Biochemical and Biophysical Research Communications 432 (2013) 626–631

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Human P2X7 receptor activation induces the rapid shedding of CXCL16

Aleta Pupovac [a,b], Christopher M. Foster [a], Ronald Sluyter [a,b,*]

[a] School of Biological Sciences, University of Wollongong, Wollongong, NSW, Australia
[b] Illawarra Health and Medical Research Institute, Wollongong, NSW, Australia

ARTICLE INFO

Article history:
Received 24 January 2013
Available online 18 February 2013

Keywords:
Purinergic receptor
Extracellular ATP
Chemokine
IgE receptor
ADAM10
B cell

ABSTRACT

Activation of the purinergic P2X7 receptor by extracellular ATP induces the shedding of cell-surface molecules including the low-affinity IgE receptor, CD23 from leukocytes. CD23 is a known substrate of a disintegrin and metalloprotease (ADAM)10. The aim of the current study was to determine if P2X7 activation induced the shedding of the chemokine CXCL16, an ADAM10 substrate. Using immunolabelling and flow cytometry we demonstrate that human RPMI 8226 multiple myeloma B cells, which have been previously shown to express P2X7, also express CXCL16. Flow cytometric and ELISA measurements of ATP-induced loss of cell-surface CXCL16 showed that ATP treatment of RPMI 8226 cells induced the rapid shedding of CXCL16. Treatment of RPMI 8226 cells with the specific P2X7 antagonists, AZ10606120 and KN-62 impaired ATP-induced CXCL16 shedding by ~86% and ~90% respectively. RT-PCR demonstrated that ADAM10 is expressed in these cells and treatment of cells with the ADAM10 inhibitor, GI254023X, impaired ATP-induced CXCL16 shedding by ~87%. GI254023X also impaired P2X7-induced CD23 shedding by ~57%. This data indicates that human P2X7 activation induces the rapid shedding of CXCL16 and that this process involves ADAM10.

1. Introduction

The CXCR6 ligand and chemokine, CXCL16 is present on B cells and other leukocytes and exists in both transmembrane and soluble forms [1]. Membrane CXCL16 functions as an adhesion molecule for CXCR6-expressing cells and a scavenger receptor for oxidised low density lipoprotein, while the soluble form functions as a chemoattractant for lymphocytes [1]. CXCL16 cleavage from the cell surface is mediated by membrane metalloproteases of the ADAM (a disintegrin and metalloprotease) family. ADAM10 is principally responsible for the constitutive and ionomycin-induced shedding of the membrane CXCL16 [2,3].

P2X7 is a trimeric, ATP-gated cation channel which plays important roles in health and disease [4]. Activation of P2X7 by extracellular ATP causes the uptake of organic cations such as ethidium$^+$ [4]. P2X7 activation also stimulates various downstream events including the rapid shedding of CD23 from malignant B cells [5,6] and normal dendritic cells [7,8], and the shedding of interleukin-6 receptor from murine splenic T cells and P2X7/interleukin-6 receptor co-transfected HEK293 and NIH323 cells [9]. P2X7-induced shedding of the interleukin-6 receptor has been attributed to ADAM10 [9]. P2X7-induced CD23 shedding from leukemic B cells and RPMI 8226 myeloma B cells is mediated by yet an unidentified metalloprotease [5,6], however ADAM10 is a likely candidate [10,11]. The aim of the current study was to determine if P2X7 activation induces CXCL16 shedding from human RPMI 8226 cells. We demonstrate that P2X7 activation induces the rapid shedding of CXCL16 from these cells, and that this process involves ADAM10. In addition, we demonstrate that P2X7-induced CD23 shedding from RPMI 8226 cells is also mediated by ADAM10.

