

2012

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Safina Gadeock
University of Wollongong, sg699@uow.edu.au

Aleta Pupovac
University of Wollongong, ap251@uow.edu.au

Vanessa Sluyter
University of Wollongong

Mari Spildrejorde
University of Wollongong, mgs840@uow.edu.au

Ronald Sluyter
University of Wollongong, rsluyter@uow.edu.au

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Recommended Citation

Gadeock, Safina; Pupovac, Aleta; Sluyter, Vanessa; Spildrejorde, Mari; and Sluyter, Ronald: P2X7 receptor activation mediates organic cation uptake into human myeloid leukaemic KG-1 cells 2012, 669-676.
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Abstract

The P2X7 purinergic receptor is an ATP-gated cation channel with an emerging role in neoplasia. In this study we demonstrate that the human KG-1 cell line, a model of acute myelogenous leukaemia, expresses functional P2X7. RT-PCR and immunochemical techniques demonstrated the presence of P2X7 mRNA and protein respectively in KG-1 cells, as well as in positive control multiple myeloma RPMI 8226 cells. Flow cytometric measurements demonstrated that ATP induced ethidium(+) uptake into KG-1 cells suspended in sucrose medium (EC(50) of ~ 3 μM), but not into cells in NaCl medium. In contrast, ATP induced ethidium(+) uptake into RPMI 8226 cells suspended in either sucrose or NaCl medium (EC(50) of ~ 3 or ~ 99 μM , respectively), as well as into RPMI 8226 cells in KCl medium (EC(50) of ~ 18 μM). BzATP and to a lesser extent ATP γ S and $\alpha\beta$ -methylene ATP, but not ADP or UTP, also induced ethidium(+) uptake into KG-1 cells. ATP-induced ethidium(+) uptake was completely impaired by the P2X7 antagonists, AZ10606120 and A-438079. ATP-induced ethidium(+) uptake was also impaired by probenecid but not by carbenoxolone, both pannexin-1 antagonists. ATP induced YO-PRO-1(2+) and propidium(2+) uptake into KG-1 cells. Finally, sequencing of full-length P2X7 cDNA identified several single nucleotide polymorphisms (SNPs) in KG-1 cells including H155Y, A348T, T357S and Q460R. RPMI 8226 cells contained A348T, A433V and H521Q SNPs. In conclusion, the KG-1 cell line expresses functional P2X7. This cell line may help elucidate the signalling pathways involved in P2X7-induced survival and invasiveness of myeloid leukaemic cells.

Keywords

kg, p2x7, receptor, activation, mediates, organic, cells, cation, 1, uptake, into, human, myeloid, leukaemic, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

Publication Details

Gadeock, S., Pupovac, A., Sluyter, V., Spildrejorde, M. & Sluyter, R. (2012). P2X7 receptor activation mediates organic cation uptake into human myeloid leukaemic KG-1 cells. *Purinergic Signalling*, 8 (4), 669-676.

P2X7 receptor activation mediates organic cation uptake into human myeloid leukaemic KG-1 cells

Safina Gadeock^{1,2}, Aleta Pupovac^{1,2}, Vanessa Sluyter^{1,2}, Mari Spildrejorde^{1,2}, Ronald Sluyter^{1,2*}

¹School of Biological Sciences, University of Wollongong, Wollongong, NSW, 2522, Australia

²Institute of Illawarra Health and Medical Research Institute, Wollongong, NSW, 2522, Australia

*Correspondence to Dr Ronald Sluyter, Email: rsluyter@uow.edu.au; Telephone: +61 2 4221 5508;

