Expression of P2X<sub>7</sub> purinoceptors on human lymphocytes and monocytes: evidence for nonfunctional P2X<sub>7</sub> receptors

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Gu, B. J., W. Y. Zhang, L. J. Bendall, I. P. Chessell, G. N. Buell, and J. S. Wiley. Expression of P2X<sub>7</sub> purinoceptors on human lymphocytes and monocytes: evidence for nonfunctional P2X<sub>7</sub> receptors. Am J Physiol Cell Physiol 279:C1189–C1197, 2000.—Lymphocytes from normal subjects and patients with B-chronic lymphocytic leukemia (B-CLL) show functional responses to extracellular ATP characteristic of the P2X<sub>7</sub> receptor (previously termed P2Z). These responses include opening of a cation-selective channel/pore that allows entry of the fluorescent dye ethidium and activation of a membrane metalloprotease that sheds the adhesion molecule L-selectin. The surface expression of P2X<sub>7</sub> receptors was measured in normal leucocytes, platelets, and B-CLL lymphocytes and correlated with their functional responses. Monocytes showed four- to fivefold greater expression of P2X<sub>7</sub> than B, T, and NK lymphocytes, whereas P2X<sub>7</sub> expression on neutrophils and platelets was weak. All cell types demonstrated abundant intracellular expression of this receptor. All 12 subjects with B-CLL expressed lymphocyte P2X<sub>7</sub> at about the same level as B lymphocytes from normal subjects. P2X<sub>7</sub> function, measured by ATP-induced uptake of ethidium, correlated closely with surface expression of this receptor in normal and B-CLL lymphocytes and monocytes (n = 47, r = 0.70; P < 0.0001). However, in three patients the ATP-induced uptake of ethidium into the malignant B lymphocytes was low or absent. The lack of P2X<sub>7</sub> function in these B-CLL lymphocytes was confirmed by the failure of ATP to induce Ba<sup>2+</sup> uptake into their lymphocytes. This lack of function of the P2X<sub>7</sub> receptor resulted in a failure of ATP-induced shedding of L-selectin, an adhesion molecule that directs the recirculation of lymphocytes from blood into the lymph node.

extracellular adenosine 5′-triphosphate; adenosine 5′-triphosphate-induced ethidium uptake; lymphocyte P2X<sub>7</sub>; B cell chronic lymphocytic leukemia

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Activation of the P2X<sub>7</sub> receptor in B lymphocytes causes immediate opening of a cation-selective channel and also leads to shedding of the adhesion molecules L-selectin and CD23 via activation of a membrane metalloprotease(s) (23). Moreover, ATP also stimulates the activity of both Ca<sup>2+</sup>-dependent and -independent phospholipase D, and all these effects occur within minutes of ATP exposure (12, 18). In contrast, ATP has been shown to induce apoptotic death or cytolysis of human macrophages or murine thymocytes only after a delay of several hours (1, 35, 51). The cytolytic effect of even brief P2X<sub>7</sub>-receptor activation has been proposed to result from sustained activity of intracellular phospholipase D, the action of which generates a delayed permeability lesion in the cell membrane (13). These delayed effects show a time frame in keeping with ATP-induced activation of the transcription factor nuclear factor-κB (15), and there is strong evidence that activation of P2X<sub>7</sub> also stimulates the activity of intracellular caspases before ATP-induced apoptosis (14). This stimulation of caspase activity may also be responsible for ATP-induced processing of pro-interleukin (IL)-1β to the cytokine IL-1β in activated macrophages (34).

This study of the human P2X<sub>7</sub> receptor has taken advantage of the recent development of a monoclonal antibody that is both species and subtype specific and is directed against an extracellular domain on the molecule (2). This latter property makes it suitable for flow cytometric analysis of P2X<sub>7</sub>-expression in leukocyte subpopulations, and P2X<sub>7</sub> function also can be measured from ethidium uptake by flow cytometry. The results indicate that purinoceptors of the P2X<sub>7</sub> class are strongly expressed on both normal and leukemic lymphocytes but that, in some patients, this receptor is functionally inactive.

