Involvement of Sodium in Early Phosphatidylserine Exposure and Phospholipid Scrambling Induced by P2X7 Purinoceptor Activation in Thymocytes*

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Marie-Pierre Courageot‡§, Sandrine Lépine‡, Michel Hours§, Françoise Giraud‡, and Jean-Claude Sulpine‡

From the ‡Laboratoire des Biomembranes et Messagers Cellulaires, CNRS UMR 8619 and §Service de cytométrie, Institut Fédératif de Recherches 46, bât 440, Université Paris XI, 91405 Orsay Cedex, France

Extracellular ATP (ATP\textsubscript{ec}), a possible effector in thymocyte selection, induces thymocyte death via purinoceptor activation. We show that ATP\textsubscript{ec} induced cell death by apoptosis, rather than lysis, and early phosphatidylserine (PS) exposure and phospholipid scrambling in a limited thymocyte population (35–40%). PS externalization resulted from the activation of the cationic channel P2X7 (formerly P2Z) receptor and was triggered in all thymocyte subsets although to different proportions in each one. Phospholipid movement was dependent on ATP\textsubscript{ec}-induced Ca\textsuperscript{2+} and/or Na\textsuperscript{+} influx. At physiological external Na\textsuperscript{+} concentration, without external Ca\textsuperscript{2+}, PS was exposed in all ATP\textsubscript{ec}-responsive cells. In contrast, without external Na\textsuperscript{+}, physiological external Ca\textsuperscript{2+} concentration promoted a submaximal response. Altogether these data show that Na\textsuperscript{+} influx plays a major role in the rapid PS exposure induced by P2X7 receptor activation in thymocytes.

Extracellular ATP (ATP\textsubscript{ec})\textsuperscript{1} induces thymocyte apoptosis and may play a role in thymocyte maturation (1–4). A major change of cell membrane during apoptosis, slightly preceding nuclear condensation (5), is the exposure on the exoplasmic cell surface of phosphatidylserine (PS) (6–9), a phospholipid normally maintained on the inner membrane leaflet by the aminophospholipid translocase (APLT) (10). This process is associated with a general loss of the asymmetric distribution (scrambling) of all the other major phospholipids (6, 7, 9). The presence of specific PS receptors on macrophages potentiates allowing a rapid elimination of apoptotic cells by phagocytosis before their lysis (11). Rapid PS exposure and scrambling are generally observed after platelet activation or treatment of cells with a Ca\textsuperscript{2+} ionophore, both conditions promoting a drastic and rapid rise in cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) (7). An involvement of Ca\textsuperscript{2+} has been also reported in PS exposure during apoptosis (6, 7, 12). Ca\textsuperscript{2+} acts by inhibiting APLT and by targeting scramblase (6, 7, 13) or other molecular complexes, which could contain phosphoinositides (14, 15). Recent studies show that the rapid Ca\textsuperscript{2+}-induced PS exposure is correlated with scramblase expression (16), in contrast to the delayed PS exposure associated with apoptosis (17, 18), in agreement with the involvement of other phospholipid transporters in apoptotic pathways.

ATP\textsubscript{ec} able to evoke physiological responses in a wide variety of cells and tissues. ATP is concentrated in cell cytosol and in exocytic vesicles, reaching 2–4 and 100 mM, respectively (19), and can be merely released from damaged cells, transported through carriers or channels, or secreted (19–21). In the extracellular medium, its lifetime is very short due to its rapid degradation by ectonucleotidases and ecto-ATPases (19). ATP\textsubscript{ec} binds to distinct types of purinergic receptors classified into two subclasses: metabotropic P2Y receptors coupled to G-protein (P2Y1–6 and P2Y11) and ligand-gated ion channel P2X receptors (P2X1–7) (22). P2X7 receptor, previously designated as P2Z receptor (23), utilizes extracellular ATP\textsuperscript{2+} to increase cationic permeability with consecutive plasma membrane depolarization. Another feature of the P2X7 receptor is its ability to form or activate a non-selective pore, permeable to molecules with molecular mass <400 Da in thymocytes, when the ATP\textsubscript{ec} application is prolonged or repeated (24, 25). Activation of the P2X7 receptor triggers various physiological processes, including cell death by apoptosis or necrosis (26) or interleukin-1β release (22, 27, 28). PS exposure, Ca\textsuperscript{2+} influx, and pore formation are dependent on the cytoplasmic domain of the P2X7 receptor, which harbors a putative tumor necrosis factor receptor-related death domain and a Src homology 3-binding domain (29).