Fax: +61 2 4221 8130

Abstract

The P2X7 purinergic receptor is an ATP-gated cation channel with an emerging role in neoplasia. In this study we demonstrate that the human KG-1 cell line, a model of acute myelogenous leukaemia, expresses functional P2X7. RT-PCR and immunochemical techniques demonstrated the presence of P2X7 mRNA and protein respectively in KG-1 cells, as well as in positive control multiple myeloma RPMI 8226 cells. Flow cytometric measurements demonstrated that ATP induced ethidium⁺ uptake into KG-1 cells suspended in sucrose medium (EC₅₀ of ~3 μM), but not into cells in NaCl medium. In contrast, ATP induced ethidium⁺ uptake into RPMI 8226 cells suspended in either sucrose or NaCl medium (EC₅₀ of ~3 or ~99 μM, respectively), as well as into RPMI 8226 cells in KCl medium (EC₅₀ of ~18 μM). BzATP and to a lesser extent ATPγS and αβ-methylene ATP, but not ADP or UTP, also induced ethidium⁺ uptake into KG-1 cells. ATP-induced ethidium⁺ uptake was completely impaired by the P2X7 antagonists, AZ10606120 and A-438079. ATP-induced ethidium⁺ uptake was also impaired by probenecid but not by carbenoxolone, both pannexin-1 antagonists. ATP also induced YO-PRO-1²⁺ and propidium²⁺ uptake into KG-1 cells. Finally, sequencing of full-length P2X7 cDNA identified several single nucleotide polymorphisms (SNPs) in KG-1 cells including H155Y, A348T, T357S and Q460R. RPMI 8226 cells contained A348T, A433V and H521Q SNPs. In conclusion, the KG-1 cell line expresses functional P2X7. This cell line may help elucidate the signalling pathways involved in P2X7-induced survival and invasiveness of myeloid leukaemic cells.

Keywords Purinergic receptor; Extracellular ATP; Acute myelogenous leukaemia; Cation channel; Single nucleotide polymorphism

Introduction

The P2X7 purinergic receptor is a trimeric ATP-gated cation (Ca^{2+} , Na^+ and K^+) channel present on various cell types [1]. Prolonged activation of P2X7 by extracellular ATP causes the uptake of organic cations, including ethidium⁺, and various downstream signalling events [1]. P2X7 plays important roles in inflammation and immunity, as well as in bone, epithelial and neuronal homeostasis [2, 3]. P2X7 also has an emerging role in neoplasia [4, 5]. Down-regulation of P2X7 may act as a tumour escape mechanism by preventing ATP-induced cell death [6]. Alternatively, P2X7 activation may promote ATP-induced tumour cell survival and growth [7, 8]. P2X7 activation may also facilitate tumour invasiveness and metastases [9] by inducing the release of matrix membrane metalloproteases [10] and cysteine cathepsins [11], and the shedding of cell adhesion molecules [12, 13]. Despite these findings however, the role of P2X7 in neoplasia remains poorly defined. This, in large part, is due to the lack of human tumour cell lines suitable for the study of P2X7, with many cell lines failing to express this receptor [14] despite the detection of P2X7 in various malignant tissues [15, 16]. Therefore, using molecular, immunochemical and pharmacological approaches we investigated whether the human KG-1 cell line, a commonly used model of acute myelogenous leukaemia [17], expresses functional P2X7 receptors.

Materials and methods

Materials RPMI-1640 medium, GlutaMAX, YO-PRO-1 iodide and probenecid were from Invitrogen (Grand Island, NJ, USA). Foetal calf serum was from Lonza (Basel, Switzerland) or Bovogen Biologicals (East Kellior, Australia). Bovine serum albumin (BSA) and ethidium bromide were from Amresco (Solon, OH, USA). Nucleotides, propidium iodide and carbenoxolone were

from Sigma Chemical Co (St Louis, MO, USA). AZ10606120 and A-438079 were from Tocris Bioscience (Ellisville, MO, USA).

Cell lines KG-1 and RPMI 8226 cells (European Collection of Cell Cultures, Porton Down, UK) were maintained in complete culture medium (RPMI-1640 medium containing 2 mM GlutaMAX, and 20% or 10% foetal calf serum, respectively) at 37°C/5% CO₂.

P2X7 expression Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-PCR of P2X7 mRNA was performed using Superscript III One-Step RT-PCR System Platinum Taq DNA polymerase (Invitrogen) as described [18]. Immunoblotting of whole cell lysates (the equivalent of 2×10^5 cells per lane) was performed using a rabbit anti-rat P2X7 polyclonal antibody (pAb) (Alomone Labs, Jerusalem, Israel) as described [18]. A rabbit anti-actin pAb (Sigma Chemical Co) was used to confirm equal protein loading as described [18]. Mean fluorescence intensity (MFI) of cell-surface P2X7 expression was detected using an Alexa Fluor 647-conjugated murine anti-human P2X7 monoclonal antibody (mAb) (clone L4) and flow cytometry as described [14].