2. Materials and methods

2.1. Reagents

RPMI-1640 medium, ATP, 2′- and 3′-0(4-benzoylbenzoyl) ATP (BzATP), ADP and UTP were from Sigma (St. Louis, MO). Foetal bovine serum was from Bovogen Biologicals (East Keilor, Australia) or Lonza (Basal, Switzerland). Dimethyl sulphoxide (DMSO) and ethidium bromide were from Amresco (Solon, OH). Phycoerythrin (PE)-conjugated murine anti-human CD23 (clone EBVCS2) and isotype control (clone P3.6.2.8.1) monoclonal antibodies (mAb) were from eBioscience (San Diego, CA). Rabbit anti-human CXCL16 and control IgG antibodies (Ab) were from Peprotech (Rocky Hill, NJ),

Abbreviations: ADAM, a disintegrin and metalloprotease; BzATP, 2′- and 3′-0(4-benzoylbenzoyl) ATP; DMSO, dimethyl sulphoxide; PE, phycoerythrin; mAb, monoclonal antibody; Ab, antibody; FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity.

* Corresponding author at: School of Biological Sciences, Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, NSW 2522, Australia. Fax: +61 2 4221 8130.
E-mail address: rsluyter@uow.edu.au (R. Sluyter).

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http://dx.doi.org/10.1016/j.bbrc.2013.01.134

and the fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit Ab was from Silenus Laboratories (Melbourne, Australia). GI254023X was kindly provided by GlaxoSmithKline (Stevenage, United Kingdom). AZ10606120 and BB-94 (Batimastat) were from Tocris Bioscience (Ellisville, MO). KN-62 was from Alexis Biochemicals (Lausen, Switzerland). GM6001 was from Calbiochem (Darmstadt, Germany).

2.2. Cells

RPMI 8226 cells were maintained in complete RPMI-1640 medium (RPMI-1640 medium containing 10% foetal bovine serum and 2 mM GlutaMAX) at 37 °C and 95% air/5% CO_2 as described [6].

2.3. Measurement of P2X7-induced CXCL16 and CD23 shedding by flow cytometry

Cells, suspended in NaCl medium (145 mM NaCl, 5 mM KCl, 5 mM glucose, 0.1% bovine serum albumin, 10 mM HEPES, pH 7.4) (1×10^6 cells/ml), were incubated in the absence or presence of nucleotide (as indicated) for up to 30 min at 37 °C. In some experiments, cells in NaCl medium were pre-incubated at 37 °C for 15 min in the absence or presence of antagonist, and then in the absence or presence of 1 mM ATP (as indicated). Incubations with nucleotide were stopped by addition of an equal volume of ice-cold $MgCl_2$ medium (NaCl medium containing 20 mM $MgCl_2$) and centrifugation. Cells were then washed once with NaCl medium and incubated with anti-CXCL16 or IgG control Ab, then washed twice and finally incubated with a FITC-conjugated anti-rabbit IgG Ab. Alternatively, washed cells were incubated with PE-conjugated anti-CD23 or isotype control mAb. The mean fluorescence intensity (MFI) of cell-surface CXCL16 or CD23 expression was determined using a BD (San Jose, CA) LSR II flow cytometer and FlowJo software (Tree Star, Ashland, OR).

2.4. Measurement of soluble CXCL16 by ELISA

Cells, suspended in NaCl medium (1×10^7 cells/ml), were incubated in the absence or presence of 1 mM ATP for 10 min at 37 °C. Incubations were stopped by centrifugation (11,000×*g* for 10 s). Cell-free supernatants were stored at −80 °C until required. Soluble CXCL16 was quantified using the Human CXCL16 Mini ELISA Development Kit (Peprotech) according to the manufacturer's instructions.

2.5. Detection of ADAM10 by RT-PCR

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Hilden, Germay) according to the manufacturer's instructions. Primers to ADAM10 [12] were obtained from GeneWorks (Hindmarsh, Australia). PCR amplification was performed using the MyTaq One-Step RT-PCR Kit (Bioline, Sydney, Australia) according to the manufacturer's instructions. PCR cycling conditions were 45 °C for 20 min, 30 cycles of 94 °C for 10 s, 49 °C for 1 min 30 s and 72 °C for 1 min, and a final step of 72 °C for 10 min. Products were separated on a 2% agarose gel and visualised using ethidium bromide staining.

2.6. Measurement of P2X7-induced pore formation by flow cytometry

P2X7-induced pore formation was assessed by flow cytometric measurements of ATP-induced ethidium$^+$ uptake into cells suspended in NaCl medium as described [13].

2.7. Presentation of data and statistics

Data is presented as mean ± 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 (Windows version 5.01; GraphPad Software, San Diego, CA) with $P < 0.05$ considered significant.

