An ATP-Activated Channel Is Involved in Mitogenic Stimulation of Human T Lymphocytes

By Olevio R. Baricordi, Davide Ferrari, Loredana Melchiorri, Paola Chiozzi, Stefania Hanau, Elisabetta Chiari, Michele Rubini, and Francesco Di Virgilio

We investigated the effect of pharmacologic modulation of the ATP receptor on intracellular ion changes and proliferative response of human peripheral blood lymphocytes (PBLs) and purified T lymphocytes. Extracellular ATP (ATP) triggered these cells an increase in the cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and plasma membrane depolarization. Whereas both Ca\(^{2+}\) release from intracellular stores and influx across the plasma membrane were detected in the whole PBL population, only Ca\(^{2+}\) influx was observed in T cells. In the presence of near physiologic extracellular Na\(^{+}\) concentrations (125 mmol/L), Ca\(^{2+}\) permeability through the ATP-gated channel was very low, suggesting a higher selectivity for monovalent over divalent cations. The selective P\(_2\_\) agonist benzoylbenzoic ATP (BzATP) increased [Ca\(^{2+}\)]\(_i\) in the presence but not the absence of extracellular Ca\(^{2+}\) and also caused plasma membrane depolarization. The covalent blocker oxidized ATP (oATP), an inhibitor of P\(_2\_\) and P\(_2\_\) receptors, prevented Ca\(^{2+}\) influx and plasma membrane depolarization, but had no effect on Ca\(^{2+}\) release from stores. Stimulation with ATP, alone had no significant effects on PBL \(^3\)H-thymidine incorporation. On the contrary, ATP, or BzATP had a synergistic effect on DNA synthesis stimulated by selective T-cell mitogens such as phytohemagglutinin, anti-CD3 monoclonal antibody, or allogeneic PBLs (mixed lymphocyte cultures). Treatment with oATP inhibited mitogenic stimulation by these receptor-directed agents but not by the combined application of the Ca\(^{2+}\) ionophore ionomycin and phorbol myristate acetate. Interleukin-2 partially relieved inhibition by oATP. These results suggest that human T lymphocytes express a plasma membrane channel gated by ATP, that is involved in mitogenic stimulation.

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EXTRACELLULAR ATP (ATP) can modulate responses of diverse lymphocyte cell populations via specific cell membrane purinergic receptors.\(^1\) At least two functionally distinct receptor subtypes have been described in lymphocytes: (1) a G-protein-coupled receptor linked to inositol 1, 4, 5-trisphosphate generation and Ca\(^{2+}\) mobilization from intracellular stores, also known as P\(_2\_\); and (2) an ion channel permeable to Na\(^+\) and Ca\(^{2+}\) directly gated by ATP, without involvement of known intracellular second messengers, tentatively identified as a P\(_2\_\)/P\(_2\_\) receptor.\(^4\) The P\(_2\_\) receptor has been described in human B lymphocytes and has been reported to be absent from T cells.\(^7\) The nucleotide-activated ion channel has been shown to be expressed at a low level in normal human B lymphocytes\(^8\) and to be upregulated in resting mouse T and B lymphocytes, leukemic peripheral blood lymphocytes, and B-lymphoblastoid cells isolated from individuals affected by Duchenne muscular dystrophy.\(^9\) Whether receptors for ATP, are also expressed by normal human T lymphocyte cells is an open question.

Despite compelling evidence for the presence of purinergic receptors in lymphoid cells, very little is known about their possible physiologic role. It has been suggested that they may be involved in the modulation of mitogenic responses\(^1\) and cytotoxic reactions\(^6\) but the cellular mechanism and the in vivo significance of these actions are still dubious.

A relevant obstacle to understanding the role of ATP, receptors is the lack of specific agonists or antagonists. In recent years, we have characterized a compound, periodate-oxidized ATP (oATP), also known as dialdehyde ATP, that showed a good selectivity for the ATP-gated channel/pore of mouse macrophage and human lymphoblastoid cells.\(^1\) This reagent turns out to be very interesting because it (1) inhibited plasma membrane ion fluxes or permeability changes without decreasing Ca\(^{2+}\) mobilization from intracellular stores, (2) abolished all cytotoxic effects due to oATP, stimulation of macrophage or lymphoblastoid cells, and (3) was devoided of any toxic effects even after prolonged (2 to 3 days) in vitro culture. Furthermore, because oATP forms a Schiff base or a dihydromorpholino derivative with unprotonated lysins in the vicinity of the ATP, binding site,\(^1\) it allows covalent (irreversible) modification of the ATP-gated channel.