Organic cation uptake measurements Nucleotide-induced organic cation uptake into cells suspended in either sucrose (280 mM sucrose, 5 mM KCl, 10 mM *N*-methyl-D-glucamine, 5 mM glucose, 0.1% BSA, 10 mM HEPES, pH 7.4), NaCl (145 mM NaCl, 5 mM KCl, 5 mM glucose, 0.1% BSA, 10 mM HEPES, pH 7.4) or KCl (150 mM KCl, 5 mM glucose, 0.1% BSA, 10 mM HEPES, pH 7.4) medium was determined using a fixed-time flow cytometric assay as described [14]. The fluorescence of the organic cations ethidium⁺, YO-PRO-1²⁺ and propidium²⁺ were collected with band pass filters 575/26, 515/20 and 695/40, respectively.

cDNA sequencing Total RNA was isolated as above. RT-PCR was performed using the MyTaq One-Step RT-PCR Kit (Bioline, Sydney, Australia) according to the manufacturer's instructions using primer pairs (GeneWorks, Hindmarsh, Australia) specific for four overlapping regions of full length P2X7 cDNA including parts of the untranslated 5' and 3' ends. The primer pairs (forward and reverse, respectively) were: (1) 5'-tggccctgtcaggaagagta-3' and 5'-caccaggcagagacttcaca-3'; (2) 5'-ttgtaaaaagggatggatgga-3' and 5'-aaatatgggagcgacagcag-3'; (3) 5'-tacatcggctcaaccctctc-3' and 5'-gaacagctctgaggtggtga-3'; and (4) 5'-gtctggtgccagtgtggaa-3' and 5'-actcccgacctcaggtgat-3'. The PCR cycling conditions were 45°C for 20 min, 95°C for 1 min, 40 cycles of 95°C for 10 s, 63°C (primer pair 1), 59°C (primer pair 2), 62°C (primer pair 3) or 61°C (primer pair 4) for 10 s, and 72°C for 30 s, and a final step of 72°C for 5 min. Amplicons were run on 2% agarose gels, visualized by ethidium bromide staining, excised and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Purified amplicons were sequenced using the above primers with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and an Applied Biosystems 3130xl Genetic Analyzer. PCR cycling conditions for sequencing were 95°C for 2 min, and then 25 cycles of 96°C for 30 s, 55°C for 15 s and 60°C for 4 min.

Data presentation and statistics Data are presented as mean \pm SD. Differences between treatments were compared using either the unpaired Student's t-test for single comparisons to control samples or ANOVA for multiple comparisons (using Tukey's post test) using Prism 5 (Mac OS X Version 5.0a; GraphPad Software, San Diego, CA, USA). Concentration response curves of log(agonist) vs. normalized response were fitted using the least squares (ordinary) fit method using Prism 5.

Results

RT-PCR demonstrated P2X7 mRNA expression in KG-1 cells, as well as in positive control RPMI 8226 cells (Fig. 1A), a human multiple myeloma cell line which expresses high amounts of functional P2X7 [14]. Immunoblotting with an anti-P2X7 pAb revealed the presence of a major band at 75 kDa, the predicted size of glycosylated P2X7, in both cell types (Fig. 1B). A minor band at 42 kDa was also observed in both cell lines (Fig. 1B). The amount of P2X7 was lower in KG-1 cells compared to RPMI 8226 cells, despite equal protein loading as demonstrated using an anti-actin pAb (Fig. 1B). Finally, immunolabelling with an anti-P2X7 mAb and flow cytometric analysis demonstrated cell-surface P2X7 expression on KG-1 cells, which was significantly lower than that on RPMI 8226 cells (MFI of 3.3 ± 0.4 and 17.0 ± 3.2 respectively, $n=3$, $P<0.01$; Fig. 1C).

Preliminary experiments demonstrated that incubation of KG-1 cells suspended in NaCl medium with 1 mM ATP for up to 20 min failed to induce ethidium⁺ uptake into these cells, despite significant ethidium⁺ uptake into RPMI 8226 cells in NaCl medium after 5 min ATP incubation (*results not shown*). Therefore, ATP-induced ethidium⁺ uptake into KG-1 cells was next assessed in sucrose medium, which can facilitate P2X7-induced cation uptake in other cell types [19, 20]. A 20 min incubation with ATP induced significant ethidium⁺ uptake into KG-1 cells in a concentration-dependent manner, with a maximal uptake at 100 μ M ATP and with an EC₅₀ of 3.3 ± 1.3 μ M (Fig. 2A). This EC₅₀ value is typical of cation fluxes mediated by recombinant human P2X7 expressed in cell lines in sucrose medium [19]. Moreover, this EC₅₀ value was similar to the EC₅₀ value (2.7 ± 2.7 μ M) for ATP-induced ethidium⁺ uptake into RPMI 8226 cells suspended in sucrose medium (Fig. 2B). In contrast, the EC₅₀ values for ATP-induced ethidium⁺ uptake into RPMI 8226 cells suspended in either KCl or NaCl medium were higher (17.8 ± 8.2 or 99.3 ± 19.6 μ M respectively) (Fig. 2B). The latter EC₅₀ value was similar to that previously observed for RPMI 8226 cells in NaCl medium [14].