3. Results

3.1. P2X7 activation induces rapid shedding of CXCL16 from RPMI 8226 cells

To determine whether RPMI 8226 cells express CXCL16, cells were labelled with an anti-CXCL16 or control IgG Ab. CXCL16 was present on RPMI 8226 cells (MFI of 14 ± 1, Fig. 1A). Therefore, to determine if ATP induces CXCL16 shedding, cells were incubated with 1 mM ATP which resulted in an 83 ± 8% ($n = 3$) loss of cell-surface CXCL16 at 30 min, with a similar loss of CXCL16 observed at 10 and 20 min (results not shown). ATP-induced cell-surface

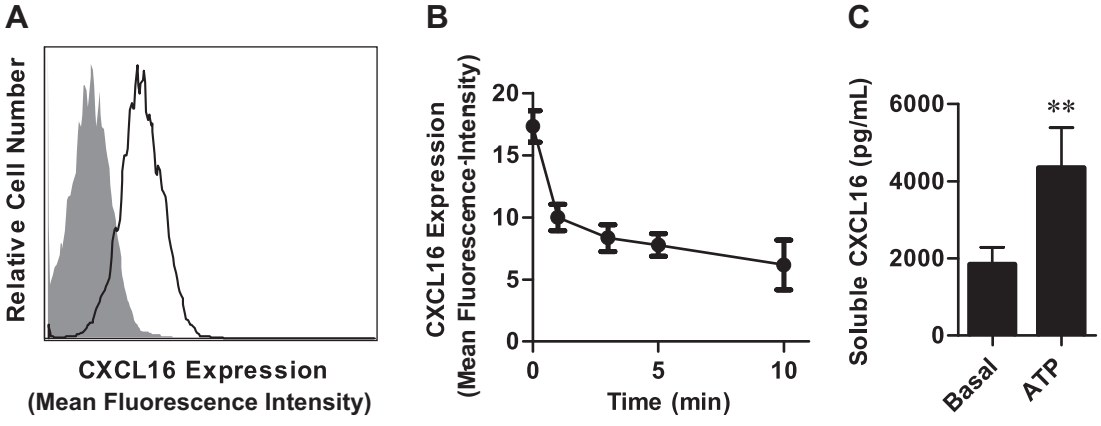

Fig. 1. ATP induces rapid CXCL16 shedding from RPMI 8226 cells. (A) RPMI 8226 cells in NaCl medium were labelled with anti-CXCL16 (solid line) or isotype control (shaded) Ab, and then with a FITC-conjugated secondary Ab. The mean fluorescence intensity (MFI) of CXCL16 cell-surface expression was determined by flow cytometry. A representative result from three experiments is shown. (B) Cells in NaCl medium were incubated for up to 10 min at 37 °C in the presence of 1 mM ATP as indicated. Incubations were stopped and the MFI of cell-surface CXCL16 expression was determined by flow cytometry. (C) Cells in NaCl medium were incubated in the absence (basal) or presence of 1 mM ATP at 37 °C for 10 min and the amount of soluble CXCL16 in cell-free supernatants determined by ELISA. Results are mean ± SD ($n = 3$–4); $^{**}P < 0.01$ compared with corresponding basal.