MATERIALS AND METHODS

Materials. ATP, 3'-O-(4 benzoylbenzoyl)-ATP (BzATP), ethidium bromide, barium chloride, d-glucose, bovine serum albumin (BSA), RPMI 1640 medium, and the FluoroTag FITC conjugation kit were purchased from Sigma Chemical (St. Louis, MO). Fura 2-acetoxymethyl ester and the Alexa 488 protein labeling kits were obtained from Molecular Probes (Eugene, OR). AffiGel 10 was from Bio-Rad (Hercules, CA). Ficoll-Hypaque (density 1.077) was obtained from Amersham Pharmacia (Uppsala, Sweden). Fluorescein isothiocyanate (FITC)- and R-phycocerythrin (RPE)-conjugated negative control antibodies, mouse anti-human CD41, CD3, CD14, CD16 antibodies, and RPE-Cy5-conjugated mouse anti-human CD19 antibody were from Dako (Carpinteria, CA). The FITC-conjugated mouse anti-human monoclonal antibody to L-selectin (CD62L) was the DREG.55 clone (Bender MedSystems, Vienna, Austria).

Preparation of leukocytes and platelets. Peripheral blood from 9 normal subjects and 12 B-CLL patients was collected and diluted with an equal volume of RPMI 1640 medium. Mononuclear cells were separated by density gradient centrifugation over Ficoll-Hypaque, washed once in RPMI 1640 medium, and then resuspended in HEPES-buffered NaCl medium (145 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 0.1 mM EDTA, pH 7.0) for 10 min at 4°C. The neutrophils were washed twice and resuspended in HEPES-buffered NaCl medium. Platelet-rich plasma (PRP) was obtained from normal subjects by low-speed centrifugation (150 g for 5 min) of whole blood. PRP was then centrifuged again at 200 g for 10 min to remove small contaminating leukocytes. Immunofluorescent staining showed that 99.9% of cells were strongly CD41 positive.

Antibody production and preparation. Mouse anti-human P2X<sub>7</sub>-receptor monoclonal antibody was prepared from hybridoma L4 supernate by chromatography on protein A-Sepharose Fast Flow as described previously (2). Either FITC or Alexa 488 antibody labeling kits were used to conjugate the P2X<sub>7</sub>-antibody according to the manufacturer's instructions. The conjugated antibody had 1.2 FITC per IgG and was stored at 0.64 mg/ml at 4°C. Anti-P2X<sub>7</sub>-antibody showed no binding to the surface or cytoplasm of HEK-293 cells, a cell line that does not express this receptor in subconfluent conditions.

Immunofluorescent staining for P2X<sub>7</sub>-receptor. Suspensions of leukocytes (50 μl of 1.0 × 10<sup>7</sup> cells/ml in HEPES-buffered NaCl medium) or PRP (100 μl of 1–2 × 10<sup>9</sup> cells/ml) were incubated with fluorescence-conjugated anti-P2X<sub>7</sub>-antibody (60 μg/ml) at room temperature for 20 min and washed once with PBS (0.15 M NaCl and 0.01 M phosphate, pH 7.2). RPE-labeled anti-CD41, anti-CD14, anti-CD16, or anti-CD3 together with RPE-Cy5-labeled anti-CD19 monoclonal antibodies were used as the second or third color. Labeled cells were then resuspended in 1 ml of PBS and analyzed on a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA). The isotype control was a murine FITC-conjugated IgG2b. For intracellular staining, cells were first labeled with RPE-conjugated anti CD41, CD14, CD16, or CD3 plus CD19 and then fixed and permeabilized with the use of a Fix & Perm kit (Cal Tag, Jarfalla, Sweden) according to the manufacturer's instructions before they were labeled with the FITC-conjugated anti-P2X<sub>7</sub>-antibody.

