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Abstract

B lymphocytes are known to synthesise the P2X(7) subtype of the P2X purinergic receptor family; however, the identification of the other six P2X subtypes on these cells has been limited by the absence of specific antibodies. In this study, we used a panel of anti-P2X polyclonal antibodies and confocal microscopy to examine the presence of each P2X receptor on human B lymphocytes. We observed that P2X(1), P2X(2), P2X(4) and P2X(7) subtypes, but not P2X(3), P2X(5) and P2X(6) subtypes, are present on B lymphocytes.

Keywords

purinergic, b, lymphocytes, human, detection, receptors, p2x, CMMB

Disciplines

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Detection of P2X purinergic receptors on human B-lymphocytes

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Key words P2X purinoceptor, ATP-gated ion channel, B-lymphocyte, polyclonal antibody, confocal microscopy

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Abstract B-lymphocytes are known to synthesise the P2X₇ subtype of the P2X purinergic receptor family, however the identification of the other six P2X subtypes on these cells has been limited by the absence of specific antibodies. In this study, we used a panel of anti-P2X polyclonal antibodies and confocal microscopy to examine the presence of each P2X receptor on human B-lymphocytes. We observed that P2X₁, P2X₂, P2X₄ and P2X₇ subtypes, but not P2X₃, P2X₅ and P2X₆ subtypes, are present on B-lymphocytes.

Introduction

P2X-type purinergic receptors are ligand-gated cation channels which are activated by extracellular adenosine 5'-triphosphate (ATP). To date seven subtypes of P2X receptors, P2X₁ to P2X₇, have been cloned (Buell et al. 1996a). These receptors are well recognised to have a role in nervous and muscle tissues where they function in cell-cell communication (Ralevic and Burnstock 1998), and although they have been identified on cells of haematopoietic origin their function in these cells is less clearly defined. P2X₇ receptor immunoreactivity on human lymphocytes and monocytes has been demonstrated by binding of a monoclonal antibody against this receptor (Gu et al. 2000). The P2X₇ receptor has an established role in the apoptosis of haematopoietic cells such as macrophages and dendritic cells (Lammas et al. 1997; Coutinho-Silva et al. 1999). Both the distribution and function of P2X₁ receptors on haematopoietic cells are less well defined, although there is some evidence that this receptor is required for human blood platelets to function normally (Oury et al. 2000). Moreover the presence of other P2X receptors on lymphocytes is not known. In this study, confocal microscopy was used to identify which P2X subtypes are present on human B-lymphocytes from patients with chronic lymphocytic leukemia (CLL) using a panel of polyclonal antibodies raised against extracellular epitopes of the P2X₁ to P2X₇ receptors.

Materials and methods

Antibodies

Rabbit polyclonal antibodies produced against non-homologous extracellular epitopes of P2X receptors have been previously described (Dutton et al. 1999; Worthington et al. 1999b; Yunaev et al. 2000). Each epitope was selected in part to ensure no cross-reactivity with any other P2X subtype (Dutton et al. 1999). Each P2X polyclonal antibody binds selectively and specifically as shown by Western blots in a range of tissue although not all subtypes are present in all tissues (Worthington et al. 1999a; Worthington et al. 1999b; Li et al. 2000). In addition, specificity was confirmed using specific P2X cRNA transfected into *Xenopus* oocytes (Dutton et al. 1999). Specific P2X polyclonal antibody was added to transfected cells in the presence or absence of specific P2X epitope or non-specific epitope to demonstrate only the specific antibody reacted with the specific epitope (Dutton et al. 1999). Moreover, specificity was also evident in that only sub-populations of cells were labelled.

Murine anti-human P2X₇ receptor monoclonal antibody (clone L4) (Buell et al. 1998) was purified from hybridoma supernatant using a Protein A-Sepharose Fast Flow Column (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Murine anti-human CD19 (clone HD37) and isotype control antibodies were purchased from Dako A/S (Glostrup, Denmark). Cy2- or Cy5-conjugated donkey

anti-rabbit antibodies and Cy2-conjugated donkey anti-murine antibody were obtained from Jackson ImmunoResearch (West Grove, PE).

Preparation of mononuclear cells

Heparinised venous blood from 8 B-CLL subjects was diluted with an equal volume of RPMI-1640 (Sigma, St Louis, MO) and the mononuclear cells separated by density-gradient centrifugation over Ficoll-Paque (Pharmacia Biotech). The mononuclear cells were washed twice with RPMI-1640. The mononuclear cells were resuspended at 1×10^6 cells/mL in RPMI-1640 containing 10% heat inactivated foetal calf serum (Life Technologies, Grand Island, NY) and 25 μ g/mL gentamycin (Delta West, Perth, Australia). Cells (5×10^5 cells/well) were incubated for 60 min in wells of 48-well plates (Falcon, Becton Dickinson, Franklin Lakes, NJ) containing poly-L-lysine-coated (0.1 mg/ml; Sigma) glass coverslips. Adherent cells were fixed with cold 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) (pH 7.2) for 4 min and washed twice with PBS.