In this report, we show that human T lymphocytes from PBLs express an ATP-gated plasma membrane channel and investigate the effect of pharmacologic modulation of this channel on T-cell--specific mitogenic stimulation.

Our results suggest that the ATP receptor expressed by human T lymphocytes may be involved in the control of mitogenic stimulation by different stimuli.

MATERIALS AND METHODS

Cells and solutions. PBLs were isolated by Ficoll gradient from samples withdrawn from healthy volunteers. Plastic nonadherent cells were passed through a nylon-wool column to isolate purified T cells. CD4\(^+\) and CD8\(^+\) cells were separated by positive selection with the Dynabead-Detachabead technique (Dynal AS., Oslo, Norway). In brief, PBLs were resuspended in RPMI medium at a concentration of 10\(^7\)/mL and incubated with mouse anti-human CD4 or CD8 monoclonal antibody (MoAb)-coated magnetic beads at a ratio of...
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5:1 (beads:lymphocytes). The bead:cell suspension was gently rotated for 60 minutes at 4°C. Rossetted beads were selected on a magnet, carefully washed 5 times to remove unbound cells, and resuspended in RPMI medium at a concentration of 10^7 beads/100 μL. Selected cells were released from the beads by coincubation with goat-antimouse IgG detachable bead at a concentration of 1:10^7 beads. The suspension was gently rotated for 45 minutes at room temperature, the beads were isolated with a magnet, and the cells were pelleted. Viability was greater than 90%. The purity of the T cells obtained was assessed by indirect immunofluorescence by using a FACS Vantage cytometer (Becton Dickinson, San Jose, CA) and MoAb anti-CD3 (kindly provided by Dr. Fabio Malavasi, University of Torino, Torino, Italy) or anti-CD4 and CD8 MoAbs (Dakopatts, Copenhagen, Denmark) and was found to be in all cases greater than 95%. Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 2 mMol/L glutamine, hereafter abbreviated as RPMI10F. [H]Thymidine incorporation was performed in RPMI10F medium. Fluorimetric measurements were performed in saline or sucrose solution. Saline solution had the following composition: 125 mMol/L NaCl, 5 mMol/L KCl, 1 mMol/L MgSO4, 1 mMol/L Na2HPO4, 5.5 mMol/L glucose, 5 mMol/L NaHCO3, 1 mMol/L CaCl2, and 20 mMol/L HEPES (pH 7.4). Sucrose solution contained 300 mMol/L sucrose, 1 mMol/L MgSO4, 1 mMol/L K2HPO4, 5 mMol/L KHCO3, 1 mMol/L CaCl2, 5.5 mMol/L glucose, and 20 mMol/L HEPES and the pH was adjusted to 7.4 with Tris-HCl.

Measurement of plasma membrane potential and [Ca2+]. Changes in plasma membrane potential were measured with the fluorescent dye bis 1, 3-diethyliodoarbiturite trimethineoxonal (bisoxonol; Molecular Probes, Inc, Eugene, OR) at the wavelength pair 540/580 nm, as previously described. Changes in [Ca2+] were measured with the fluorescent indicator fura-2/AM, as described previously. Briefly, cells were loaded with 2 μmol/L fura-2/AM and incubated in a thermostatted-control (37°C) and magnetically stirred fluorimeter cuvette (Perkin-Elmer LS50; Perkin-Elmer Ltd, Beaconsfield, Buckinghamshire, UK). Sucroxynpirazone (250 μmol/L; Sigma-Aldrich Sr, Milan, Italy) was present throughout the experiment to prevent fura-2 leakage and/or sequestration. Levels were determined with the 340/380 excitation ratio, at an emission wavelength of 500 nm. When indicated, cells were also pre-treated with apyrase (Sigma) at the concentration of 0.2 U/mL.