In this work, we show that ATP\textsubscript{ec} induced early PS exposure, 1-oleoyl-2-(6-(7-nitro-2-1,3-benzoxadiazol-4-yl)amino)caproyl-sn-glycery-3-phosphocholine (NBD-PC) scrambling, and apoptosis in 35–40% of the thymocytes. PS exposure and NBD-PC scrambling were dependent on an increase in Ca\textsuperscript{2+} and/or Na\textsuperscript{+} influx resulting from P2X7 activation. At physiological external concentrations, Na\textsuperscript{+} was more potent than Ca\textsuperscript{2+} to induce PS exposure. Portions of this work have appeared in a preliminary report (31).

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To whom correspondence should be addressed. Tel.: 33-01-69-15-49-59; Fax: 33-01-69-15-49-61; E-mail: Jean-Claude.Sulpice@ibaic.u-psud.fr.

The abbreviations used are: ATP\textsubscript{ec}, extracellular ATP; PS, phosphatidylserine; APLT, aminophospholipid translocase; FCS, fetal calf serum; αβμATP, αβω-methyleneATP; BaATP, 2′- and 3′-O-(4-benzoyl- benzoyl)ATP; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfo- nic acid; PI, phosphatidylinositol; FITC, fluorescein isothiocyanate; NBD-PC, 1-oleoyl-2-(6-(7-nitro-2-1,3-benzoxadiazol-4-yl)amino)caproyl-sn-glycery-3-phosphocholine; DN, double negative CD4−CD8− cells; DP, double positive CD4+CD8+ cells; SPCD4+ and SPCD8+, single positive CD4+CD8− and CD8+CD4− cells; AM, acetoxymethyl ester; DiBac4(3), bis(1,3-dibutylbarbituric acid)trimethine oxonol.
**ATP<sub>e</sub>-induced PS Exposure Is Mediated by Na<sup>+</sup> Influx**

## EXPERIMENTAL PROCEDURES

### Reagents

RPMI 1640 medium and fetal calf serum (FCS) were purchased from Invitrogen, and Ca<sup>2+</sup>-free RPMI 1640 medium was from BIO MEDIA. ATP, ADP, AMP, adenosine, UTP, α,β-methylene ATP (αβmATP), 2',3'-dideoxyadenosine (ddA), fluo-4 (Fluo-4), and fluorescent plasma membrane potential probe DiBac<sub>4</sub>(3) were purchased from Molecular Probes. FITC-annexin-V in the presence of PI (50 μg/ml), a marker of plasma membrane integrity used to evaluate the percentage of necrotic cells. When cells were incubated in a medium devoid of Ca<sup>2+</sup>, factor Va was detected by indirect immunofluorescence. The fixation of factor Va was from Hematologic Technologies Inc. The fluorescent analog NBG-PC was purchased from Avanti Polar Lipids. The fluorescent Ca<sup>2+</sup> probes (fluo4-AM and fura2-AM) and fluorescent plasma membrane potential probe DiBac<sub>4</sub>(3) were purchased from Molecular Probes.

### Cell Preparation

Thymocytes were isolated from thymus of 3–6-week-old BALB/c male mice. Cell suspensions with viability, as estimated by trypan blue exclusion, lower than 95% were discarded. Thymocytes (2×10<sup>6</sup>/ml) were cultured at 37 °C in RPMI 1640 medium supplemented with 5% heat-inactivated FCS (5% FCS/RPMI) in a humidified atmosphere containing 5% CO<sub>2</sub>. When required, cells were incubated in Ca<sup>2+</sup>-free RPMI 1640 medium or in saline media (choline media) containing 5 mM KCl, 150 mM NaCl, 25 mM Na<sub>2</sub>HPO<sub>4</sub>·4H<sub>2</sub>O, 10 mM HEPES (pH 7.4), 0.1% bovine serum albumin, 150 μM EGTA, and various concentrations of NaCl (0–139 mM) or CaCl<sub>2</sub> (0–5 mM) supplemented with choline chloride to maintain the osmolarity to 300 mOs M.

