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The role of P2X7 in red blood cell biology

Bin Wang
University of Wollongong

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Faculty of Sciences
School of Biological Sciences

The Role of P2X7 in Red Blood Cell Biology

Bin WANG, Bachelor of Sciences

"This thesis is presented as part of the requirements for the award of the Degree of Master of Sciences (Research) of the University of Wollongong"

May 2011
DECLARATION

The work described in this thesis was carried out on a full-time basis by the candidate under the supervision of Dr. Ronald Sluyter in the Department of Biological Sciences, University of Wollongong. The studies described in this thesis have not previously been submitted for a degree at this or any other university. The experiments undertaken are my own original work unless otherwise referenced or acknowledged.

Bin WANG
Red blood cells (RBCs) are essential for human health, and defects in RBC development and death give rise to various disorders including anaemia and erythrocytosis. Mature RBCs or erythrocytes contain high amounts of adenosine 5’-triphosphate (ATP) which can be released under various physiological and pathophysiological conditions. Once released, ATP triggers the activation of purinergic P2 receptors in an autocrine or paracrine fashion. The P2X7 receptor is a ligand-gated ion channel belonging to the P2X receptor family. P2X7 activation induces a number of downstream effects including reactive oxygen species (ROS) formation and cell death. P2X7 is predominantly expressed on haematopoietic cells including RBCs. However, the physiological and pathophysiological roles of P2X7 on RBCs remain poorly defined. Recent works from our laboratory have identified the presence of P2X7 on an immature RBC model, murine erythroleukemia (MEL) cells. Activation of P2X7 in this cell line induces ethidium+ uptake, phosphatidylinerine exposure, and cell death. This study aims to 1) to determine if activation of P2X7 induces ROS formation in MEL cells; 2) to determine the mechanism(s) of P2X7-induced death of MEL cells.

RT-PCR confirmed the presence of P2X7 in MEL cells. Cytofluorometric cation uptake assay confirmed the function of P2X7 in MEL cells. ATP induced the uptake of ethidium+ and Yo-Pro-12+ in MEL cells, and this uptake was blocked by the P2X7 antagonist, A-438079. A cytofluorometric assay using the ROS sensitive probe 2’, 7’-dichlorodihydrofluorescein diacetate demonstrated that ATP induced ROS formation.
formation in MEL cells in a time- and concentration-dependent fashion with an EC$_{50}$ of ~150 μM. The most potent P2X7 agonist 3′-O-(4-benzoyl)benzoyl-adenosine 5′-triphosphate, but not adenosine 5′-diphosphate or uridine 5′-triphosphate, also induced ROS formation. Moreover, ATP-induced ROS formation was impaired by the P2X7 antagonist, A-438079. ATP-induced ROS formation was impaired by the broad spectrum ROS inhibitors, N-acetyl-l-cysteine (NAC) and diphenyleneiodonium (DPI), and the mitochondrial complex I inhibitor, rotenone, but not by the NADPH oxidase inhibitor, apocynin. None of these compounds impaired ATP-induced ethidium$^+$ uptake. Finally, ATP-induced ROS formation was not dependent on extracellular Ca$^{2+}$ influx or intracellular K$^+$ efflux.

The colourmetric MTT assay indirectly confirmed that ATP impaired MEL cell growth. Moreover, cytofluorometric measurements of Annexin-V binding and 7-aminoactinomycin staining directly confirmed that ATP induced death of MEL cell. A-438079 impaired ATP-induced cell death in both of these assays. Flow cytometry also showed that MEL cells pre-treated with ATP were phagocytosed by J774A.1 macrophage and that this process was impaired by incubation of MEL cells with A-438079 prior to ATP treatment. ATP-induced death of MEL cells was impaired by the broad spectrum caspase inhibitor, Z-VAD-FMK and mitogen-activated protein kinase (MAPK) p38 inhibitors, SB202190 and SB203580. None of these compounds impaired ATP-induced ethidium$^+$ uptake. Moreover, a cytofluorometric assay and phospho-immunoblotting demonstrated that ATP induced caspase and p38 MAPK activation in MEL cells respectively, and that these processes were impaired by A-438079. In contrast, NAC did not impair the ATP-induced death of MEL cells.
In conclusion, activation of P2X7 induces ROS formation via a mitochondrial pathway that is independent of extracellular Ca$^{2+}$ influx and K$^+$ efflux. Moreover, P2X7 induces death of MEL cells, which can result in their phagocytosis by J774A.1 macrophages. Finally, P2X7 induced MEL cell death is mediated by apoptotic pathways involving p38 MAPK and caspases, but not ROS formation. This study supports a role of P2X7 in RBC development and homeostasis.
ACKNOWLEDGEMENTS

I wish to sincerely thank my supervisor Dr. Ronald Sluyter for his support and guidance both before and during my master candidature. His never-failing sense of enthusiasm has been a continuing source of inspiration.

