PLD activation. Experiments were performed as described in **A** during 45 min. Data are means ± SEM of 3 or 4 independent experiments performed in duplicate.

**Figure 6 : Implication of P2X7R on PC-PLD activation triggered by ATPe treatment of murine thymocytes.** 

**A** Effect of different purinergic receptor agonists or antagonists on PC-PLD activation. After 15 min pretreatment with 0.3% butanol-1, thymocytes from Balb/c mice labeled with [³H]myristic acid were stimulated with 1 mM ATPe, 0.25 mM BzATP or 1mM UTP and the incubations were continued for 45 min. Antagonists were tested by preincubating thymocytes for
2 h with 0.25 mM oATP and for 15 min with 0.3 mM PPADS. B) Effect of ATPe on PC-PLD activity in thymocytes from P2X7R-deficient mice. After a 15 min pretreatment with 0.3% butanol-1, thymocytes from wild-type BDF1 (WT) or P2X7R\(^{-/-}\) mice, labeled with \(^{[3]}\text{H}\)myristic acid, were stimulated with 1 mM ATPe for 45 min. C, D) Effect of ATPe on PC-PLD activity of thymocytes from C57BL/6 mice and BW5147 cells. After a 15 min pretreatment with 0.3% butanol-1, Balb/c and C57BL/6 thymocytes (C) and BW5147 cells (D) labeled with \(^{[3]}\text{H}\)myristic acid were stimulated with 1 mM ATPe and the incubations were continued for 45 min. The amounts of \(^{[3]}\text{H}\)PBut formed are expressed as a percentage of the amounts of total radiolabeled lipids. Data are means ± SEM of 3 independent experiments performed in duplicate.

Figure 7: Effect of Ca\(^{2+}\) on PC-PLD activation triggered by ATPe treatment of murine thymocytes. A, B, and C) Effect of ionomycin on PC-PLD activation. After a 15 min pretreatment with 0.3% butanol-1, thymocytes from Balb/c and C57BL/6 mice (A), thymocytes from wild-type BDF1 (WT) mice and P2X7R\(^{-/-}\) mice (B) and BW2147 cells (C) labeled with \(^{[3]}\text{H}\)myristic acid were stimulated with 2 \(\mu\)M ionomycin for 45 min. D) Dose-dependent effect of calcium on PC-PLD activation. After 15 min pretreatment with 0.3% butanol-1, Balb/c thymocytes labeled with \(^{[3]}\text{H}\)myristic acid were stimulated with 1 mM ATPe for 45 min in the presence of various concentrations of extracellular CaCl\(_2\). The amounts of \(^{[3]}\text{H}\)PBut formed are expressed as a percentage of the amounts of total radiolabeled lipids. Data are means ± SEM of 2
or 3 independent experiments performed in duplicate.

**Figure 8: Effect of PC-PLD inhibition on ATPe-triggered LDH release of Balb/c thymocytes.**

A) Effect of extracellular calcium on LDH release. Balb/c thymocytes were treated with 1 mM ATPe, in the absence or presence of 0.925 mM CaCl2. After a 3 h-incubation, supernatants were collected for LDH content determination. Data are means ± SEM of 4 independent experiments.

B) Inhibition of PC-PLD activation by ATPe. After a 15 min pretreatment with 0.3% butanol-1 in the absence or the presence of 5 mM 2,3 DPG or 2 µM U73122, Balb/c thymocytes labeled with [3H]myristic acid were stimulated with 1 mM ATPe for 45 min. The amounts of [3H]PBut formed are expressed as percentages of the amounts of total radiolabeled lipids. Data are means ± SEM of 3 independent experiments performed in duplicate. C) Effect of PLD inhibition on LDH release. Balb/c thymocytes were stimulated with 1 mM ATPe in the presence of 0.3% butanol-1, 5 mM 2,3 DPG or 2 µM U73122. After 3 h of incubation, supernatants were collected for LDH content determination. Data are means ± SEM of 4 independent experiments performed in duplicate.
Fig. 1

A: Graph showing the release of LDH (% of total LDH) over time (min) for ATPe and none.

B: Graph showing the release of LDH (% of total LDH) vs. ATPe concentration (mM).

C: Bar graph showing LDH released (% of total LDH) for none, ATPe 30' + 4 h 30', and ATPe 5 h.

D: Bar graph showing LDH released (% of total LDH) for none, ATPe, z-VAD, z-VAD + ATPe.
Fig. 2

A

LDH released (% of total LDH)

| Condition          | Value |
|--------------------|-------|
| none               | 5     |
| ATPe               | 30    |
| BzATP              | 20    |
| UTP                | 10    |
| none + c-ATP       | 5     |
| ATPe + c-ATP       | 25    |
| none + PPADS       | 10    |
| ATPe + PPADS       | 15    |

B

LDH released (% of total LDH)

| Condition          | Value |
|--------------------|-------|
| WT                 | 15    |
| P2X7R<sup>−/−</sup> | 20    |
| none               | 5     |
| ATPe               | 20    |

C

LDH released (% of total LDH)

| Condition          | Value |
|--------------------|-------|
| Balb/c             | 5     |
| C57BL/6            | 40    |
| BW5147             | 10    |

none
t

ATPe
Fig. 4

A

![Bar chart showing fragmented DNA (% of total) for different conditions:
- none
- + but-1
- + z-VAD

B

![Bar chart showing fragmented DNA (% of total) for different mouse strains:
- Balb/c
- C57BL/6
- B6147]
Fig. 5

A

\[ \text{\% of total radio-labeled lipids} \]

Time (min)

B

\[ \text{\% of total radio-labeled lipids} \]

ATP (mM)
Fig. 6

A

\[ ^{3}H \text{IPB}_{\text{But}} \] (% of total radiolabeled lipids)

\begin{align*}
\text{none} & \quad \text{ATP}e \\
\text{BzATP} & \quad \text{UTP} \\
\text{none} & \quad \text{ATP}e \\
\text{none} & \quad \text{ATP}e
\end{align*}

+ o-ATP

+ PPADS

B

\[ ^{3}H \text{IPB}_{\text{But}} \] (% of total radiolabeled lipids)

\begin{align*}
\text{WT} & \quad \text{P2X7R}^{-/-} \\
\text{none} & \quad \text{ATP}e
\end{align*}

C

\[ ^{3}H \text{IPB}_{\text{But}} \] (% of total radiolabeled lipids)

\begin{align*}
\text{Balb/c} & \quad \text{C57BL/6} \\
\text{none} & \quad \text{ATP}e
\end{align*}

D

\[ ^{3}H \text{IPB}_{\text{But}} \] (% of total radiolabeled lipids)

\begin{align*}
\text{none} & \quad \text{ATP}e
\end{align*}
The Pro-451 to Leu polymorphism within the C-terminal tail of P2X7 receptor impairs cell death but not phospholipase D activation in murine thymocytes
Hervé Le Stunff, Rodolphe ;Auger, Jean Kanellopoulos and Marie-Ñoëlle Raymond

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