### Flow Cytometry Analysis

**Endogenous PS Externalization**—When cells were incubated in Ca<sup>2+</sup>-containing medium, PS exposure was assayed from the binding of FITC-annexin-V in the presence of PI (50 μg/ml), a marker of plasma membrane integrity used to evaluate the percentage of necrotic cells. PS+/PI− cell population represents non-necrotic cells with externalized PS. Alternatively, when cells were incubated in a medium devoid of Ca<sup>2+</sup>, factor Va, another specific ligand of PS whose binding is Ca<sup>2+</sup>-dependent, was used as described previously (32). Binding of factor Va was detected by indirect immunofluorescence. The fixation of factor Va on the cell surface altered the plasma membrane integrity as reflected by the uptake of PI in all PS+ cells. Consequently the responsive cells were expressed as total PS+ cells. However, the proportion of the necrotic cells, estimated in independent assays by measurement of PI uptake, was found to be less than 10% after ATP<sub>e</sub> treatment, a proportion similar to that observed in control cells. The percentage of PS+ cells determined by factor Va binding was always slightly higher than that obtained by FITC-annexin-V staining, suggesting that the affinity of PS for PS was higher than that of FITC-annexin-V.

**NBG-PC Internalization**—The transenriched distribution of the PC analog was assayed as described previously (6, 33). Briefly, after incubation of thymocytes for 10 min with 1 mM ATP<sub>e</sub>, the fluorescent analog NBG-PC (62.5 mM final concentration representing about 1.5% of cellular phospholipids) was loaded into the cells by 6-min incubation at room temperature. The percentage of cells with internalized PC was assayed after extraction by fatty acid-free bovine serum albumin (2 min at 4 °C) of the fraction of the analog present in the external leaflet. Samples were sorted by flow cytometry after addition of PI to evaluate the proportion of necrotic cells.

### Plasma Membrane Depolarization—Thymocytes (2×10<sup>6</sup>/ml) were stained with 300 nM DiBac<sub>4</sub>(3) for 20 min at 37 °C. Fluorescence intensity of the DiBac<sub>4</sub>(3) probe increased when cells were depolarized. In controls, the responsive cells were comparable to that promoted by 10 μg/ml proteinase K for 10 min. Approximately 200 randomly selected cells were scored for each experimental condition. Cells exhibiting blue or red condensed or fragmented nuclei were considered apoptotic. Cells with red nuclei without signs of condensation or fragmentation were considered necrotic.

For detection of DNA fragmentation, cells (3×10<sup>6</sup>/ml) were washed and lysed in 10 mM Tris-HCl, pH 7.4, 0.2% Triton X-100, 1 mM EDTA, 100 μg/ml proteinase K for 10 min on ice followed by 60 min at room temperature. After centrifugation (30 min, 13,000 g, 4 °C), DNA in the supernatant was precipitated overnight in cold absolute ethanol containing 0.3 M sodium acetate at −20 °C. After centrifugation (30 min, 13,000 g, 4 °C), the pellet was resuspended in water and incubated for 30 min at 37 °C with 50 μg/ml DNase-free RNase. DNA concentration was quantified by UV spectrometry at 260 nm. About 5 μg of DNA was mixed with gel loading solution and incubated for 5 min at 65 °C before electrophoresis in a 1.5% agarose gel containing ethidium bromide. DNA was visualized by UV light.

### Data Analysis

Data are means ± S.E. of a minimum of three independent experiments. Differences between means were evaluated by paired t test with p < 0.05 being taken as the level of significance.