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Much gratitude also goes to the many other members of the School of Biological Sciences, and the Illawarra Health and Medical Research Institute.

Finally, I would like to thank my family and Fan Zhang who have supported and cared for me during the study and lifelong.
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α,β-meATP</td>
<td>α,β-methylene adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>μM</td>
<td>10^{-6} M</td>
</tr>
<tr>
<td>2-MeSADP</td>
<td>2-methylthio- adenosine 5’-diphosphate</td>
</tr>
<tr>
<td>2-meSATP</td>
<td>2-methylthio- adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>7AAD</td>
<td>7-amino-actinomycin-D</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5’-diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine 5’-monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BzATP</td>
<td>2(3’)-O-(4-benzoylbenzoyl) adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3’, 5’-cyclic monophosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DCF</td>
<td>2’,7’-dichlorofluorescein</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DPI</td>
<td>Diphenyleneiodonium</td>
</tr>
<tr>
<td>EC_{50}</td>
<td>concentration of a drug that produce 50% of the maximum Response</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis-(2-aminoethyl)-N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>Fig</td>
<td>figure</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>H_2DCFDA</td>
<td>2’,7’-dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEL</td>
<td>Murine erythroleukemia</td>
</tr>
<tr>
<td>mM</td>
<td>$10^{-3}$ M</td>
</tr>
<tr>
<td>mRNA</td>
<td>message RNA</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>Min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>OxATP</td>
<td>adenosine 5'-triphosphate-2',3'-dialdehyde</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>RBCs</td>
<td>Mature red blood cells</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5'-triphosphate</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine 5'-diphosphate</td>
</tr>
</tbody>
</table>
1 GENERAL INTRODUCTION

1.1 Red blood cells

1.1.1 Red blood cells and diseases

Mature red blood cells (RBCs) or erythrocytes are biconcave discs devoid of a nucleus, mitochondria and other internal organelles (Klinken 2002). Erythrocytes are essential for transporting O$_2$ and CO$_2$ throughout the body and regulating vascular tone (Ellsworth et al. 2009). The erythrocyte bilayer membrane is composed of protein, lipid and carbohydrate. More than 95% of cytoplasmic protein is hemoglobin, which is crucial for gaseous exchange by the erythrocytes.

Defects in erythrocytes result in a number of haematological disorders. The most common disorders associated with RBCs are the anaemias, characterized by a decrease in erythrocyte number or haemoglobin concentration, which are caused by impaired erythropoiesis, increased erythrocyte destruction, decreased haemoglobin synthesis or combination of these (Means Jr. 1999). In contrast, the overproduction of RBCs causes erythrocytosis (Prchal and Sokol 1996).

1.1.2 Life cycle of red blood cells

RBCs are produced from haemopoietic stem cells originating from the bone marrow. RBCs proliferate and differentiate through a series of stages, consisting of progenitor cells, proerythroblasts, erythroblasts, reticulocytes then eventually erythrocytes. This process (erythropoiesis) is tightly regulated by the primary hormone, erythropoietin.
(Klinken 2002). Human RBCs have a life-span of approximately 120 days after which senescent RBCs are removed from the circulation by the spleen and liver. The process of programmed death of RBCs (eryptosis) is characterized by cell shrinkage, membrane blebbing, activation of proteases, phosphatidylserine (PS) exposure on the outer membrane leaflet and haemolysis. Exposed PS is recognized by macrophages and subsequently phagocytosed (Bratosin et al. 2001; Lang et al. 2008). Eryptosis is similar to apoptosis of nucleated cell (Foeller et al. 2008). Defect in eryptosis lead to development of anemia or erythrocytosis (Klinken 2002).

1.1.3 Murine reticulocyte-like erythroleukemia cells

Murine erythroleukemia (MEL) cells originate from virus-transformed spleen hematopoietic cells, which grow in suspension cultures and differentiate into terminally differentiated orthochromatophilic erythroblasts following activation by a variety of compounds including dimethyl sulfoxide (Friend et al. 1971). MEL cells have been extensively used as a model of immature RBCs and RBCs (Tsiftsoglou et al. 2003).