Tyrosine phosphorylation. PBLs (1 × 10^6/mL) were incubated in 10% FCS Iscove’s modified Dulbecco’s medium in the presence and absence of 200 μmol/L ATP. After 2 hours of incubation, they were stimulated with the anti-CD3 MoAb CBT3 (1 μg/mL) and 5 minutes later rinsed three times with cold Hank’s medium and lysed in a buffer containing 150 mMol/L NaCl, 1.5 mMol/L MgCl2, 1 mMol/L EGTA, 10% glycerol, 1% Triton X-100, 100 mMol/L NaF, 0.2 mMol/L Na2VO4, 10 mMol/L phenylmethylsulphonyl fluoride (PMSF), 0.1 mg/mL aprotinin, 50 mMol/L HEPES, pH 7.5. The lysates were placed in ice for 4 minutes, collected, and centrifuged (2 minutes at 13,000 rpm in Beckman microfuge; Beckman Instruments, Inc, Fullerton, CA) to sediment the nuclear fraction. The supernatants containing the lysates (400 μg) were then diluted with HNTG (150 mMol/L NaCl, 10% glycerol, 1% Triton X-100, 100 mMol/L NaF, 0.2 mMol/L Na2VO4, 0.1% Triton X-100, 100 mMol/L PMSF, 0.1 mg/mL aprotinin, and 20 mMol/L HEPES, pH 7.5) supplemented with 100 μL anti-CD3 MoAb CBT3 and 20 μL of agarose-conjugated protein-A (Oncogene Science, Uniondale, NY) in a total volume of 1.5 mL. Ab/Ag complexes were allowed to form for 4 hours at 4°C and were then precipitated by centrifugation in the cold for 4 minutes at 13,000 rpm (Beckman microfuge). Immunoprecipitated complexes were washed three times in HNTG and resuspended in 20 μL of Laemli buffer (20% glycerol, 3% sodium dodecylsulfate, 3% β-mercaptoethanol, 10 mMol/L EDTA, and 0.05% bromophenol blue). Samples were boiled for 4 minutes and run on a 4% to 20% gradient polyacrylamide gel (BioRad, Hercules, CA). Proteins were electrophoretically onto a nitrocellulose filter. Phosphorylated proteins were detected by western immunoblot analysis by standard technique with an anti-phosphotyrosine antibody (PY20) conjugated with horseradish peroxidase (Transduction Lab, Lexington, KY) and visualization by enhanced chemiluminescence (ECL) detection system (Amersham, Little Chalfont, Buckinghamshire, UK).

Thymidine incorporation. For [H]Thymidine incorporation, PBLs were resuspended in RPMI10F medium at a concentration of 10^5/mL; 100 μL was plated in Falcon 96-well 3172 plates and either challenged with the different stimulants (phytohemagglutinin [PHA], anti-CD3, or allogeneic cells), or left untreated (controls). After 72 hours (when the challenge was PHA or anti-CD3) or 120 hours (when the challenge was allogeneic cells), [H]Thymidine (1 μCi/well) was added to the culture medium and the incubation was performed for an additional 16 to 24 hours. Allogeneic cells were previously irradiated at 2,500 Rads in a volume of 100 μL. After mitogenic stimulation, cells were harvested and radioactivity was determined by liquid scintillation counting.

RESULTS

Characterization of ATP,-dependent ion changes in human PBLs. Few data are currently available on ATP-dependent [Ca2+] responses of PBLs and human T lymphocytes. In fact, it has been suggested that human lymphocytes from healthy individuals lack Ca2+ mobilization in response to this nucleotide.4,8 A recent study of ours in B-lymphoblastoid cells obtained from Duchenne patients showed that extracellular Ca2+ and Na+ compete for entry through the ATP-gated receptor/channel1; thus, under physiologic extracellular Na+ and Ca2+ concentrations, Ca2+ influx is obliterated by the simultaneous Na+ influx. Figure 1 shows that ATP, stimulation of fura-2—loaded PBLs suspended in standard saline (trace a) triggered a small but clearly detectable increase in [Ca2+]i, whereas a much larger increase was observed in Na+-free, sucrose-supplemented medium (trace b). Although the sus-
tained phase of the \([Ca^{2+}]_i\) increase was fully dependent on influx across the plasma membrane, as it was obliterated by chelation of extracellular \(Ca^{2+}\), the fast initial \([Ca^{2+}]_i\) spike likely reflected release of \(Ca^{2+}\) from intracellular stores (trace c). Besides ATP, UTP, also released a small but clearly detectable amount of \(Ca^{2+}\) from intracellular stores (trace d).

\(Ca^{2+}\) release from intracellular stores was likely due to activation of B lymphocytes or residual contaminating monocytes, because these cells were previously shown to mobilize \(Ca^{2+}\) from intracellular stores in response to extracellular nucleotides; therefore, we investigated the response to ATP, in a purified T-cell population. As shown in Fig 2, trace a, ATP, caused no mobilization of intracellular \(Ca^{2+}\) in T cells incubated in the absence of this extracellular cation and in the presence of EGTA. Nonetheless, this nucleotide activated a plasma membrane channel, because re-addition of ATP triggered a rapid increase in \([Ca^{2+}]_i\). A similar increase was triggered by stimulation of T cells in \(Ca^{2+}\)-containing medium (trace b). Preliminary experiments in purified resting CD4+ and CD8+ lymphocytes suggests that an ATP-gated plasma membrane channel is present in both cell populations (O.R. Baricordi and F. Di Virgilio, manuscript in preparation).