### RESULTS

**ATP<sub>e</sub> Induces Apoptosis and PS Exposure in a Limited Thy-mocyte Population**—Cell death was measured after 5-h incubation of freshly isolated thymocytes in the presence of ATP<sub>e</sub>. Nuclear condensation was significant at 0.5 mM ATP<sub>e</sub> in 20–25% of the cells and maximal at 1 mM affecting 35% of the total population (Fig. 1A). Thymocyte apoptosis was confirmed by visualization of DNA fragmentation on agarose gel electrophoresis. Oligonucleosome formation induced by 1 mM ATP<sub>e</sub> was comparable to that promoted by 1 μM dexamethasone, a well known inducer of thymocyte apoptosis (Fig. 1B). When compared with spontaneous thymocyte apoptosis, the effect of 1 mM ATP<sub>e</sub> on nuclear condensation was significantly increased after 4-h treatment and reached a maximum (35–40%) after 6 h (Fig. 1C). Incubation for up to 9 h did not change significantly the percentage of cells with condensed nuclei and marginally increased the percentage of necrotic cells (11%), indicating that under these experimental conditions ATP<sub>e</sub> induced apoptosis preferentially to necrosis in a limited population of thymocytes.

During the apoptotic process, PS exposure was reported to be concomitant or to precede slightly nuclear events such as nuclear condensation (5). ATP<sub>e</sub> induced PS exposure and nuclear
condensation within the same range of concentrations (0.5–3 mM) with a maximal effect at 1 mM (Figs. 1A and 2A). Surprisingly PS exposure was already significant in 15% of cells after 6-min treatment with 1 mM ATP_{ec} and was maximal after 20–30 min, affecting about 40% of the thymocytes (Fig. 2B). These results show that PS exposure was a very early event in thymocytes, preceding nuclear alterations. After 5-h incubation with 1 mM ATP_{ec}, both PS exposure and nuclear condensation, observed by light microscopy after FITC-annexin-V binding and Hoechst staining, were detected in the same cell population (data not shown).
Differentiation of immature thymocytes into mature T cells takes place in the thymus and proceeds via an ordered sequence of developmental steps characterized notably by variable expression of CD4 and CD8. Early precursor cells are initially double negative CD4−CD8− (DN) and go through a double positive CD4+CD8+ (DP) intermediate state before differentiating into single positive CD4+CD8− or CD4−CD8+ (SPCD4+ or SPCD8+, respectively). The distribution of the thymocyte population and the percentage of PS-exposing cells in each subset (DN, DP, and SP, either CD4+ or CD8+) were analyzed by flow cytometry. As previously reported (2), DP in each subset (DN, DP, and SP, either CD4+ or CD8+) was accounted for about 80% of the population (Fig. 2C). ATPec induced PS externalization in each thymocyte subset. The most mature thymocytes (SPCD4+ or SPCD8+) were also those in which the percentage of cells exposing PS was significantly the highest (68 and 73% versus 43 and 29% in DN and DP, respectively) (Fig. 2C). These data show that, as far as PS exposure is concerned, ATPec responsiveness was dependent on the maturation state of thymocytes.

P2X7 Purinoceptor Is Involved in ATPec-induced PS Exposure—Thymocytes express different classes of membrane receptors activated by purine ligands (1, 3). To identify which purinoceptor(s) was mediating early PS exposure in thymocytes, the effect of various purine agonists and antagonists was investigated. Neither adenosine (a P1 receptor agonist) nor UTP (a P2Y1, P2Y4, and P2Y6 agonist) (22) was able to induce PS exposure. αβmATP, a P2X1 or P2X3 receptor agonist (25), was unable to induce PS exposure (Fig. 3A). In contrast, 0.1 mM BzATP, a P2X7 agonist with a 10-fold higher affinity than ATPec (22, 25), promoted PS externalization to the same extent as did 1 mM ATPec (Fig. 3A). Furthermore antagonists of P2X7 (suramin or PPADS) (22, 25), significantly inhibited ATPec-induced PS exposure (Fig. 3B), whereas oxidized ATP, a P2X7 antagonist (22, 25), significantly inhibited ATPec-induced PS externalization (Fig. 3B). As P2X7 is activated by ATP4− but not by Mg-ATP (22, 25), chelation of ATPec by high Mg2+ concentrations antagonizes P2X7 activation. Pretreatment of thymocytes with 15 mM Mg2+ significantly inhibited PS exposure induced by ATPec without affecting PS exposure in control cells (Fig. 3B). Furthermore ADP induced PS exposure in a significantly smaller percentage of cells than did ATPec whereas 1 mM AMP was without effect (Fig. 3A) as expected from their respective affinities for the P2X7 purinoceptor (2, 23, 35). PS exposure induced by 1 mM ADP was inhibited by oxidized ATP confirming that this effect was mediated through P2X7 activation (data not shown). PS exposure required ATPec concentrations above 0.1 mM (Fig. 3A) in agreement with the range of concentrations reported to activate P2X7, excluding a role for P2X1 purinoceptor, whose activation occurs at concentrations below 0.1 mM (25). Altogether these data provide evidence for the implication of P2X7 in ATPec-induced PS exposure.