Immunofluorescence staining and confocal microscopy

Fixed cells on poly-L-lysine-coated glass coverslips in 48-well plates were incubated with 20% normal horse serum in PBS for 60 min, then incubated with primary antibody for 60 min, washed once with PBS and finally labelled with secondary antibody for 60

min. Cells were then washed twice with PBS before mounting the coverslips on slides in 50% glycerol in PBS. The identity of B-CLL cells was confirmed by double labelling with anti-CD19 antibody. Cells were visualised with a Leica TCS NT UV laser confocal microscope system with the pinhole set at 1.0. Preimmune sera and murine isotype control antibodies were used routinely as negative controls and showed no staining.

Results

Human B-lymphocytes, identified by anti-CD19 monoclonal antibody binding (Fig. 1A), were stained with an anti-P2X₇ receptor polyclonal antibody (Fig. 1B). Two-colour confocal microscopy demonstrated that B- lymphocytes stained positively for the P2X₇ receptor with staining intensity varying from weak to strong. There was clear evidence of puncta formation of P2X₇ receptors on the cell surface (Fig. 1C,D). Heavily stained cells were characterised by overlapping puncta (results not shown). A similar distribution of P2X₇ receptors on the cell surface was observed with the anti-P2X₇ receptor monoclonal antibody (Fig. 1E).

The anti-P2X₁ receptor polyclonal antibody labelled approximately half the B-lymphocyte population with weak to moderate staining intensity (Fig 2A,B,C), except in one subject (CLL 1) in whom the staining was weak and confined to only a small number of cells (results not shown). P2X₁ receptor immunoreactivity was characterised by puncta formation on the cell surface similar to that observed for the P2X₇ receptor (Fig. 2C,D). However at lower levels of P2X₁ receptor staining the distribution was more diffuse, while at high levels the puncta began to merge (results not shown). B-lymphocytes from all subjects examined had moderate levels of P2X₂ receptors but this was restricted to less than half the population of cells. Puncta of P2X₂ receptors were also observed on the surface of B-lymphocytes (Fig. 2E). P2X₄ receptor staining was the most variable of the different P2X subtypes. Cells positive for the P2X₄ receptor had weak to moderate levels of staining in a large proportion on B-lymphocytes from three

subjects (CLL 1, 5 and 6) (Fig. 2F) but weaker levels and only in a small minority of cells from others (CLL 2, 3 and 4) (results not shown). The P2X₃, P2X₅ and P2X₆ receptors were absent from human B-lymphocytes, although occasional examples of cells with negligible to moderate staining showing either a diffuse distribution or puncta formation were observed (results not shown). Thus P2X₁, P2X₂, P2X₄ and P2X₇ receptors were present on human B-lymphocytes (Table 1).

Discussion

In this study, we used a panel of polyclonal antibodies raised against extracellular epitopes of the seven P2X subtypes to examine the presence of these receptors on B-lymphocytes. Subjects with CLL were studied because B-lymphocytes are the major mononuclear cell type present and are clonal in origin. The polyclonal antibodies against the P2X₁ to P2X₇ subtypes have been previously shown to bind to their respective P2X receptors in human embryonic kidney cells, human salivary gland epithelial cells and sympathetic ganglia (Worthington et al. 1999a; Barden and Bennett 2000; Li et al. 2000). In epithelial cells and ganglia there is evidence of P2X receptor puncta distributed over the cell surface, similar to that observed here in this study (Fig. 1D). The physiological significance of this punctate distribution is uncertain but it is striking that lymphoid, epithelial and nervous tissue are similar. We have previously shown P2X₇ receptor immunoreactivity on human B-lymphocytes using flow cytometry (Gu et al. 2000), and our results using confocal microscopy demonstrate the punctate distribution of these receptors on B-lymphocytes.

In this study we show for the first time that B-lymphocytes display P2X₁, P2X₂ and P2X₄ receptor immunoreactivity. The P2X₁ receptor was detected in a proportion of B-lymphocytes from all subjects examined (Fig. 2B,C,D). The presence of the P2X₁ receptor on leucocytes is controversial. P2X₁ receptor mRNA has been identified in peripheral blood leucocytes (Longhurst et al. 1996), and in cultured macrophages and dendritic cells (Berchtold et al. 1999). Moreover polyclonal antibodies have been used

to identify the P2X₁ receptor in the HL-60 myeloid leukaemic cell line (Buell et al. 1996b) and rodent thymocytes (Chvatchko et al. 1996; Glass et al. 2000). However these positive observations are in contrast with previous results in which the P2X₁ receptor was detected in mononuclear cell preparations from normal subjects by immunoblotting but its presence on leucocytes was attributed to contaminating platelets (Clifford et al. 1998).