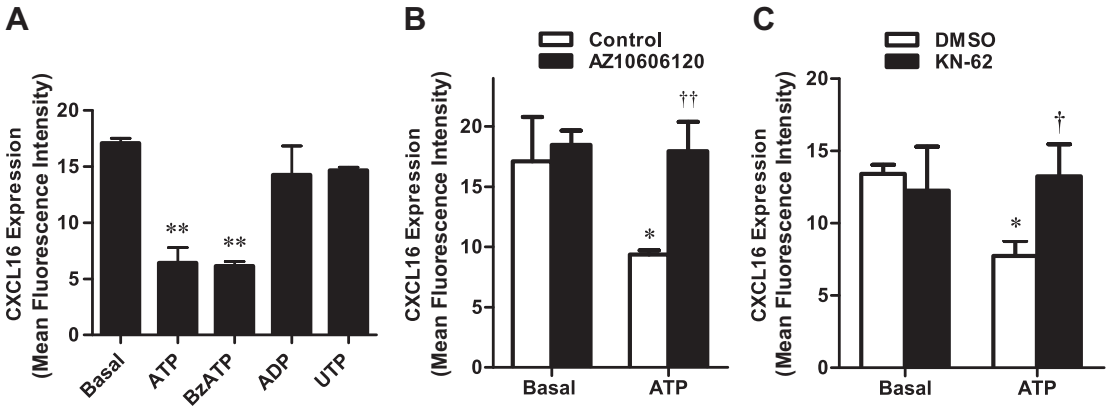

Fig. 2. P2X7 activation induces CXCL16 shedding from RPMI 8226 cells. (A) RPMI 8226 cells in NaCl medium were incubated in the absence (basal) or presence of ATP, BzATP, ADP or UTP (all 100 μM) at 37 °C for 1 min. (B) and (C) Cells in NaCl medium were pre-incubated at 37 °C for 15 min (B) in the absence (control) or presence of 100 nM AZ10606120, or (C) in the presence of DMSO or 1 μM KN-62, (B) and (C) and then in the absence (basal) or presence of 1 mM ATP for 1 min at 37 °C. (A)–(C) Incubations were stopped and the MFI of cell-surface CXCL16 expression was determined by flow cytometry. Results are mean ± SD ($n = 3$); $^{*}P < 0.05$ or $^{**}P < 0.01$ compared with corresponding basal; $^{\dagger}P < 0.05$ or $^{\dagger\dagger}P < 0.01$ compared with corresponding ATP without antagonist.

CXCL16 loss was then determined over shorter time points. ATP induced the rapid loss of cell-surface CXCL16 in a time-dependent fashion, with a $t_{1/2}$ of approximately 1 min (Fig. 1B). To directly determine if ATP-induced loss of cell-surface CXCL16 was due to CXCL16 shedding, cells were incubated in the absence or presence of 1 mM ATP for 10 min, and the relative amount of soluble CXCL16 in cell-free supernatants quantified by ELISA. Incubation of cells with ATP resulted in a significantly higher release of soluble CXCL16 compared with cells incubated in the absence of ATP (Fig. 1C).

To determine if the ATP-induced CXCL16 shedding was mediated by P2X7, cells were incubated for 1 min (the $t_{1/2}$) in the absence or presence of ATP, the most potent P2X7 agonist BzATP, or the non-P2X7 agonists ADP and UTP (all at 100 μM). ATP and BzATP induced a 62 ± 8% and 64 ± 1% loss of cell-surface CXCL16, respectively, while ADP and UTP had no effect compared to cells incubated in the absence of nucleotide (Fig. 2A). To confirm that ATP-induced shedding of CXCL16 was mediated by P2X7, cells were pre-incubated in the absence or presence of the P2X7 antagonists AZ10606120 [14] and KN-62 [15], and ATP-induced CXCL16 shedding determined by flow cytometry. Both 100 nM AZ10606120 and 1 μM KN-62 impaired 1 mM ATP-induced CXCL16 shedding by 86 ± 24% and 90 ± 9%, respectively (Fig. 2B and C). In the absence of ATP, neither antagonist significantly altered CXCL16 shedding (Fig. 2B and C).

3.2. ADAM10 is involved in P2X7-induced CXCL16 and CD23 shedding from RPMI 8226 cells

To determine a role for metalloproteases in P2X7-induced CXCL16 shedding, cells were pre-incubated in the absence or presence of broad spectrum metalloprotease antagonists BB-94 [16] and GM6001 [17], and the 1 mM ATP-induced CXCL16 shedding determined by flow cytometry. Both 1 μM BB-94 and 1 μM GM6001 impaired P2X7-induced CXCL16 shedding by 77 ± 20% and 52 ± 30%, respectively (Fig. 3A and B). To determine if RPMI 8226 cells express ADAM10, RNA was isolated from RPMI 8226 cells and examined by RT-PCR. RT-PCR showed the expression of ADAM10 mRNA in these cells (Fig. 3C). No PCR products were observed in the H_2O control (Fig. 3C). To determine a role for ADAM10 in P2X7-induced CXCL16 shedding, cells were pre-incubated in the presence of DMSO or the ADAM10 antagonist GI254023X [18] and ATP-induced CXCL16 shedding determined as above. GI254023X (3 μM) impaired ATP-induced CXCL16 shedding by 87 ± 15% (Fig. 3D). To exclude a direct inhibitory role of GI254023X on P2X7 itself, cells were pre-incubated in the presence of DMSO or GI254023X for 15 min and ATP-induced ethidium^{+} uptake was measured by flow cytometry. GI254023X (3 μM) did not affect 1 mM ATP-induced ethidium^{+} uptake into cells (Fig. 3E). In the absence of ATP, GI254023X did not significantly alter CXCL16 shedding or ethidium^{+} uptake compared with cells incubated with DMSO alone (Fig. 3D and E).