P2X7 Receptor Is Initially in Channel Conformation during PS Exposure—To determine whether P2X7 was in a channel or pore conformation under the conditions leading to early PS exposure, we measured the uptake of two impermeant nucleus markers of different molecular sizes, ethidium (314 Da) and propidium (414 Da), in response to 1 mM ATPec. Fig. 4A shows that, during the first 20 min of incubation, there was no uptake of propidium, and the uptake of ethidium, observed in less than 15% of cells, was not significantly different with or without ATPec. Furthermore the level of fluorescence intensity was the same in control or ATPec-treated cells (data not shown). These data indicate that P2X7 was not in a pore conformation at least...
for the period during which PS became externalized in thymocytes upon treatment with 1 mM ATP_ec. Nevertheless, after 40 min of ATP_ec treatment, only ethidium and not propidium uptake was observed in a significant percentage of cells (Fig. 4A), showing that the pore formation was a time-dependent and size-limited process in thymocytes.

P2X7 is an ATP-gated nonspecific cation channel mediating fast permeability changes to Na^+ /H^+, K^+ /H^+, and Ca^{2+}/H^+ (22). When thymocytes were incubated in a Ca^{2+}-free medium, there was no change in [Ca^{2+}]_i upon addition of 1 mM ATP_ec showing that this treatment did not induce any Ca^{2+} release from intracellular stores (Fig. 4B). Nevertheless subsequent addition of Ca^{2+} to the medium triggered a significant increase in [Ca^{2+}]_i, reflecting Ca^{2+} influx in ATP_ec-treated cells (Fig. 4B). Attempts to measure Na^+ influx by using Sodium Green-AM or sodium-binding benzofuran isophthalate-AM (Molecular Probes), two
specific probes for Na⁺ detection, were unsuccessful, presumably as a result of inefficient probe loading in thymocytes. However, addition of 1 mM ATPec induced a rapid depolarization of the plasma membrane detected from the increase in DiBac4(3) fluorescence (Fig. 4C), providing indirect evidence for the occurrence of ATPec-induced Na⁺ influx (35). Altogether these data confirm that P2X7 in thymocytes is a rapidly gated cation channel.

**ATPec Induces Ca²⁺-dependent or Na⁺-dependent PS Exposure**—To determine a possible involvement of ATPec-induced Ca²⁺ influx in PS exposure, the percentage of cells with externalized PS (PS⁺ cells) was determined after 20-min incubation with 1 mM ATPec in Na⁺-free choline media containing different Ca²⁺ concentrations. Increasing external Ca²⁺ concentration resulted in a progressive elevation in [Ca²⁺], detectable as soon as 1 mM external Ca²⁺, in a maximum of about 40% of thymocytes (data not shown), named high Ca²⁺ cells. Similarly the percentage of PS⁺ cells induced by ATPec increased with external Ca²⁺, reaching a maximum as soon as 2.5 mM Ca²⁺, not significantly different from the percentage detected in 5% FCS/RPMI (Fig. 5A, dotted line). Remarkably, at 0.5 mM Ca²⁺ in choline medium, PS exposure was observed in only a significantly smaller percentage of cells than that detected in 5% FCS/RPMI, although the latter medium contained about 0.5 mM Ca²⁺ (Fig. 5A, dotted line). This difference suggests that an additional mechanism, independent of Ca²⁺, was involved in PS exposure in 5% FCS/RPMI.