1.1.4 Red blood cells and ATP release

Erythrocytes contain high levels of adenosine 5’-triphosphate (ATP) that are produced primarily by membrane-bound glycolytic pathways. Erythrocytes release ATP upon various physiological stimuli including decreased oxygen tension, shear-stress, hypoxia and hypercapnia and infection (Bergfeld and Forrester 1992; Subasinghe and Spence 2008; Wan et al. 2008). The ATP release pathway involves the activity of heterotrimeric G proteins Gs and Gi and the adenylyl cyclase, cycle
adenosine 5’-monophosphat (cAMP) and protein kinase A activity (Olearczyk et al. 2001; Sprague et al. 2002; Olearczyk et al. 2004; Sprague et al. 2006). ATP release also involves the cystic fibrosis transmembrane conductance regulator (Sprague et al. 1998). However, the gap junction protein, pannexin-1, has recently been suggested to have a role in ATP release from erythrocytes (Locove et al. 2006). In erythrocytes, pannexin-1 forms a mechanosensitive ATP-permeable channel that mediates osmotic-induced ATP release (Bao et al. 2004). Once released, extracellular ATP can interact with purinergic P2 receptors present on the endothelium to elicit both endothelium-dependent and smooth muscle cell-dependent vasoactive responses, result in relaxation and dilation (Ellsworth et al. 2009). Apart from physiological ATP release from erythrocytes, lysis of erythrocytes provides another mechanism of extracellular ATP, which may activate P2 receptor on leukocytes. For example, ATP released from lysed erythrocytes is sufficient to activate P2X7 receptor on murine T cells (Scheuplein et al. 2009).

1.2 P2 receptor on red blood cells

1.2.1 P2X and P2Y receptors

Purinoceptors are classified as P1 purinoceptors, which are activated most potently by adenosine and P2 purinoceptors which are activated preferentially by ATP or other nucleotides. P2 receptors can be sub-divided into P2X and P2Y receptors, which are trimeric ligand-gated ion channels and G protein-coupled receptors, respectively. (Burnstock 2008).
Figure 1-1 Extracellular nucleotide signaling via P2X and P2Y receptor. Schematic structure of P2X (Left) and P2Y (Right) receptor are shown. Adenosine 5’-triphosphate (ATP) is converted to adenosine 5’-diphosphate (ADP) as well as adenosine 5’-monophosphate (AMP) and adenosine (not shown) by ecto-enzymes. ATP acts on P2X and some P2Y receptors; most P2Y receptors are activated by either ADP, uridine 5’-triphosphate (UTP) or uridine 5’-triphosphate-glucose (UDP-glucose).

The P2X receptor family consists of seven subunits, which assemble as homomeric or heteromeric trimeric channels. Each subunit has two hydrophobic transmembrane domains, an extracellular loop and intracellular amino and carboxy terminus (Khakh and North 2006) (Fig.1-1). P2X receptors are selectively permeable to cations (Ca\(^{2+}\), Na\(^{+}\) and K\(^{-}\)) with each subunit displaying unique pharmacological profile (Table 1-1). The influx of extracellular Ca\(^{2+}\) through these channels constitutes a significant source of increase in intracellular Ca\(^{2+}\). However, membrane depolarization leads to the secondary activation of voltage-dependent Ca\(^{2+}\) channels, which probably makes the major contribution to Ca\(^{2+}\) influx and to the increase in intracellular Ca\(^{2+}\). Unlike signal transduction pathways which utilize G-protein coupled receptors, this transduction mechanism does not depend on the production and diffusion of second
Table 1-1 General nomenclature and pharmacology of P2 receptors