ATP-induced shedding of L-selectin. Aliquots of lymphocytes (1.0 × 10<sup>6</sup> cells/ml) were incubated for up to 30 min in 1 ml of HEPES-buffered KCl medium (10 mM HEPES, 150 mM KCl, 5 mM d-glucose, and 0.1% BSA, pH 7.5) at 37°C with 0.1 mM BzATP or 1.0 mM ATP. The incubation was stopped by the addition of an equal volume of cold isotonic nonphysiologic lysis in lysing buffer (0.7% NH<sub>4</sub>Cl, 5 mM KHCO<sub>3</sub>, and 0.1 mM EDTA, pH 7.0) at 37°C. Cytometric analysis of whole blood. PRP was then centrifuged again at 150 g for 10 min at 4°C. The neutrophils were washed twice and resuspended in HEPES-buffered NaCl medium. Platelet-rich plasma (PRP) was obtained from normal subjects by low-speed centrifugation (150 g for 5 min) of whole blood. PRP was then centrifuged again at 200 g for 10 min to remove small contaminating leukocytes. Immunofluorescent staining showed that 99.9% of cells were strongly CD41 positive.

Ethidium cation influx measurement by time-resolved flow cytometry. Mononuclear cells (2 × 10<sup>6</sup>) prelabeled with fluorophore-conjugated anti-CD3, anti-CD4, anti-CD16, or anti-CD19 were washed once and resuspended in 1.0 ml of HEPES-buffered KCl medium at 37°C. Cells were gated by forward and side scatter and by cell type-specific antibodies. Ethidium (25 μM) was added, followed 20 s later by the addition of 1.0 mM ATP. Mononuclear cells were analyzed at 1,000 events/s by flow cytometry, and the linear mean channel fluorescence intensity for each gated subpopulation over successive 5-s intervals was analyzed with the use of WinMDI software (Joseph Trotter, version 2.7) and plotted against time (48).
Cytosolic Ba\textsuperscript{2+} measurements by fluorometry. Lymphocytes (1 × 10\textsuperscript{7} cells/ml) were washed once and loaded with 2 μM fura 2-acetoxyethyl ester by incubation at 37°C for 30 min in Ca\textsuperscript{2+}-free HEPES-buffered NaCl medium. Cells were washed once and left in HEPES-buffered NaCl with 1 mM Ca\textsuperscript{2+} for another 30 min. Lymphocytes were then washed twice and resuspended in 3 ml of HEPES-buffered KCl medium at a concentration of 2 × 10\textsuperscript{6} cells/ml. These samples were stirred at 37°C and stimulated with 1 mM ATP after the addition of 1.0 mM BaCl\textsubscript{2}. Entry of Ba\textsuperscript{2+} into cells loaded with fura 2 produces changes almost identical to those produced by Ca\textsuperscript{2+} in the excitation and emission spectra of fura 2 (37, 50). Fluorescence signals were recorded by a Johnson Foundation fluorometer with excitation at 340 nm and emission at 500 nm. Calibration of maximal and minimal fluorescence intensities was performed after each run by the addition of 25 μM digitonin followed by 50 mM EGTA. Control experiments showed that addition of ATP did not release Ca\textsuperscript{2+} from the internal stores of lymphocytes suspended in medium containing EGTA.

RT-PCR analysis for P2X\textsubscript{7}. Total RNA was isolated from cells using an RNA isolation reagent (Advanced Biotechnologies, Epsom, UK) according to the manufacturer’s recommendations. For complementary DNA (cDNA) synthesis, 1 μg of total RNA was used in the RT reaction with 0.5 μg of oligo(dT) primer, heated for 10 min at 70°C, and then 1× PCR buffer, 2.5 mM MgCl\textsubscript{2}, 0.5 mM dNTP (dATP, dGTP, dCTP, and dTTP), 10 pmol each of P2X\textsubscript{7} primer and 0.75 U Taq DNA polymerase (Promega), and 100 ng of cDNA sample. The PCR was carried out with a thermocycling program as follows: initial denaturation at 95°C for 5 min and then 35 cycles of 95°C for 45 s, 52°C for 45 s, and 72°C for 1 min; the final extension step was performed at 72°C for 10 min. Detection of PCR products was performed by electrophoresis in a 2% agarose gel and ethidium bromide staining. The size of the PCR fragments was determined with a 100-bp DNA molecular weight ladder and the nature of the products was confirmed by DNA sequencing.