Both the P2X₂ and P2X₄ receptors were observed on B-lymphocytes (Fig. 2E,F). P2X₂ receptor mRNA has been observed in murine thymocytes (Freedman et al. 1999) but was not detected in human mononuclear cells (Adrian et al. 2000). However, at the protein level, others using a polyclonal antibody have detected the P2X₄ receptor in rat thymocytes but found specific mRNA to be absent suggesting that P2X₄ receptor mRNA may be short-lived (Glass et al. 2000).

Previous studies (Bretschneider et al. 1995, Markwardt et al. 1997) using the voltage-clamp technique provided evidence that human B-lymphocytes have only non-desensitising ATP-activated cation channels, a feature characteristic of the P2X₇ receptor but not P2X₁ or P2X₄ receptors. The contrasting results between these functional studies and those observed in this immunocytochemical study may relate to differences in the B-lymphocytes used. Markwardt and coworkers used tonsillar and Epstein-Barr virus-transformed tonsillar B lymphocytes, while malignant B-lymphocytes were used here. Alternatively, the state of lymphocyte differentiation maybe a controlling determinant of P2X synthesis.

The P2X₃, P2X₅ and P2X₆ receptors were absent from B-lymphocytes, although we did find traces of these receptors in cells from some subjects. The absence of the P2X₃ receptor is consistent with the inability to find mRNA for this receptor in mononuclear cells from normal subjects (Adrian et al. 2000), however this same group reported that mononuclear cells contain high levels of P2X₅ receptor mRNA. Given the absence of the P2X₅ subtype in B-lymphocytes observed here, the results suggests that T cells, which are the predominant population in mononuclear cell preparations from normal subjects, may have accounted for the P2X₅ receptor transcript detected by Adrian et al. (2000). The detection of the P2X₆ receptor on human lymphocytes was negative in agreement with studies using an anti-P2X₆ polyclonal antibody which failed to detect this receptor in rat thymocytes (Glass et al. 2000).

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Table 1 Immunoreactivities for P2X receptor subtypes on B-lymphocytes

CLL	P2X ₁	P2X ₂	P2X ₃	P2X ₄	P2X ₅	P2X ₆	P2X ₇
1	±	+	-	+	-	-	+
2	+	+	-	±	-	-	+
3	+	+	-	±	-	-	+
4	+	+	-	±	-	-	+
5	+	+	-	+	-	-	+
6	+	nd	nd	+	nd	nd	+
7	nd	nd	nd	nd	nd	nd	+
8	nd	nd	nd	nd	nd	nd	+

-, absent; ±, present but on a small minority of cells; +, present; nd, not determined

Figure Legends

Fig. 1 Immunoreactivity for P2X₇ receptors on B-lymphocytes. Confocal images (from the same low power field of view) using two-colour immunofluorescence staining show anti-CD19 monoclonal antibody (**A**) and anti-P2X₇ receptor polyclonal antibody (**B**) labelling of B-lymphocytes. Note the presence of a cell (*arrow*) which labels for the P2X₇ receptor but not for CD19. Confocal images of B-lymphocytes labelled with the anti-P2X₇ receptor polyclonal (**C,D**) or monoclonal (**E**) antibody demonstrate the distribution of P2X₇ receptors. Sections of lymphocytes (**C,E**) show similar surface staining with either antibody. A superior view of a lymphocyte (**D**) demonstrates P2X₇ receptor puncta on the cell surface. Preimmune sera and murine isotype control antibody were included as negative controls and showed no staining (**F**). *Scale bars* 5 μm (**A,B,F**) and 1 μm (**C,D,E**).

Fig. 2 Immunoreactivities for P2X₁, P2X₂ and P2X₄ receptors on B-lymphocytes. Confocal images (from the same low power field of view) using two-colour immunofluorescence staining show anti-CD19 monoclonal antibody (**A**) and anti-P2X₁ receptor polyclonal antibody (**B**) labelling of B-lymphocytes. Note the presence of cells (*arrows*) which label for CD19 but not for the P2X₁ receptor. Section (**C**) or superior views (**D,E,F**) of B-lymphocytes labelled with the anti-P2X₁ (**C,D**), -P2X₂ (**E**) or -P2X₄ (**F**) receptor polyclonal antibody. *Scale bars* 5 μm (**A,B**), 2 μm (**C**) and 1 μm (**D,E,F**).