In addition to Ca²⁺ uptake, P2X7 activation triggers K⁺ efflux and Na⁺ influx (22). Interestingly P2X7 channel can be activated in the absence of external Ca²⁺ and Na⁺ (36). Thus, in the choline medium devoid of both Ca²⁺ and Na⁺, ATPec could induce K⁺ efflux but was unable to promote significant PS exposure (Fig. 5, A and B), suggesting that K⁺ efflux alone could not be responsible for PS exposure. To study the possible involvement of Na⁺ in ATPec-induced PS exposure, we determined the percentage of PS⁺ cells in a Ca²⁺- and Na⁺-free choline media containing different Na⁺ concentrations. The percentage of PS⁺ cells, induced by 20-min treatment with 1 mM ATPec, augmented at external Na⁺ concentrations above 70 mM, affecting 38% of cells at 139 mM Na⁺, a percentage not significantly different from that observed in 5% FCS/RPMI (Fig. 5B, dotted line). Under these conditions, the effect of external Na⁺ on PS exposure can be attributed to ATPec-mediated Na⁺ influx.

To provide further evidence for a Na⁺-dependent mechanism of PS exposure, we tested the effect of gramicidin D and monensin, ionophores that induce Na⁺ influx in cells suspended in media containing exclusively Na⁺. Fig. 5C shows that incubation of thymocytes for 15 min in NaCl medium, but not in choline medium, with 1.5 μM gramicidin D induced PS exposure in about 25% of cells. Increasing the time of incubation or the gramicidin D concentration induced thymocyte necrosis as detected by PI uptake (data not shown). In the same way, PS exposure was induced by monensin in the presence of Na⁺ in the medium (Fig. 5C).

**ATPec Induces Ca²⁺-dependent or Na⁺-dependent Phospholipid Scrambling**—PS exposure is generally accompanied by the transmembrane redistribution of the other major phospholipids, a process known as scrambling. To investigate whether this scrambling process also occurs in thymocytes activated by ATPec, PC redistribution was studied using a fluorescent analog of PC (NBD-PC). This method allows the determination of the percentage of the cells exhibiting a transbilayer movement of PC at the time of measurement, whereas the factor Va binding assay provides the percentage of cells having exposed PS over the duration of the ATPec treatment. In choline medium containing 5 mM Ca²⁺, 10 min after addition of 1 mM ATPec NBD-PC was internalized in about 35–40% of thymocytes, a percentage similar to that of PS⁺ cells under the same conditions (Fig. 6A). Addition of 15 mM MgCl₂ after 10-min incuba-
ATP<sub>ec</sub>-induced PS Exposure Is Mediated by Na<sup>+</sup> Influx

**DISCUSSION**

ATP<sub>ec</sub> has been reported to induce cell death by lysis or apoptosis in a variety of cells, including immune cells, such as dendritic cells, macrophages, and thymocytes (22). Our data show that ATP<sub>ec</sub> induced a rapid PS exposure in thymocytes, which was already significant after 10–15 min. Following a longer time of incubation, PS exposure and nuclear condensation occurred in the same cell population, amounting to 35–40% of total thymocytes. Cells exhibiting PS exposure and nuclear condensation remained impermeable to PI, and apoptosis was confirmed by DNA fragmentation observed in cellular extracts. Consequently, under these experimental conditions, thymocyte death induced by ATP<sub>ec</sub> occurred by apoptosis rather than by lysis. ATP<sub>ec</sub>-induced apoptosis of thymocytes has been reported to be mediated through activation of P2X1 and P2X7 (1–4). Our pharmacological studies provide evidence for the involvement of P2X7 in PS exposure. We confirm also that, in thymocytes, ATP<sub>ec</sub> triggers a significant and sustained Ca<sup>2+</sup> influx without releasing intracellular Ca<sup>2+</sup> (3, 24, 35, 37), a rapid membrane depolarization (4, 24, 35), and the progressive formation of a limited size pore permeable to ethidium and not to PI (22, 24, 35). All these characteristics are hallmarks of P2X7 activation.