To confirm that P2X7 activation induces CD23 shedding from RPMI 8226 cells as previously observed [6], cells were incubated in the absence or presence of 1 mM ATP for up to 30 min and the P2X7-induced loss of cell-surface CD23 was assessed using an anti-CD23 mAb and flow cytometry. Incubation of cells with ATP induced rapid CD23 shedding in a time-dependent fashion, with a $t_{1/2}$ of approximately 7 min (Fig. 4A). To assess a potential role for ADAM10 in P2X7-induced shedding of CD23, cells were pre-incubated with DMSO or the specific ADAM10 inhibitor GI254023X, and ATP-induced CD23 shedding assessed as above using the $t_{1/2}$ of 7 min. GI254023X (3 μM) impaired P2X7-induced CD23 shedding by 57 ± 11% (Fig. 4B).

4. Discussion

In the current study, we show for the first time that P2X7 activation induces the rapid shedding of CXCL16. Moreover, we also show for the first time that RPMI 8226 cells express CXCL16, a known ADAM10 substrate [2,3], and that P2X7-induced CXCL16 shedding involves ADAM10. The P2X7 agonists ATP and BzATP induced the rapid cell-surface loss of CXCL16 from RPMI 8226 cells and measurements of soluble CXCL16 indicated that the ATP-induced loss was a result of shedding. Specific P2X7 antagonists AZ10606120 [14] and KN-62 [15] almost completely impaired ATP-induced CXCL16 shedding, while the non P2X7 agonists ADP and UTP had no effect on CXCL16 expression. Moreover, P2X7-induced CXCL16 shedding could be impaired by broadspectrum metalloprotease antagonists, BB-94 [16] and GM6001 [17], outlining a role for metalloproteases in this process. The specific involvement of ADAM10 in this process was established by the ADAM10 antagonist GI254023X [18]. Of note, GI254023X impaired P2X7-induced CXCL16 and CD23 shedding by ~87% and ~57%, respectively. This reduced capacity for GI254023X to block P2X7-induced CD23 shedding may reflect differences in cell-surface expression and/or rates of shedding between CXCL16 and CD23.

P2X7 activation induces the shedding of CD23 from malignant B cells [5,6] and normal dendritic cells [7,8]. In the current study, we confirm that P2X7 activation induces the rapid shedding of CD23