To determine whether the ATP-induced ethidium⁺ uptake into KG-1 cells was mediated by P2X7, cells suspended in sucrose medium were incubated in the absence or presence of ATP, the most potent P2X7 agonist BzATP, the partial P2X7 agonist ATP γ S, or agonists of other P2 receptors. As above, 100 μ M ATP induced a significant amount of ethidium⁺ uptake (Fig. 3A). Similarly, 100 μ M BzATP, and to a lesser extent 100 μ M ATP γ S also induced significant ethidium⁺ uptake (Fig. 3A). Surprisingly, 100 μ M $\alpha\beta$ -methylene ATP, which is not a recognized P2X7 agonist, also induced a small but significant uptake of ethidium⁺ (Fig. 3A). In contrast, neither 100 μ M ADP or UTP caused a significant amount of ethidium⁺ uptake into KG-1 cells (Fig. 3A). To confirm that this ATP-induced ethidium⁺ uptake was mediated by P2X7, KG-1 cells were pre-incubated in the absence or presence of the P2X7 antagonists, AZ10606120 or A-438079, and the relative amounts of ATP-induced ethidium⁺ uptake into KG-1 cells suspended in sucrose medium measured. Pre-incubation of cells with 100 nM AZ10606120 or 10 μ M A-438079 completely inhibited 100 μ M ATP-induced ethidium⁺ uptake (Fig. 3B). Neither antagonist significantly altered ethidium⁺ uptake in the absence of ATP (Fig. 3B).

Debate exists over the cut-off size of the P2X7 pore. Therefore, in addition to ethidium⁺ (314 Da) above, the effect of ATP incubation on YO-PRO-1²⁺ (375 Da) and propidium²⁺ (415 Da) uptake into KG-1 cells suspended in sucrose medium was assessed. ATP induced significant uptake of both YO-PRO-1²⁺ and propidium²⁺ (Fig. 3C). Debate also exists over the molecular identity of the P2X7 pore; in particular the hemichannel pannexin-1 may function as the P2X7 pore [21]. Therefore, KG-1 cells were pre-incubated in the absence or presence of the pannexin-1 antagonists, probenecid or carbenoxolone, and the relative amounts of ATP-induced ethidium⁺ uptake into KG-1 cells suspended in sucrose medium measured. Cells were pre-incubated with probenecid or carbenoxolone at concentrations reported to block pannexin-1 [21, 22]. Pre-incubation of cells with 2 mM probenecid significantly inhibited 100 μ M ATP-induced ethidium⁺ uptake (Fig. 3D). In contrast, 20 μ M carbenoxolone failed to alter 100 μ M ATP-induced ethidium⁺ uptake (Fig. 3D).

Neither antagonist significantly altered ethidium⁺ uptake in the absence of ATP (Fig. 3D).

The above data indicates that the low amount of P2X7 function in KG-1 cells, compared to RPMI 8226 cells, corresponds to low P2X7 expression. To determine if this low P2X7 function in KG-1 cells may be attributed to single nucleotide polymorphisms (SNPs) that alter P2X7 expression or function, full length P2X7 cDNA from these cells was amplified by RT-PCR and sequenced. Full length P2X7 cDNA from RPMI 8226 cells was also amplified and sequenced as a comparison. The *p2x7* gene of KG-1 cells was heterozygous for six non-synonymous and three synonymous SNPs (Table 1). Of note, these cells contained the loss-of-function SNP, T357S [23], two gain-of-function SNPs, H155Y [24] and A348T [25, 26], and the Q460R SNP, which uniquely identifies the gain-of-function haplotype variant P2X7-4 [26]. The *p2x7* gene of RPMI 8226 cells was homozygous for three non-synonymous and two synonymous SNPs (Table 1). Of note, these cells contained the A348T gain-of-function SNP, as well as the H521Q SNP, which reduces the sensitivity of the receptor to inhibition by extracellular Ca²⁺ [25]. Neither cell line encoded other P2X7 SNPs (reviewed in [1, 3] including well-characterised SNPs such as R307Q, E496A and I568N (*results not shown*)).