PS externalization is known to play a critical role in cell phagocytosis by macrophages (38–40). Although PS exposure is considered to be a hallmark of apoptosis, it can be dissociated from other features of the apoptotic process, for instance caspase activation or DNA fragmentation, as PS-dependent phagocytic recognition of cells can occur earlier or independently of these latter events (41, 42). We have observed that, after a short treatment (30 min) with ATP<sub>ec</sub>, thymocyte phagocytosis by thioglycollate-elicited peritoneal macrophages was significant and inhibited when external PS was masked by annexin-V binding. Consequently early PS exposure, mediated by ATP<sub>ec</sub> via P2X7 activation and preceding further steps of apoptosis, could lead to recognition and phagocytosis of thymocytes. Such a possibility for a rapid thymocyte elimination is supported by the observations of Surv and Sprent (43) that apoptotic cells were mainly present within phagocytic cells in the thymus. Furthermore, thymocyte elimination, studied in fetal thymic organ culture, suggests an implication of ATP<sub>ec</sub> and potentially of P2X7 in thymocyte death by neglect (3). In this context, the early P2X7-induced PS exposure could be involved in thymocyte elimination during thymic selection. From the various P2X7-mediated responses (Ca<sup>2+</sup> influx (35, 37), plasma membrane depolarization (4), or ethidium uptake (35)) within the different subsets of thymocytes or in peripheral T lymphocytes, previous studies have attempted to implicate ATP<sub>ec</sub> in thymocyte maturation and/or in peripheral T cell regulation. DN subset was the most responsive to P2X7 activation according to Nagy et al. (4), whereas, in other studies, the most mature SP subsets, notably SPCD4+, exhibited the strongest response (35, 37). This latter observation was supported by the persistence of the P2X7 response in peripheral T cells (30, 35). We show that P2X7 activation induced PS exposure in all subsets and predominantly in the most differentiated SP cells. This characteristic is likely to be due to the higher level of P2X7 expression in SP subsets in relation to the thymocyte maturation state (35). Furthermore P2X7 activation, induced by ATP<sub>ec</sub> in splenic T cells (29) or by ADP-ribosylation in lymph node T cells (30), leads to apoptosis with an early PS exposure.
suggesting a role of P2X7 in T cell death, for instance to
eliminate potentially autoreactive T cells during inflammatory
reaction (30). Thus, early PS externalization induced by P2X7
activation could be essential in the rapid elimination of periph-
eral T cells by phagocytosis.

Our data show that ATP\textsubscript{ec} induced both early PS exposure
and NBD-PC internalization, reflecting phospholipid scram-
bling, a process generally attributed to an increase in [Ca\textsuperscript{2+}]\textsubscript{i} in
cell activation (7) and apoptosis (6, 7, 12). However, in many
instances there was no correlation between scrambling and
increased [Ca\textsuperscript{2+}]\textsubscript{i}. In Jurkat T cells, the increase in [Ca\textsuperscript{2+}]\textsubscript{i}
induced by Ca\textsuperscript{2+} ionophore was not sufficient by itself to trigger
scrambling, leading to the hypothesis that the ionophore has an
additional effect, possibly implicating mitochondria (44). In
thymocytes, induction of apoptosis and PS exposure by dexa-
methasone, gliofoxin, and thapsigargin was not correlated with
an increase in [Ca\textsuperscript{2+}]\textsubscript{i} (45). In the same way, during in vitro
attack of hepatocytes by natural killer cells, there was no
evidence for a straightforward relationship between elevation
of [Ca\textsuperscript{2+}]\textsubscript{i}, and PS externalization (46). Ca\textsuperscript{2+} is not the only
known inducer of phospholipid scrambling: a decrease in pH
stimulated NBD-phospholipid scrambling in inside-out vesicles
(13, 47), and polyamines were implicated in PS exposure in
apoptosis (48). In platelets stimulated with collagen, Na\textsuperscript{+}
influx, mediated through activation of the Na\textsuperscript{+}/H\textsuperscript{+}
exchanger, was involved in the generation of procoagulant activity (49), a
process generally initiated by PS externalization.