A \([Ca^{2+}]_i\) increase, larger in Na+-free medium, was also caused in PBLs by the ATP analog BzATP, an agonist specific for purinergic receptors of the P2X/P2X subtype (Fig 3, traces a and c). Pretreatment with oATP blocked the \([Ca^{2+}]_i\) increase (trace b), thus suggesting involvement of a P2Y/P2X purinergic receptor subtype. As shown in trace c, the kinetics of the \([Ca^{2+}]_i\) increase triggered by BzATP was slower than that caused by ATP (see Fig 1, trace a), suggesting that the fast initial discharge of \(Ca^{2+}\) from intracellular stores was lacking in response to stimulation with this ATP analog. To confirm this suggestion, PBLs were stimulated with BzATP in the absence of extracellular \(Ca^{2+}\) and in the presence of EGTA. Under these conditions, BzATP triggered no \([Ca^{2+}]_i\) increase, whereas UTP, was still active (trace d). These results show that BzATP and oATP allow selective pharmacologic manipulation of the ATP-gated channel (P2Y/P2X receptor) expressed by T lymphocytes while leaving unaltered responses mediated by the nucleotide receptor of B lymphocytes. To further strengthen this finding, we investigated the ability of BzATP to trigger a \([Ca^{2+}]_i\) increase in oATP-
inhibited PBLs. As shown in trace e, incubation in the presence of oATP prevented the BzATP- but not the UTP-, dependent \([Ca^{2+}]_i\) increase.

As previously reported in mouse lymphocytes and human Duchenne B lymphocytes, ATP, caused BzATP plasma membrane depolarization that was potentiated by chelation of extracellular \(Ca^{2+}\) (Fig 4, upper panel, traces a and b). Like the \(Ca^{2+}\) response, membrane depolarization was also triggered by BzATP (trace c) and blocked by preincubation with oATP (trace d). Quite interestingly, UTP, was ineffective (trace c), suggesting that depolarization was mainly, if not entirely, due to activation of the ATP-gated channel of T lymphocytes. To confirm this suggestion, T lymphocytes were examined. Trace e shows that ATP, caused a near-maximal depolarization in T lymphocytes. Depolarization was also triggered by BzATP but not by UTP (trace f) and was inhibited by pretreatment with oATP (trace g). ATP, dose-dependency for activation of the ATP-gated channel, measured as membrane plasma membrane depolarization, was close to that reported for mouse thymocytes, showing a threshold and an \(EC_{50}\) of 100 and 400 \(\mu\)mol/L, respectively (Fig 4, lower panel).21 Besides plasma membrane depolarization, we did not observe a non selective increase in plasma membrane permeability due to ATP, (data not shown).

Effects of ATP, on T lymphocyte proliferation. ATP, has been shown to modulate diverse lymphocyte functional responses, such as DNA synthesis, blastogenesis, and cell-mediated killing, but a clear-cut physiologic role (if any) has never been identified. In our hands, ATP, at concentrations ranging from 100 to 300 \(\mu\)mol/L, was unable to stimulate PBL proliferation (data not shown). However, this nucleotide showed a strong costimulatory effect when added together with PHA or anti-CD3 at suboptimal concentrations (Table 1). The costimulatory effect was stronger at 100 \(\mu\)mol/L than 300 \(\mu\)mol/L ATP, both in the case of PHA- and anti-CD3-driven DNA synthesis. In agreement with the \([Ca^{2+}]_i\), in-
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Fig 4. PBLs and T lymphocytes are depolarized by ATP_e. PBLs (upper panel) and T lymphocytes (middle panel) were incubated in the fluorimeter cuvette at a concentration of 5 x 10^6/mL in the presence of 200 μmol/L bisoxonol. Traces a, c, and d, standard saline; traces e, f, and g, Ca^2+-free, 0.5 mmol/L EGTA-supplemented saline. In traces d and g, cells were incubated for 2 hours in the presence of 300 μmol/L oATP before being stimulated. ATP_e, UTP_e, and BzATP were at 1.1, and 0.1 mmol/L, respectively. KCI was used to depolarize the plasma membrane and allow internal calibration of ATP_e-dependent potential changes. Each KCI addition was 30 μmol/mL. The ATP_e dose-dependence shown in lower panel was performed in Ca^2+-free, 0.5 mmol/L EGTA-supplemented saline solution. In lower panel, depolarization is expressed as the percentage of maximal depolarization induced by 3 μmol/L ATP_e.