RESULTS

Surface expression of P2X\textsubscript{7} on normal lymphocytes and monocytes. In Fig. 1, the surface expression of
P2X7 was measured by flow cytometry of normal white cells stained with directly conjugated P2X7 antibodies. Monocytes showed the highest expression of surface P2X7 receptors, which was four- to fivefold higher than P2X7 values for normal lymphocytes (Table 1, P < 0.001). Lymphocytes of B, T, and NK cell lineage showed lower and similar expression. Polymorphonuclear neutrophils showed weak expression for P2X7 when gated to exclude those cells that expressed CD41 as a result of platelet adhesion. Platelets were also weakly positive for P2X7, whereas erythrocytes (data not shown) were negative. As previously shown (2), the cell line HEK-293 was also negative for surface P2X7 expression.

Intracellular expression of P2X7 on leukocytes and platelets. Large amounts of P2X7 protein were found in an intracellular location in monocytes, lymphocytes of all subtypes, neutrophils, and platelets (Fig. 1B). Precise comparison of intracellular to cell surface P2X7 expression was not possible because of varying values of the isotype (IgG2b) control. However, Fig. 1 clearly shows that intracellular P2X7 expression is approximately an order of magnitude greater than expression on the cell surface. The cell line HEK-293 showed no intracellular staining with the P2X7 antibody (data not shown).

P2X7 functional responses in normal monocytes and lymphocyte subsets. ATP-induced uptake of ethidium into mononuclear preparations from six normal subjects was measured by flow cytometry (48). Monocytes and lymphocyte subsets were identified by using FITC- or RPE-Cy5-conjugated antibodies that allowed the relative ethidium uptakes to be compared among different cell types. Figure 2 shows uptake curves for monocytes, NK, B, and T lymphocytes from one normal subject and confirms that ethidium permeates via the P2X7 pathway given that uptake by lymphocytes was almost abolished in the presence of the P2X7 monoclonal antibody. When the area under the uptake curve was used as an estimate of P2X7 permeability for six normal subjects, there were no differences among these three lymphocyte subsets (Table 1). However, ethidium uptake through the P2X7 channel/pore was fivefold greater for monocytes than for B lymphocytes of normal origin (Table 1, P < 0.001). ATP-induced ethidium uptake into resting neutrophils and platelets was negligible (data not shown).

Surface and intracellular P2X7 in B-CLL lymphocytes. Our previous work showed that ATP-induced ethidium uptake is mediated by the P2X7 receptor in B-CLL lymphocytes (46). Fluorescence histograms of the P2X7 antibody binding shows that B-CLL lymphocytes express P2X7 on their surface at about the same level as observed for normal B lymphocytes (Fig. 3A and Table 1). Moreover, intracellular levels of P2X7 were far higher than those found on the cell surface (Fig. 3B) by approximately an order of magnitude. The expression of P2X7 receptors on monocytes from the B-CLL patients was fourfold greater than that on B-CLL lymphocytes (Table 1, P < 0.001).

Functional and nonfunctional P2X7 channels. As previously reported (46), lymphocytes from most patients with B-CLL showed strong ATP-induced uptake of both Ba2+ and ethidium cation (Table 1 and Fig. 4A). However, B lymphocytes from three patients failed to show these ATP-induced permeability responses. Representative data are shown in Fig. 4A from subjects

Table 1. Expression and function of P2X7 receptor in mononuclear cells from normal and B-CLL subjects