We investigated the respective roles of Ca\textsuperscript{2+} and Na\textsuperscript{+} in
ATP\textsubscript{ec}-induced PS externalization by manipulating the cation
concentration of the incubation media. First, we show that PS
exposure was correlated with an increase in [Ca\textsuperscript{2+}]\textsubscript{i}, itself
resulting from a stimulation of Ca\textsuperscript{2+} influx, mediated by ATP-
gated channel P2X7. In our experimental conditions, we con-
firm that ATP\textsubscript{ec} did not induce any Ca\textsuperscript{2+} release (Refs. 3, 24, 35,
and 37 and the present data). Consequently the store-operated
Ca\textsuperscript{2+} influx, previously implicated in PS exposure in erythro-
leukemia cells treated with Ca\textsuperscript{2+} ionophore (34), could not be
involved in ATP\textsubscript{ec}-induced PS exposure. Second, in a medium
devoid of Ca\textsuperscript{2+}, PS externalization was correlated with the
external Na\textsuperscript{+} concentration. This effect is attributable to Na\textsuperscript{+}
influx mediated by ATP-gated channel P2X7 and expected to
increase intracellular Na\textsuperscript{+} concentration. The role of Na\textsuperscript{+}
influx in PS exposure was confirmed by using the Na\textsuperscript{+} ionophore,
gramicidin D or monensin. Third, a ta2m M external Ca\textsuperscript{2+}
ionophore was not sufficient by itself to trigger
scrambling, allowing relocation of endogenous PS. Following ATP\textsubscript{ec}
chelation, intracellular Na\textsuperscript{+} could be actively extruded through the
Na\textsuperscript{+}/K\textsuperscript{+} pump, returning to a concentration below
the threshold level required to maintain scrambling. Thus, APLT
could be directly inhibited either by Na\textsuperscript{+} or by ATP consump-
tion by the Na\textsuperscript{+}/K\textsuperscript{+} pump. Interestingly the presence of extern-
al PS in a subpopulation of sickle cells was correlated with
APLT inhibition rather than with an elevated [Ca\textsuperscript{2+}]\textsubscript{i} (52).
Furthermore this cell population had a low K\textsuperscript{+} and high Na\textsuperscript{+}
content (52) in agreement with a potential role of Na\textsuperscript{+} in PS
exposure and APLT inhibition. Although the possibility of reg-
ulation of PS externalization in apoptosis by modulating APLT
activity is attractive (18, 46, 51, 52), it cannot account for the
attacked scrambling of the other phospholipids. Consequently
the most likely hypothesis could be that Na\textsuperscript{+}, as already
described for Ca\textsuperscript{2+}, acts by both inhibiting APLT and activating
scramblase or other molecular complexes able to promote phos-
pholipid transport. A role for intracellular Na\textsuperscript{+} has been pro-
posed to explain other cellular processes such as reverse trans-
port of dopamine (53), stress-activated protein kinase/c-Jun
N-terminal kinase activation (54), P2X7-mediated membrane
blebbing (36), and platelet procoagulant properties (49). In
apoptosis, an early Na\textsuperscript{+} influx is involved in the control of cell
shrinkage and activation of the apoptotic program (55). How-
ever, the mechanisms by which Na\textsuperscript{+} exerts its action remain
deliberately unknown and would deserve to be investigated.

Here we present evidence that ATP\textsubscript{ec} induces early PS ex-
ternalization and phospholipid scrambling, which precede
other events of apoptosis, in a maximum of 35–40% of thymo-
cytes. Phospholipid movements are dependent on an increase
in intracellular Ca\textsuperscript{2+} and/or Na\textsuperscript{+} concentrations resulting from
activation of the P2X7 channel. We suggest that Na\textsuperscript{+}, rather than
Ca\textsuperscript{2+}, is the major mediator of the early PS externalization
induced by ATP\textsubscript{ec}. The role of Na\textsuperscript{+} in PS externalization,
and consequently cell elimination, would require to be investig-
ated in other physiological or pathological conditions, particularly
during apoptosis in which the role of Ca\textsuperscript{2+} in mediating delayed
PS exposure has been challenged.

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