Discussion

In the current study, we demonstrate that the acute myelogenous leukaemic KG-1 cell line expresses functional P2X7 receptors. First, RT-PCR, and immunoblotting and immunolabelling demonstrate the presence of P2X7 mRNA and protein in KG-1 cells, respectively. Second, ATP induced ethidium⁺ uptake into KG-1 cells with an EC₅₀ of ~3 μM, a value typical of cation fluxes mediated by recombinant human P2X7 expressed in cells in sucrose medium [19] or endogenous P2X7 in RPMI 8226 cells in sucrose medium (this study). Third, the most potent P2X7 agonist BzATP, as well as the partial P2X7 agonist ATPγS, induced ethidium⁺ into KG-1 cells. Fourth, the

P2X7 antagonists, AZ10606120 and A-438079, prevented ATP-induced ethidium⁺ uptake into KG-1 cells.

The amount of functional P2X7 on KG-1 cells is relatively low compared to other cell lines. Previously, we have shown that 5 min ATP incubation of either RPMI 8226 cells, murine erythroleukaemia cells or murine RAW264.7 macrophages in NaCl medium results in significant ethidium⁺ uptake [14, 27]. In comparison, up to 20 min incubation with ATP failed to induce significant ethidium⁺ uptake into KG-1 cells in NaCl medium. This relatively low amount of functional P2X7 in KG-1 cells is most likely attributed to the low amounts of cell-surface P2X7, as observed when compared to RPMI 8226 cells. This low amount of cell-surface P2X7 corresponded with reduced P2X7 mRNA and total protein expression in KG-1 cells compared to RPMI 8226 cells. The RT-PCR assay used however was not quantitative nor did it include a house-keeping gene.

In addition to the low amount of P2X7 expression in KG-1 cells, these cells were heterozygous for the loss-of-function T357S SNP, which may further explain the reduced P2X7 function in KG-1 cells. The KG-1 cells were also heterozygous for the partial loss-of-function Q460R SNP, although the negative effect of this SNP on P2X7 function [26] has not been observed by others [25]. Moreover, in Caucasians (from which KG-1 cells originate), the Q460R SNP uniquely identifies a haplotype variant termed P2X7-4, which contains the gain-of-function SNPs, H115Y and A348T (but is wild-type at residue 357), and results in a net gain of function [26]. Nevertheless, given that the T357S SNP in heterozygous dosage reduces P2X7 function by at least 50% in primary leukocytes from all subjects studied [23], it remains likely that the allele coding for S357 (and uniquely associated with haplotype variant P2X7-5) may have a dominant negative effect on other P2X7 alleles (including that containing P2X7-4) resulting in a net loss of function. KG-1 cells also contained two other non-synonymous SNPs, L320P and P582L, however their effect on P2X7 expression or function remains unknown. A study of rat P2X7, demonstrated that a P582G

mutation fails to alter P2X7 channel or pore formation, although using truncated P2X7 variants this same study identified P582 as part of a pore-enabling region (551-582) which regulates cell-surface P2X7 expression and thereby P2X7 function [28]. As a result, these authors hypothesised that mutations within this region may prevent cell-surface P2X7 expression [28]. Thus the possibility remains that P582L may reduce P2X7 function as a result of reduced cell-surface P2X7 expression. Alternatively, the L320P SNP, which resides within the ATP-binding site of P2X receptors [29], may result in an atypical pharmacology and contribute to the low P2X7 function in KG-1 cells. Atypical P2X7 pharmacology has also been observed in human osteosarcoma MG63 and SaOS2 cells [30]. Moreover, $\alpha\beta$ -methylene ATP, which is not a recognised P2X7 agonist, induced a small but significant uptake of ethidium⁺ into KG-1 cells. Nucleotide preparations however can contain small amounts of contaminating ATP [31]. Thus, comparison of the ATP concentration response and agonist data suggests that as little as a 1% contaminating ATP may have contributed to the $\alpha\beta$ -methylene-induced ethidium⁺ uptake into KG-1 cells. Finally, we cannot exclude that KG-1 cells contain an alternate P2X7 isoform, rather than a SNP, that reduces P2X7 function as observed in various human epithelial cervical cancer cell lines [6].