<table>
<thead>
<tr>
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<th>Relative P2X7 Expression, mean channels of fluorescence intensity</th>
<th>Relative ATP-Induced Ethidium Uptake, arbitrary units of area</th>
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<td></td>
<td>n</td>
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<tr>
<td>Normal Subjects</td>
<td></td>
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<tr>
<td>Monocytes</td>
<td>58.5 ± 16.2</td>
<td>8</td>
</tr>
<tr>
<td>B lymphocytes</td>
<td>13.1 ± 2.4</td>
<td>9</td>
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<tr>
<td>T lymphocytes</td>
<td>5.6 ± 1.6</td>
<td>9</td>
</tr>
<tr>
<td>NK cells</td>
<td>9.7 ± 2.7</td>
<td>8</td>
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<tr>
<td>B-CLL Subjects (With Functional P2X7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocytes</td>
<td>69.9 ± 15.0</td>
<td>9</td>
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<tr>
<td>B lymphocytes</td>
<td>21.7 ± 4.3</td>
<td>9</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>9.3 ± 1.7</td>
<td>9</td>
</tr>
<tr>
<td>NK cells</td>
<td>19.6 ± 2.7</td>
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Values are means ± SE; n is no. of subjects, each studied once. Mononuclear cell preparations (1 × 10^6 cells/ml) from normal and B-chronic lymphocytic leukemia (B-CLL) subjects were incubated for 20 min at 20°C with fluorescein-conjugated P2X7 antibody plus R-phycocerythrin-labeled surface marker antibody in HEPES-buffered isotonic KCl medium at 37°C. Ethidium was added, and lymphocyte subsets were identified by using FITC- or RPE-Cy5-conjugated antibodies that allowed the relative ethidium uptakes to be compared among different cell types. Figure 2 shows uptake curves for monocytes, NK, B, and T lymphocytes from one normal subject and confirms that ethidium permeates via the P2X7 pathway given that uptake by lymphocytes was almost abolished in the presence of the P2X7 monoclonal antibody. When the area under the uptake curve was used as an estimate of P2X7 permeability for six normal subjects, there were no differences among these three lymphocyte subsets (Table 1). However, ethidium uptake through the P2X7 channel/pore was fivefold greater for monocytes than for B lymphocytes of normal origin (Table 1, P < 0.001). ATP-induced ethidium uptake into resting neutrophils and platelets was negligible (data not shown).
CLL 1 and CLL 2, whose lymphocytes showed strong ATP-induced ethidium uptake, but this response was absent in B-CLL lymphocytes from subjects CLL 3 and CLL 4. Similarly, lymphocytes from subjects CLL 1 and CLL 2 showed strong ATP-induced uptake of Ba2+, whereas lymphocytes from subjects CLL 3 and CLL 4 showed only minimal ATP-induced uptake of Ba2+ in K+ medium (Fig. 4B). The function of the P2X7 receptor was also studied in monocytes from the B-CLL subjects, because most studies of P2X7 function have employed cells of monocytic origin. Figure 4C shows that ATP-induced ethidium uptake into monocytes from subjects CLL 3 and CLL 4 was below the range for monocytes from either normal subjects or subjects CLL 1 or CLL 2. Recent data have suggested that removal of extracellular Cl− as well as extracellular Na+ enhances permeability responses and stimulates the function of the P2X7 receptor (30). Thus ethidium uptake into B lymphocytes was measured in a buffered sucrose medium with and without addition of BzATP, the complete and most potent agonist for the P2X7 receptor (19). BzATP-induced ethidium cation influx was greater in this Na+-free, low-Cl− medium than in KCl medium used in Fig. 4A (data not shown). However, Fig. 4D shows that the difference between functional (CLL-2) and nonfunctional (CLL-4) responses was still evident in the sucrose medium.

**Correlation of surface P2X7 expression and ATP-induced ethidium uptake.** The expression of P2X7 receptors on the surface of normal leucocyte subsets and on B lymphocytes and monocytes from P2X7 functional B-CLL patients was compared with the ATP-induced uptake of ethidium (Table 1). A close correlation was found between receptor expression and function judged by ethidium uptake (n = 47, r = 0.70; P < 0.0001).