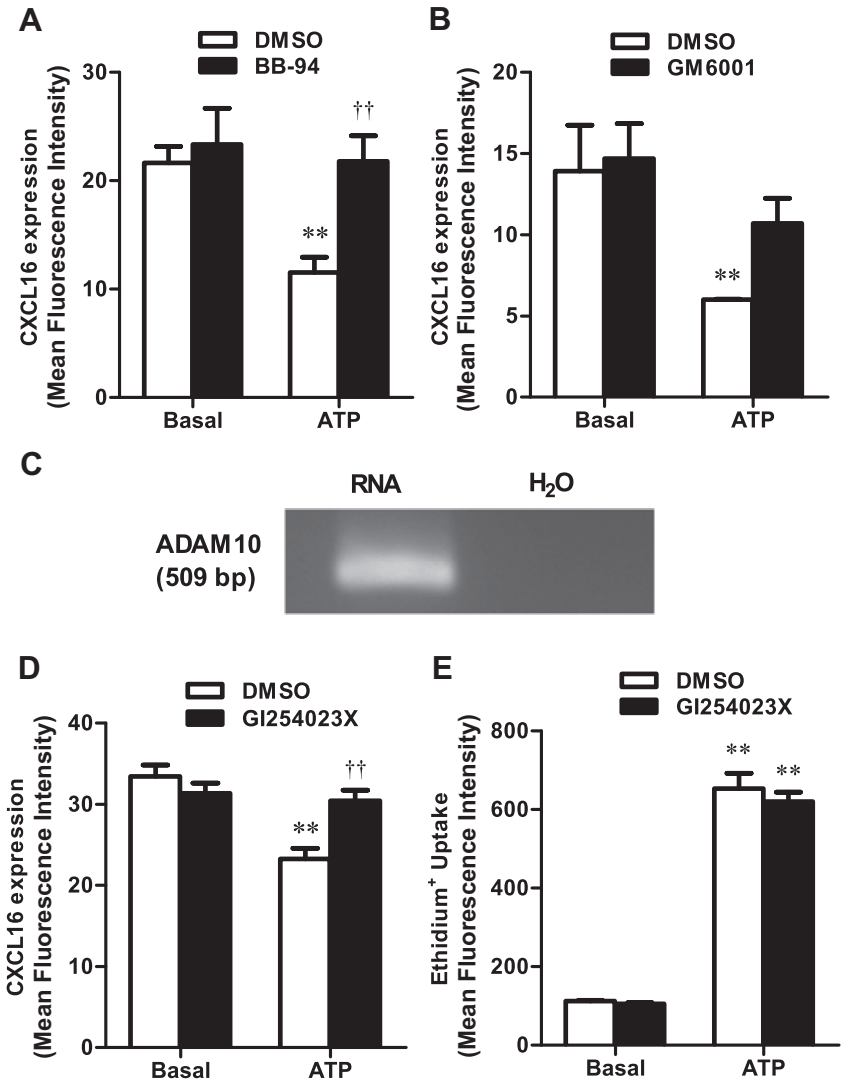

Fig. 3. ADAM10 is involved in P2X7-induced CXCL16 shedding from RPMI 8226 cells. (A, B, and D) RPMI 8226 cells in NaCl medium were pre-incubated at 37 °C for 15 min in the presence of (A, B, and D) DMSO, (A) 1 μM BB-94, (B) 1 μM GM6001, or (D) 3 μM GI254023X and (A, B, and D) then in the absence (basal) or presence of 1 mM ATP for 1 min at 37 °C. (A, B, and D) Incubations were stopped and cells labelled with anti-CXCL16 or isotype control Ab, and then with a FITC-conjugated secondary Ab and the MFI of CXCL16 expression was determined by flow cytometry. (C) RNA was isolated from RPMI 8226 cells, and then analysed by RT-PCR using primers for ADAM10. RNA substituted with H_2O was used as a negative control. PCR products were visualised using ethidium bromide. A representative result from three experiments is shown. (E) Cells in NaCl medium were pre-incubated at 37 °C for 15 min in the presence of DMSO or 3 μM GI254023X, and then with 25 μM ethidium$^+$ in the absence or presence of 1 mM ATP at 37 °C for 5 min. Incubations were stopped and the MFI of ethidium$^+$ uptake was determined by flow cytometry. Results are mean ± SD ($n = 3$); $^{**}P < 0.01$ compared with corresponding basal; $^{††}P < 0.01$ compared with corresponding ATP without antagonist.

from RPMI 8226 cells. Broad-spectrum metalloprotease antagonists have implicated a role for metalloproteases in P2X7-induced CD23 shedding from leukemic B cells [5] and RPMI 8226 cells [6], but the identity of the metalloprotease involved has remained inconclusive. A role for ADAM10 in nucleotide-induced CD23 shedding was described for human leukemic monocytic U937 cells using an inhibitory prodomain construct of ADAM10, A10-(23-213) [11], and for CD23-transfected Chinese hamster ovary cells or murine B cells using GI254023X [19,20]. However, a direct role for P2X7 in this process was not established in any of these studies. Therefore, using the ADAM10 inhibitor, GI254023X [18], we show for the first time that ADAM10 mediates P2X7-induced CD23 shedding.