These experiments suggested that a P2 receptor, probably of the P2x subtype, could play a role in mitogen-induced proliferation of human T lymphocytes. However, although 100 μmol/L was an optimal ATP_e concentration as a comitogenic stimulus, it caused only a barely detectable activation of the ATP_e-gated channel, even in the absence of Ca^2+ as acomplexing cation (see dose-dependency curve in Fig 4). One possible reason for this discrepancy could be that, during the in vitro incubation, ATP_e receptors are desensitized by the continuous leakage of endogenously released ATP. To test this hypothesis, we treated purified T lymphocytes with the ATP-hydrolysing enzyme apyrase before the challenge with ATP_e. Figure 5A shows that 100 μmol/L ATP_e triggered in apyrase-treated T cells incubated in Ca^2+ and Mg^2+-containing saline solution a clear and sustained plasma membrane depolarization that was further stimulated by a subsequent 300 μmol/L ATP_e addition. An ATP_e dose-dependency curve performed under these conditions shows that the EC50 was not significantly shifted with respect that determined in Ca^2+-free medium (Figs 4 and 5), but the threshold for receptor activation was lowered to approximately 30 μmol/L ATP_e.

To further test the involvement of P2x receptors in mitogenic stimulation, we examined the effect of the specific receptor blocker oATP. Figure 6 shows that oATP inhibited in a dose-dependent fashion T-lymphocyte stimulation by three different stimuli in PBL cultures, ie, PHA, anti-CD3, and allogeneic cells. Stimulation by anti-CD3 was extremely sensitive to inhibition because, at a concentration of 25 μmol/L oATP, an 80% reduction of 3H-thymidine incorporation was observed. On the contrary, PHA stimulation was inhibited by only about 40% at the same oATP concentration. Even less sensitive to inhibition was stimulation by allogeneic cells, as DNA synthesis was decreased by only 5%. However, near full inhibition of DNA synthesis irrespectively of the stimulant used was achieved at 300 μmol/L oATP, an inhibitor concentration that we have previously shown to fully block the ATP receptor of mouse and human macrophages and human B-lymphoblastoid cells. We think that the different sensitivity to oATP inhibition observed with the three different stimuli might depend on two reasons. On the one hand, CD3 is known to be a weaker stimulus than PHA, thus presumably more susceptible to inhibitory agents. On the other hand, in the experiments in which allogeneic cells were a stimulus, a double cell concentration was present in the sample (10^6 stimulator plus 10^6 responder cells), thus increasing the rate of degradation of the oxidized nucleotide.

oATP also inhibited DNA synthesis when added after the mitogenic stimulus, albeit with a lesser efficiency (Table 2). The addition of oATP 24 or 72 hours after mitogenic stimulation reduced 3H-thymidine incorporation by about 80% and 50%, respectively. Quite interestingly, sensitivity to stimulation by PHA, but not anti-CD3, was partially restored by supplementation of the lymphocyte growth factor interleukin-2 (IL-2) to the culture medium.

It cannot be excluded that oATP, besides blocking the ATP_e-gated channel, also caused a nonspecific inhibition of early events associated with TCR activation. To rule out this
possibility, we investigated the pattern of tyrosine phosphorylation and [Ca\textsuperscript{2+}], changes in T lymphocytes challenged with anti-CD3 MoAbs in the absence and presence of oATP. Figure 7A shows that treatment with oATP did not alter the pattern of protein tyrosine phosphorylation triggered by activation of the TCR/CD3 complex; in fact, the oxidized nucleotide, if anything, slightly increased phosphate incorporation. Figure 7B shows that [Ca\textsuperscript{2+}], changes due to TCR/CD3 activation were also unmodified by oATP treatment. Note that the Ca\textsuperscript{2+} experiments were performed in Jurkat cells, because it is known that anti-CD3 MoAbs are a weak stimulus for [Ca\textsuperscript{2+}], mobilization in peripheral T lymphocytes.\textsuperscript{22}

Because oATP is a substrate for plasma membrane ectoATPases, it is likely that oxidized adenosine is generated during incubation of cell cultures in the presence of oATP. To rule out a possible inhibitory effect of oxidized adenosine, control experiments were performed in the presence of this compound (300 \(\mu\)mol/L) without observing any decrease in thymidine incorporation (data not shown). As a further proof that inhibition of DNA synthesis by oATP was not due to its degradation products, we tested the effect of oxidized UTP (oUTP), a compound that is also known to block plasma membrane ATP\textsubscript{gated} channel.\textsuperscript{23} oUTP turned out to be almost as good an inhibitor as oATP (O.R. Baricordi and F. Di Virgilio, unpublished observation).