**Molecular analyses of P2X7 transcripts.** It has been previously reported that truncation of the long COOH-terminal tail of the P2X7 receptor abolishes ATP-induced uptake of large fluorescent dyes such as YoPro2+ or ethidium cation (42). The presence of mRNA transcripts that included the COOH-terminal tail of P2X7 was examined in B-CLL lymphocytes that were sorted to high purity to achieve >99.8% cell purity. Lymphocytes from six patients (subjects CLL 1–CLL 5 and subject CLL 8) showed an RT-PCR product of identical size using primers specific for the long COOH-terminal tail. The PCR products migrated at the 495-bp predicted size for the P2X7 product (Fig. 5). mRNA transcripts for the P2X7 tail were uniformly present on B-CLL lymphocytes in all six patients examined, and there were no gross differences among patients with functional (subjects CLL 1, CLL 2, CLL 5, and CLL 8) and nonfunctional P2X7 receptors (subjects CLL 3 and CLL 4) (Fig. 5).

**Failure of L-selectin shedding with nonfunctional P2X7.** Our previous results (25) showed that both ATP and BzATP induce shedding of L-selectin via activation of P2Z/P2X7 receptors. Figure 6 shows that BzATP, a more potent agonist for the P2X7 receptor, caused rapid and complete shedding of L-selectin within several minutes from the surface of B-CLL lymphocytes with functionally active P2X7 receptors. In contrast, BzATP produced very little shedding of L-selectin from lymphocytes with functionally inactive P2X7 receptors. Similar results were obtained when ATP was used as agonist for the P2X7 receptor.

**DISCUSSION**

Much of the characterization of the P2X7 receptor (previously termed P2Z) has been in cells of hemopo-
etic origin that express this receptor in its native state. Both these studies and those in HEK-293 cells heterologously expressing the cDNA for P2X7 have revealed features that are most unusual for a channel. These include the slow kinetics of channel dilatation and the triggering of downstream events such as activation of membrane metalloprotease(s) and intracellular caspase activation leading to apoptosis. Recently, a

Fig. 4. A: ATP-induced ethidium uptake into B-CLL lymphocytes from 4 patients with B-CLL. Ethidium was added, followed 20 s later by the addition of 1.0 mM ATP (arrow). Shaded area indicates the range of ethidium uptake observed for B lymphocyte subpopulations from normal subjects. Mean channel of cell-associated fluorescence intensity was measured for each B cell gated population at 5-s intervals. B: Ba^{2+} influx into normal and B-CLL lymphocytes. Lymphocytes (6 × 10^6) loaded with 2 μM fura-2-AM were resuspended in 3 ml of HEPES-buffered KCl medium. Ba^{2+} (1.0 mM) was added 40 s before stimulation with 1.0 mM ATP (as indicated by arrows). Addition of the P2X_7 monoclonal antibody (P2X_7-MAb) greatly reduced the Ba^{2+} influx into the normal lymphocytes. C: ATP-induced ethidium uptake in monocytes from the same 4 patients shown in Fig. 3. Cells (2 × 10^6 cells/ml) were first marked with FITC-conjugated anti-CD14 MAb. Cells were suspended in HEPES-buffered isotonic KCl medium with no added Ca^{2+}. Ethidium (25 μM) was added 1 min before 1.0 mM ATP (arrow). Cells were gated on monocyte population by forward and side scatter, and only those positive for CD14 cells were selected for analysis. Ethidium uptake was measured at 5-s intervals. Shaded area represents the range for normal monocytes. D: 3'-O-(4 benzoylbenzoyl)-ATP (BzATP)-induced ethidium uptake into B-lymphocytes from patients CLL 2 (functional P2X_7) and CLL 4 (nonfunctional P2X_7) in Na^+ -free, low-Cl^- medium. Cells were first stained with FITC-conjugated anti-CD19 MAb and then washed and resuspended in isotonic sucrose medium (280 mM sucrose, 5 mM KCl, and 20 mM HEPES, pH 7.4) at 37°C. Ethidium was added, followed 40 s later by 100 μM BzATP (arrow). Mean channel of cell-associated fluorescence intensity was measured for gated CD19^+ lymphocyte populations at 5-s intervals.
monoclonal antibody against an extracellular epitope of the P2X7 receptor was developed (2) that was used in this study for flow cytometric analysis of receptor expression on the various cells in peripheral blood. P2X7 receptor expression was approximately four- to fivefold greater on monocytes than on normal or leukemic B lymphocytes, whereas there were no significant differences among the three main lymphocyte subsets of T, B, and NK cells. Little or no surface expression of P2X7 receptor was found on either polymorphonuclear neutrophils or blood platelets. The same rank order of cell surface P2X7 receptor function was found by measuring initial rates over 5 min of ATP-induced uptake of ethidium into these various cell types within the one mononuclear cell population. This functional assay made use of two-color flow cytometry in which monocytes and NK, B, and T lymphocytes were identified by FITC- or RPE-Cy5-labeled antibodies against CD14, CD16, CD19, and CD3, respectively, whereas ethidium uptake was measured on the red (570 nm) channel. A close correlation was found between expression of P2X7 receptors and the ATP-induced ethidium uptake into the various leucocyte types, suggesting that all P2X7 receptors expressed on the surface of normal lymphocytes and monocytes are functionally competent. Previous studies have suggested that P2X7 is not expressed in freshly isolated monocytes and that receptor expression requires differentiation of these cells to macrophages under the influence of interferon-γ or lipopolysaccharide (2, 24). However, our data for both expression (Fig. 1) and function (Fig. 2) clearly show functional P2X7 receptors on the surface of all fresh circulating blood monocytes. The large size and surface area of the monocyte (ca. 500 fl) may explain in part their four- to fivefold greater number of P2X7 receptors than are found on the smaller lymphocytes (180–210 fl). The greater surface area of monocytes is also shown by their expression of threefold more membrane sodium pumps than lymphocytes as measured by the binding of the cardiac glycoside [3H]ouabain (49). Nevertheless, monocytes have the capacity to greatly up-regulate their P2X7 function on differentiation to macrophages under the influence of interferon-γ (1, 24).