The presence of *p2x7* SNPs in cell lines is not unique to KG-1 cells. The human acute monocytic leukaemic THP-1 cell line is heterozygous for the loss-of-function E496A SNP [14, 32], while others have shown that human myelomonocytic leukaemic J6-1 cells contain the loss-of-function N187D SNP [33]. Moreover, the current study reveals that RPMI 8226 cells are homozygous for the gain-of-function A348T SNP, as well as A433V and H521Q. The presence of threonine at position 348 may account for the higher P2X7 function of RPMI 8226 cells compared to KG-1 cells. Although not formally demonstrated, A433V may also act as an additional gain-of-function SNP in these cells as the opposite amino acid exchange at position 76 of P2X7 (V76A) causes a loss of function [26]. Finally, others have shown that H521Q has no effect on ATP-induced ethidium⁺ uptake, but can reduce the sensitivity of P2X7 to inhibition by extracellular Ca²⁺

[25]. In this regard, maximal ATP-induced ethidium⁺ uptake into RPMI 8226 cells is similar in cells suspended in either NaCl medium containing Ca²⁺/Mg²⁺ or nominally free of Ca²⁺/Mg²⁺ [14].

The current study also demonstrates that P2X7 activation induces uptake of organic cations up to and including the size of propidium²⁺ (415 Da) into KG-1 cells. P2X7 activation induces ethidium⁺ (314 Da) but not propidium²⁺ uptake into human lymphocytes [34], dendritic cells [35] and THP-1 cells [18]. In contrast, P2X7 activation induces uptake of propidium²⁺ into rat bone nodule cells [36] and P2X7-transfected HEK-293 cells [37]. Reasons for differences between these cells, especially between the myeloid cell types (KG-1, dendritic and THP-1 cells) remain unknown. These differences may be due to technical reasons; both of the myeloid cell studies that failed to show P2X7-induced propidium²⁺ uptake were conducted with cells suspended in KCl medium, while P2X7-induced propidium²⁺ uptake into KG-1 cells was conducted with cells suspended in sucrose medium. Alternatively, the observed differences in P2X7-induced propidium²⁺ uptake between different cell types may reflect differences in the molecular identity of the P2X7 pore or in the propensity of P2X7 to dilate. Attempts to characterize the molecular identity of the P2X7 pore in KG-1 cells using pannexin-1 antagonists suggest that the P2X7 pore in KG-1 cells is not pannexin-1 or that pannexin-1 is dispensable for P2X7-induced pore formation. ATP-induced ethidium⁺ uptake was completely blocked by probenecid but unaffected by carbenoxolone. Although probenecid can block pannexin-1 [22], it has long been recognised that probenecid blocks other molecules within cells [38]. Thus, the possibility remains that the P2X7 pore may be a molecule other than pannexin-1. Alternatively, probenecid may directly block P2X7 to prevent pore formation. Regardless, the lack of evidence for pannexin-1 as the P2X7 pore in KG-1 cells is consistent with studies using cells from pannexin-1 knockout mice, which are permeable to organic cations following ATP incubation [39].

P2X7 has been reported in various primary lymphoid and myeloid leukaemias in humans [40-44], however the pathophysiological significance of P2X7 in these malignant cell types remains

unknown. Evidence indicates that the relative P2X7 expression and/or function directly correlates with the progression of chronic lymphocytic leukaemia [40] or acute myelogenous leukaemia [44] suggesting a role for increased P2X7 activity in promoting these leukaemias. Studies confirming these observations however are lacking. Nevertheless the detection of functional P2X7 in KG-1 cells indicates that this cell line may provide a model to study the role of P2X7 in acute myelogenous leukaemia. For example, this cell line may be useful in determining if ATP induces death or survival in acute myelogenous leukaemic cells, and identifying the signalling pathways involved in either of these processes. Alternatively, given that KG-1 cell migration can be studied *in vitro* [45], and the potential role of P2X7 in facilitating tumour invasiveness and metastases [5], the KG-1 cell line might provide a useful model to further elucidate the role of P2X7 and downstream signalling pathways in this process.

Acknowledgments

This work was kindly supported by Cure Cancer Australia and the University of Wollongong. We gratefully acknowledged helpful advice from Marie Ranson, Mark Dowton and Simon Cook (all University of Wollongong), and excellent technical assistance by Margaret Phillips (University of Wollongong) and the staff of the Illawarra Health and Medical Research Institute. The authors have no conflicts of interest.