Di-aldehyde functions of oATP react with unprotonated lysines in the vicinity of ATP binding sites, thus forming a covalent Schiff base or a dihydromorpholino derivative.\textsuperscript{16} Therefore, the ATP receptor should be permanently inactivated by oATP and functional ATP receptors should only be restored by insertion into the plasma membrane of novel receptors. Were this hypothesis true, oATP-treated PBLs should remain refractory to mitogenic stimulation once washed free of the inhibitor. Table 3 shows that this is in fact the case, as a short pretreatment of 2 hours is sufficient to inhibit by 50% and 60% DNA synthesis stimulated by PHA and anti-CD3, respectively. Furthermore, it can be anticipated that, provided PBLs are allowed enough recovery time in culture, block by oATP should be completely reversible. Table 3 shows that this anticipation is fulfilled because, 72 hours after oATP labeling and wash-out, PBLs recover near complete sensitivity to mitogenic stimulation. Rather surprisingly, oATP-pretreated PBLs showed a slightly larger DNA synthesis with respect to control cells, especially when the stimulant was anti-CD3. oATP-treated lymphocytes also recovered the Ca\textsuperscript{2+} response (data not shown).

PBL DNA synthesis can be stimulated by agents that bypass plasma membrane receptors, such as phorbol esters and the Ca\textsuperscript{2+} ionophore ionomycin. Figure 8 shows that 2 hours of incubation in the presence of oATP did not block \(^3\)H-thymidine incorporation stimulated by the combined addition of these stimuli, although an inhibitory effect was present when PMA or ionomycin were added alone. We think that it is of particular interest that oATP also blocked \(^3\)H-thymidine incorporation due to PMA alone (the effect on ionomycin-dependent stimulation is of difficult interpretation because this ionophore alone was a very weak stimulus), because this might suggest that, during in vitro culture, endogenously released ATP might play a stimulatory or costimulatory role. Lack of sensitivity to oATP inhibition of PMA plus ionomycin, stimuli that bypass surface receptors, is a further demonstration that the ATP receptor modulates the mitogenic signal at a site proximal to the plasma membrane.

**DISCUSSION**

ATP\textsubscript{p} has been variably implicated in the modulation of different lymphocyte responses such as gene expression,\textsuperscript{7} proliferation,\textsuperscript{12} and cytotoxicity\textsuperscript{6,13,14,26} through interaction with specific plasma membrane receptors. Most of these studies were performed in mouse lymphocytes, but more recently human B and T lymphocytes have also been investi-

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**Table 1. Synergistic Effect of Extracellular ATP and PHA or Anti-CD3 on PBL Proliferation**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>100 (\mu)mol/L</th>
<th>300 (\mu)mol/L</th>
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<tbody>
<tr>
<td><strong>PHA</strong></td>
<td>45,489 ± 2,022</td>
<td>75,476 ± 504 ((P&lt;.001))</td>
<td>61,913 ± 762 ((P&lt;.05))</td>
</tr>
<tr>
<td><strong>Anti-CD3</strong></td>
<td>41,304 ± 3,266</td>
<td>55,966 ± 2,111 ((P&lt;.01))</td>
<td>41,064 ± 2,392 (NS)</td>
</tr>
<tr>
<td><strong>UTP</strong></td>
<td>44,471 ± 1,864</td>
<td>39,260 ± 1,297</td>
<td>36,568 ± 1,968</td>
</tr>
<tr>
<td><strong>Anti-CD3</strong></td>
<td>10,324 ± 602</td>
<td>9,508 ± 690</td>
<td>7,867 ± 650</td>
</tr>
</tbody>
</table>

PHA and anti-CD3 were added at a concentration of 0.2 \(\mu\)g/mL. In the experiments with ATP, or UTP, the nucleotide was added together with PHA or anti-CD3, whereas in those with BzATP, this analog was either added together with anti-CD3 (time 0) or 24 or 48 hours after. Data (cpm) are the means ± SD of triplicate determinations from one experiment representative of three others that gave very similar results. Statistical significance (Student’s t-test) is expressed towards controls in the absence of nucleotide.