The high expression of intracellular P2X7 protein is of interest and appears to be a ubiquitous finding in cells of hemopoietic origin, although erythrocytes were not tested for technical reasons. The finding that most of the P2X7 receptor is in an intracellular location may have parallels to other receptors such as those for insulin and for the chemokine receptor CXCR4. Both these receptors undergo endosomal internalization following receptor occupancy by their respective agonists (9, 26, 38). Recently, it has been demonstrated that the P2Y2 nucleotide receptor can also undergo internalization following binding of its ligand, UTP (20, 39), although it is uncertain whether this receptor then undergoes recycling back to the cell surface as shown for the insulin receptor of adipose cells. Likewise, a rapid internalization of the P2X7 receptor has been demonstrated by confocal microscopy on exposure of smooth muscle cells to ATP (11). The intracellular pool of P2X7 receptors such as on neutrophils or platelets (Fig. 1) is atypical because there is little cell surface expression on these cell types. Perhaps this intracellular receptor pool forms a reserve that is able to be recruited to the surface following cellular activation.

A main finding is that lymphocytes from three B-CLL subjects showed strong P2X7 immunoreactivity on the lymphocyte surface (Fig. 3) but no ATP-induced ethidium uptake and very poor ATP-induced Ba2+ uptake (Fig. 4). There is little information on the factors that modulate the surface expression and/or function of the P2X7 channel. Sodium media are known to partially inhibit the function of P2X7 (47). However, the assays shown in Fig. 4, A and B, were performed in K+-rather than Na+-based media to avoid any inhibitory effect of Na+. Chloride ions also partially inhibit the function of P2X7 (30), but even a Na+-free, low-Cl− medium with BzATP as agonist did not correct the poorly functional P2X7. Hormones may also influence the expression and/or function of P2X7, because the cytokine interferon-γ has been shown to upregulate P2X7 receptor function during maturation of monocyte to macrophages (1, 24). A recent study of B-CLL has

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**Fig. 5.** Agarose gel electrophoresis (2% agarose) of RT-PCR products using human P2X7-specific primers. cDNA was from fluorescence-activated cell sorter (FACS)-purified B-CLL lymphocytes prepared from 6 different patients. A single 495-bp product is shown at the predicted position for P2X7.