P2X7 activation induces ADAM10-mediated CXCL16 and CD23 shedding from human RPMI 8226 cells. The role of ADAM10 in this process is consistent with ADAM10 mediating P2X7-induced shedding of the interleukin-6 receptor from murine splenic T cells, and from P2X7/interleukin-6 receptor co-transfected HEK293 and NIH323 cells [9]. Nevertheless the possibility remains, that other ADAMs may be partly involved in P2X7-induced CD23 and CXCL16 shedding. ADAM 8, 15, 28 and 33 [10,21] have been associated with CD23 shedding from CD23-transfected HEK293 cells [21] and primary murine embryonic fibroblasts [10], while ADAM17 can mediate phorbol ester-induced CXCL16 shedding in CXCL16-transfected COS-7 cells [3,18].

P2X7 plays important roles in various inflammatory and autoimmune disorders including rheumatoid arthritis [4]. The role of P2X7 in these disorders has largely been attributed to the release of the proinflammatory cytokine, interleukin-1β. However it is possible that P2X7-induced shedding of pro-inflammatory CXCL16 and CD23 may also be involved in inflammatory and autoimmune disorders. For example, P2X7, CXCL16, and CD23 have each been shown to play critical roles in murine models of inflammatory arthritis [22–24], while studies of human tissue have also indicated important roles for P2X7, CXCL16 and CD23 in rheumatoid arthritis. Functional P2X7 receptors are expressed on leukocytes from

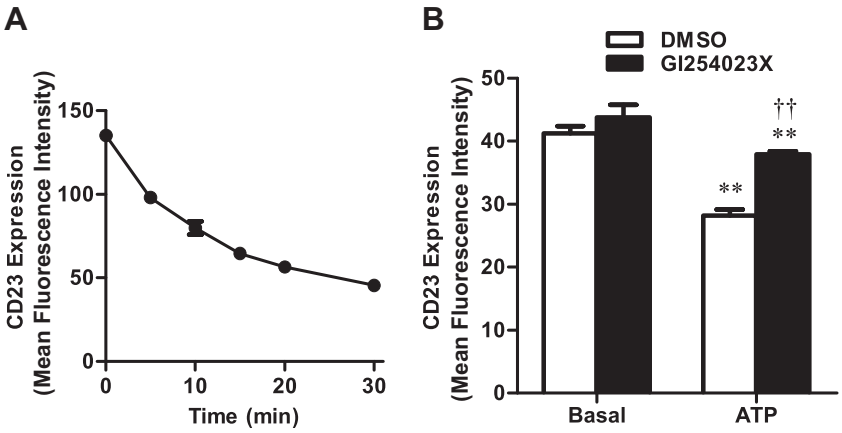

Fig. 4. ADAM10 is involved in P2X7-induced CD23 shedding from RPMI 8226 cells. (A) RPMI 8226 cells in NaCl medium were incubated for up to 30 min at 37 °C in the absence or presence of 1 mM ATP as indicated. (B) Cells in NaCl medium were pre-incubated at 37 °C for 15 min in the presence of DMSO or 3 μM GI254023X, and then in the absence (basal) or presence of 1 mM ATP at 37 °C for 7 min. (A) and (B) Incubations were stopped and cells were then labelled with PE-conjugated anti-CD23 or isotype control mAb, and the MFI of cell-surface CD23 expression determined by flow cytometry. (A, B, D–G) Results are mean ± SD ($n = 3$); $^{**}P < 0.01$ compared with corresponding basal; $^{\dagger\dagger}P < 0.01$ compared with corresponding ATP with DMSO.

rheumatoid arthritis patients [25] and human rheumatoid synoviocytes [26], while amounts of soluble CXCL16 and CD23 are increased within rheumatoid arthritis synovial fluid [24,27–29]. However, evidence directly linking these three molecules to each other in inflammatory or rheumatoid arthritis is lacking. Therefore, understanding the mechanisms and processes regulating P2X7-induced shedding of CXCL16 and CD23 may provide insight into this and other diseases.

In conclusion, this study demonstrates and confirms that human P2X7 activation induces the rapid shedding of CXCL16 and CD23, respectively. Moreover, the study indicates a role for ADAM10 in both these processes.

Acknowledgments

This project was funded by the University of Wollongong. We gratefully acknowledge GlaxoSmithKline for providing GI254023X, and the staff of Illawarra Health and Medical Research Institute for excellent technical assistance.

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