Abbreviation: NS, not significant.
It is thought that many of the effects of ATP are mediated through purinergic receptors of the P2Y subtype, coupled to a G protein-regulated phospholipase C and to Ca²⁺ release from intracellular stores. However, freshly isolated T lymphocytes do not show any detectable Ca²⁺ mobilization from intracellular stores in response to ATP, suggesting the lack of P2Y (or P2U) type of purinergic receptor. On the contrary, ATP causes Ca²⁺ influx from the extracellular medium and plasma membrane depolarization, suggesting the presence of a nucleotide-activated plasma membrane ion channel.

The ATP₂-activated channel of human T lymphocytes shares many features in common with the channel previously described in B lymphocytes isolated from patients affected by B-cell chronic lymphocytic leukemia or lymphoblastoid cell lines established from Duchenne muscular dystrophy patients. The channel is permeable to both Na⁺ and Ca²⁺, although permeability to Ca²⁺ is low in Na⁺-containing (125 mmol/L) media; thus, ATP, under physiologic conditions, triggers membrane depolarization and Ca²⁺ influx in the absence of permeabilization to low (<900 Daltons) molecular weight hydrophylic solutes.

Table 2. Time Course of oATP-Dependent Inhibition of Mitogenic Stimulation

<table>
<thead>
<tr>
<th></th>
<th>No Stimulus</th>
<th>PHA</th>
<th>Anti-CD3</th>
</tr>
</thead>
<tbody>
<tr>
<td>oATP time 0</td>
<td>198 ± 16</td>
<td>31,582 ± 1,406</td>
<td>19,073 ± 326</td>
</tr>
<tr>
<td>oATP time 3</td>
<td>48 ± 3</td>
<td>499 ± 31</td>
<td>355 ± 14</td>
</tr>
<tr>
<td>oATP 24 h</td>
<td>84 ± 4</td>
<td>5,248 ± 466</td>
<td>1,399 ± 27</td>
</tr>
<tr>
<td>oATP 48 h</td>
<td>82 ± 4</td>
<td>5,935 ± 211</td>
<td>2,354 ± 76</td>
</tr>
<tr>
<td>oATP 72 h</td>
<td>116 ± 9</td>
<td>13,819 ± 328</td>
<td>9,846 ± 176</td>
</tr>
<tr>
<td>oATP = IL-2</td>
<td>181 ± 2</td>
<td>5,906 ± 214</td>
<td>857 ± 63</td>
</tr>
</tbody>
</table>

PBLs were incubated as described in Fig 6. PHA and anti-CD3 MoAb were 1 μg/mL. oATP (300 μmol/L) was either added at time 0 or 24, 48, or 72 hours after the addition of the stimuli and kept in the incubation medium throughout the experiment. IL-2 (20 U/mL), when present, was added at time 0. Cells were harvested after 96 hours. Data (cpm) are the means ± SD of quadruplicate determinations from a single experiment representative of three others.
The ATP, threshold for cation influx is about 100 μmol/L, similar to that reported for tumoral B lymphocytes and B-lymphoblastoid Duchenne cells, and about 2 log units higher than that reported for the nucleotide-gated channel described in rat PC12 cells. An additional feature in common with lymphoblastoid cells is the lack of significant inactivation during a prolonged exposure to ATP. Furthermore, BzATP is more potent than ATP, and oATP is a very good inhibitor. These characteristics suggest that the ATP, channel of human T cells belongs to the P2x subtype, although lack of permeabilization would rather be consistent with a P2y subtype. However, we feel that, until purinergic receptors expressed in different tissues will be cloned and fully biochemically characterized, attribution to a given subtype only on the basis of functional and pharmacologic criteria can be misleading. In fact, it cannot be excluded that the same receptor subtype (ie, a P2x receptor) may show different features in different tissues.

The role of purinergic receptors in lymphocytes has remained mostly elusive. Low ATP concentrations are reported to stimulate DNA synthesis in mouse lymphocytes, but there is no direct indication for a role of purinergic receptors in the mitogenic response of human lymphocyte cells. Our data suggest that ATP, receptors may be involved in the mitogenic response to different stimulants such as PHA, anti-CD3, and allogeneic cells. In fact, oATP, a covalent and early gene expression in B-human lymphocytes, but there is no direct indication for a role of purinergic receptors in the mitogenic response of human lymphocyte cells. Our data suggest that ATP, receptors may be involved in the mitogenic response to different stimulants such as PHA, anti-CD3, and allogeneic cells. In fact, oATP, a covalent and

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**Table 3. Reversibility of oATP-Induced Inhibition of Proliferation**