**Fig. 6.** Time course of BzATP-induced loss of L-selectin. B-CLL lymphocytes with functionally active (●) and inactive (○) P2X7 receptors were incubated with 100 μM BzATP for the indicated times before being labeled with anti-L-selectin antibody. Results are means ± SE and are expressed as the percentage of initial expression (n = 5 for functional P2X7 and n = 3 for nonfunctional P2X7 patients).
shown elevated values for tumor necrosis factor (TNF)-α and IL-8 in the serum of these patients (16, 17). However, the effect of neither TNF-α nor IL-8 on P2X7 expression on lymphocytes is known. Incubation up to 48 h of lymphocytes from patients with nonfunctional P2X7 in buffered medium containing 80% plasma from patients with functional P2X7 failed to produce a P2X7 response. Also, P2X7 functional responses were not inhibited by prolonged incubation of responding B-CLL cells in plasma from patients with nonfunctional P2X7 (unpublished observations). Buell and colleagues (3) showed that ambient ATP released during incubation of HL-60 cells produces a sustained desensitization of P2X1 receptors but that apyrase added to the medium can resensitize this receptor. However, addition of apyrase (5 U/ml) during incubation of B-CLL lymphocytes failed to reactivate the functionally inactive P2X7 in these cells (unpublished observations). Although we have been unable to find evidence for a soluble inhibitory factor responsible for the decrease in P2X7 function, it is intriguing that monocytes from these same patients also showed an impaired P2X7 function (Fig. 4C). This finding strongly suggests that cell-cell contact or some short-range mediator impairs P2X7 function following the many cellular interactions that occur during the traffic of these cells through the lymph node. An alternate possibility is that genetic changes may underlie the impaired P2X7 receptor. Both point mutations and nonrandom chromosomal changes are associated with B-CLL involving trisomy of chromosome 12 in 20% of cases (8, 40). Genomic instability of chromosome 12 is common in B-CLL (27, 29) and involves the regions (12q 21–23) adjoining the location of P2X7 on 12q 24 (4). Although our RT-PCR analyses (Fig. 5) exclude a major genetic deletion causing truncation of the long COOH-terminal tail of the P2X7 receptor, it is still possible that point mutations or deletions in other parts of the receptor may underlie the impaired P2X7 function in certain patients.

Lymphocytes in the body undergo continuous recirculation between the blood and tissues (21), and the first step of lymphocyte emigration to the lymph node is its adhesion to vascular endothelial cells. The initial event in this adhesion involves the interaction of L-selectin with counterreceptors on the surface of endothelial cells followed by transendothelial migration. The central importance of L-selectin in regulating leucocyte emigration has been shown in L-selectin “knock-out mice,” which show greatly reduced lymphocyte numbers in lymph nodes and an impaired ability of leucocytes to emigrate to sites of inflammation (44). It is known that extracellular ATP acting via the lymphocyte P2X7 receptor can activate a membrane metalloproteinase that cleaves L-selectin at a membrane-proximal site to release soluble L-selectin (23, 25). However, the lymphocytes from those B-CLL subjects who have functionally inactive P2X7 receptors failed to show L-selectin downregulation with extracellular ATP (Fig. 6). These subjects have high levels of L-selectin expression on their lymphocytes, which contrasts with the low levels of L-selectin that are generally found on the surface of B-CLL lymphocytes with functional P2X7 (6). This study has shown that the functional state of P2X7 receptors regulates the expression of the homing adhesion molecule L-selectin, whereas in contrast, the B cell chemotactant SDF (stromal cell-derived factor)-1α has little effect on this adhesion molecule (Gu BJ, Dao LP, and Wiley JS, unpublished observations). It is likely that the functional status of P2X7 receptors plays a central role in regulating L-selectin expression on lymphocytes and in directing their patterns of recirculation in the body.

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