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Figure legends

Fig. 1 KG-1 cells express P2X7. (A) RNA isolated from KG-1 and RPMI 8226 cells was amplified by RT-PCR using primers to P2X7 and products examined by agarose gel electrophoresis. (B) Whole cell lysates from KG-1 and RPMI 8226 cells were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-P2X7 (*top panel*) or anti-actin (*bottom panel*) pAb. (C) KG-1 and RPMI 8226 cells were labeled with Alexa Fluor 647-conjugated P2X7 (*solid line*) or isotype control (*shaded*) mAb, and the relative cell-surface P2X7 expression measured by flow cytometry. Representative results from (A,B) two or (C) three experiments shown.

Fig. 2 ATP induces ethidium⁺ uptake into KG-1 cells. (A) KG-1 cells in sucrose medium or (B) RPMI 8226 cells in sucrose, KCl or NaCl medium were incubated with 25 μ M ethidium⁺ in the presence of varying amounts of ATP (*as indicated*) at 37°C for (A) 20 or (B) 5 min. Incubations were stopped by MgCl₂ solution and centrifugation, and ethidium⁺ uptake measured by flow cytometry. Ethidium⁺ uptake is expressed as percent maximum response compared to 1 mM ATP for each corresponding medium; results are mean \pm SD (A, *n*=3; B, *n*=9).

Fig. 3 P2X7 activation mediates organic cation uptake into KG-1 cells. (A-D) KG-1 cells were suspended in sucrose medium. (A) Cells were incubated with 25 μ M ethidium⁺ in the absence (Basal) or presence of 100 μ M nucleotide (*as indicated*; $\alpha\beta$ MethATP, $\alpha\beta$ -methylene ATP) at 37°C for 20 min. Cells were pre-incubated (B) with DMSO or 100 nM AZ10606120 (*left panel*), in the absence (Control) or presence of 10 μ M A-438079 (*right panel*), (D) in the absence (Control) or presence of 2 mM probenecid (*left panel*), or in the absence (Control) or presence of 20 μ M carbenoxolone (*right panel*) at 37°C for 15 min. Cells were then incubated with (B,D) 25 μ M ethidium⁺, (C) 1 μ M YO-PRO-1²⁺ or 25 μ M propidium²⁺ (B-D) in the absence (Basal) or presence

of 100 μM ATP at 37°C for 20 min. (A-D) Incubations were stopped by MgCl_2 solution and centrifugation, and cation uptake measured by flow cytometry. Results are mean \pm SD ($n=3-6$); * $P<0.05$ or ** $P<0.01$ compared to corresponding basal, †† $P<0.01$ compared to corresponding ATP alone.

Table 1 KG-1 and RPMI 826 cells contain several single nucleotide polymorphisms in the *p2x7* gene

dbSNP rs#	Base Change ^b	Amino Acid	Effect on	KG-1	RPMI 8226
Cluster ID ^a		Change	Function		
rs28360448	GTG>GTA	V154V	Synonymous ^c	Wild-type	Homozygous
rs208294	CAT>TAT	H155Y	Gain	Heterozygous	Wild-type
rs73403850	CTG>CCG	L320P	Unknown	Heterozygous	Wild-type
rs1718119	GCT>ACT	A348T	Gain	Heterozygous	Homozygous
rs2230911	ACT>AGT	T357S	Loss	Heterozygous	Wild-type
rs28360459	GCG>GTG	A433V	Unknown	Wild-type	Homozygous
rs2230912	CAG>CGG	Q460R	Possible loss	Heterozygous	Wild-type
rs3751144	CCC>CCT	P474P	Synonymous	Heterozygous	Wild-type
rs2230913	CAC>CAG	H521Q	Neutral ^d	Wild-type	Homozygous
rs3751142	CTG>CTT	L534L	Synonymous	Heterozygous	Wild-type
rs77111027	CCG>CTC	P582L	Unknown	Heterozygous	Wild-type
rs1621388	CCG>CCA	P582P	Synonymous	Heterozygous	Homozygous

^ahttp://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=5027.

^bFull length P2X7 cDNA from KG-1 and RPMI 8226 cells was amplified by RT-PCR and sequenced to identify potential base changes (single nucleotide polymorphisms).

^cEach synonymous mutation is predicted to have neutral effect on function.

^dNeutral effect; however the mutant receptor displays reduced sensitivity to inhibition by extracellular Ca²⁺.