<table>
<thead>
<tr>
<th>Time 0</th>
<th>No Stimulus</th>
<th>PHA</th>
<th>Anti-CD3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no oATP</td>
<td>566 ± 36</td>
<td>32,195 ± 714</td>
</tr>
<tr>
<td>+ oATP</td>
<td>263 ± 12</td>
<td>16,584 ± 326</td>
<td>6,201 ± 511</td>
</tr>
<tr>
<td>48 h</td>
<td>no oATP</td>
<td>671 ± 14</td>
<td>21,122 ± 291</td>
</tr>
<tr>
<td>+ oATP</td>
<td>1,106 ± 189</td>
<td>17,077 ± 614</td>
<td>23,060 ± 871</td>
</tr>
<tr>
<td>72 h</td>
<td>no oATP</td>
<td>1,282 ± 78</td>
<td>36,861 ± 1,415</td>
</tr>
<tr>
<td>+ oATP</td>
<td>947 ± 36</td>
<td>38,791 ± 915</td>
<td>28,856 ± 2,004</td>
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</table>

Experimental conditions are as in Fig 6. PBLs were incubated in the presence of 300 μmol/L oATP for 2 hours, rinsed and then either immediately (time 0) stimulated with PHA or anti-CD3 or allowed to recover for 48 or 72 hours before the addition of the mitogenic stimuli. Control cells were incubated in the absence of oATP for 0, 48, or 72 hours before being stimulated with PHA or anti-CD3. Data (cpm) are the means ± SD of quadruplicate determinations from a single experiment representative of three others.
specific inhibitor of P2y/P2x receptors, powerfully inhibited DNA synthesis stimulated by these various agonists. Inhibition of DNA synthesis could be overcome by mitogens that bypass plasma membrane receptors, such as the Ca2+ ionophore ionomycin plus PMA, showing that oATP blocked a pathway associated to plasma membrane receptors and did not nonspecifically inhibit cell responses. Furthermore, the block could also be partially overcome by supplementing PBL cultures with IL-2.

oATP is a covalent reagent that irreversibly binds to and inactivates ATP, receptors. However, inhibition of ATP, receptor-linked responses would be expected not to be irreversible because newly synthesized receptors are expressed on the plasma membrane. This is in fact the case, as shown by the observation that T cells became again susceptible to mitogenic stimulation 2 to 3 days after oATP wash-out. Full reversibility of mitogenic block, together with lack of biochemical and morphologic signs of cell damage, clearly shows that oATP had no untoward effects on T-cell viability.

It can be argued that oATP, being a dialdehyde reagent, might form Schiff bases with unprotonated lysine residues present on other membrane proteins unrelated to ATP, receptors. However, we do not think that this is the case under our experimental conditions. Previous studies in human monocyte/macrophage cells have shown that oATP does not alter chemotactic response and membrane adhesion molecule expression in these cells.23 Furthermore, oATP did not block [Ca2+]i mobilization triggered by anti-CD3 MoAbs or the associated tyrosine phosphorylation. Finally, the inhibitory effect of oATP is prevented by coincubation in the presence of excess ATP, (F. Di Virgilio, unpublished observation), a clear indication that it is due to specific interaction with an ATP, binding site.

Requirement for a functional ATP, receptor during mitogenic stimulation suggests that this receptor might be involved at some postreceptor step crucial for lymphocyte activation. Mitogenic or comitogenic effects of ATP, were described in several cell models28,36 as well as lymphocytes.12 Although these effects are commonly believed to be mediated by P2y type receptors, a mitogenic role for ATP, gated plasma membrane channels has been recently suggested in cultured vascular smooth muscle cells.24 The mechanism whereby the ATP, channel exerts its comitogenic effect deserves further investigation. This nucleotide triggers both Na+ and Ca2+ fluxes, thus it cannot be excluded that the increase in the intracellular Na+ concentration might be an important permissive factor for volume changes that are well known to occur in mitogenically stimulated lymphocytes.32

These observations stress the very complex role of purinergic receptors in the regulation of lymphocyte responses. We and others have shown that prolonged exposures to ATP, are cytotoxic to mouse T and B lymphocytes, Duchenne human B-lymphoblastoid cells, and mouse and human macrophages.6,11,14,26,33,34 Very recently, the potential cytotoxic role of ATP, receptors has been reinforced by the molecular cloning of two P2x subtypes that showed striking homologies with membrane receptors known to be involved in apoptotic cell death.35,36

This contrasting dual role of ATP, receptors is not surpris-
22. Grierson J, Meldolesi J: shear-stress-induced [Ca2+] transients and oscillations in mouse fibroblasts are mediated by endoge
An ATP-activated channel is involved in mitogenic stimulation of human T lymphocytes

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