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>Rank Order of Agonist Activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Antagonists&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X1</td>
<td>2-meSATP &gt; ATP &gt; α,β-meATP</td>
<td>NF449, MRS2220</td>
</tr>
<tr>
<td>P2X2</td>
<td>2-meSATP &gt; ATP</td>
<td>PPADS</td>
</tr>
<tr>
<td></td>
<td>α,β-meATP inactive</td>
<td></td>
</tr>
<tr>
<td>P2X3</td>
<td>2-meSATP &gt; ATP &gt; α,β-meATP</td>
<td>RO-3, Spinorphin</td>
</tr>
<tr>
<td>P2X4</td>
<td>ATP &gt; 2-meSATP &gt; α,β-meATP</td>
<td>5-BBDBD</td>
</tr>
<tr>
<td>P2X5</td>
<td>ATP &gt; 2-meSATP &gt; ADP</td>
<td>none</td>
</tr>
<tr>
<td>P2X6</td>
<td>ATP &gt; 2-meSATP &gt; ADP</td>
<td>none</td>
</tr>
<tr>
<td>P2X7</td>
<td>BzATP &gt; ATP &gt; 2-meSATP &gt; ADP</td>
<td>KN-62, OxATP, AZ 10606120, A438790</td>
</tr>
<tr>
<td>P2Y1</td>
<td>2-MeSADP &gt; ADP &gt; 2-meSATP &gt; ATP</td>
<td>MRS2500, MRS2179</td>
</tr>
<tr>
<td></td>
<td>UTP inactive</td>
<td></td>
</tr>
<tr>
<td>P2Y2</td>
<td>4-thioUTP &gt; UTP = ATP &gt;&gt; 2-meSATP</td>
<td>Suramin</td>
</tr>
<tr>
<td></td>
<td>ADP, UDP inactive</td>
<td></td>
</tr>
<tr>
<td>P2Y4</td>
<td>UTP = UDP &gt; GTP = ATP</td>
<td>Reactive blue-2</td>
</tr>
<tr>
<td></td>
<td>UDP, ADP inactive</td>
<td></td>
</tr>
<tr>
<td>P2Y6</td>
<td>UDP &gt;&gt; UTP &gt; ADP, ATP inactive</td>
<td>MRS2578</td>
</tr>
<tr>
<td>P2Y11</td>
<td>ATP = 2-meSATP &gt; ADP</td>
<td>NF 157</td>
</tr>
<tr>
<td>P2Y12</td>
<td>2-MeSADP &gt; ADP</td>
<td>ATP, AR-C 69931</td>
</tr>
<tr>
<td>P2Y13</td>
<td>ADP &gt; ATP &gt;&gt; UDP, UTP</td>
<td>MRS 2211</td>
</tr>
<tr>
<td>P2Y14</td>
<td>UDP-glucose &gt;&gt; UTP, ATP, UDP, ADP</td>
<td>18q</td>
</tr>
</tbody>
</table>

<sup>a</sup> Table modified from (Gu 2003), (Guay et al. 2011) and Tocris data
<sup>b</sup> Abbreviations: α,β-meATP: α,β-methylene adenosine 5’-triphosphate; 2-meSATP: 2-methylthio-adenosine 5’-triphosphate; ADP: adenosine 5’-diphosphate; BzATP: 3’-O-(4-benzoyl)benzoyl-adenosine 5’-triphosphate; OxATP: adenosine 5’-triphosphate-2’,3’-dialdehyde; UDP: uridine 5’-diphosphate; UTP: uridine 5’-triphosphate
messengers within the cytosol or cell membrane, thus the response time is therefore very rapid (North 2002). Progressive dilation of the ion-conducting pathway during prolonged activation by ATP (> 30s) is a further characteristic of some P2X receptors, especially P2X7 (North 2002). This feature of P2X7 is further discussed in Section 1.3.

The P2Y receptor family consists of eight members, which under certain conditions may form homo- and hetero-multimeric assemblies (Burnstock 2008). P2Y receptors, common as other G protein coupled receptor, have seven hydrophobic transmembrane domains with an extracellular N-terminus and a cytoplasmic carboxyl terminal. The transmembrane domains are connected by three extracellular and three cytoplasmic hydrophilic loops of unequal size (Burnstock 2008) (Fig.1-1). As for P2X receptor, each P2Y receptor displays a unique pharmacological profile (Table 1-1). Each P2Y receptor binds to a single heterotrimeric G protein. The signalling pathway common to most P2Y receptors is the activation of the phospholipase C (PLC)-inositol trisphosphate (IP₃) pathway, which leads to a rise in both of diacylglycerol (DAG) and cytosolic Ca²⁺. This stimulates a variety of signalling pathways including protein kinase C, nitric oxide synthase and subsequent endothelium-derived relaxing factor formation (Burnstock 2008). P2Y receptors can also couple positively or negatively to adenylate cyclase to alter cAMP levels, respectively (Burnstock 2008). In addition, the activation of several P2Y receptors is commonly associated with the stimulation of several mitogen-activated protein kinases (MAPKs), in particular extracellular signal-regulated protein kinase 1/2 (Nicholas et al. 1996; Sabala et al. 2001).
P2X and P2Y receptors have wide cellular distributions. P2 receptors are expressed in neural cells, glial cells, epithelia, endothelia, skeletal muscle cells, and haemopoietic cells (Burnstock and Knight 2004). Activation of these P2 receptors induces a wide range of cell responses, such as neurotransmission, endocrine and exocrine secretion, and endothelial-mediated vasodilatation, platelet aggregation, immune and inflammation responses as well as cell proliferation and cell death (Burnstock and Knight 2004).

1.2.2 Expression of P2 receptors on red blood cells

RBCs from a number of species express various P2 receptors (Table 1-2). The distribution of P2X receptors on mammalian RBCs is well established through pharmacological studies and the detection of mRNA or protein. In contrast, the distribution of P2Y receptors on mammalian RBCs is mainly limited to pharmacological studies. Moreover, the distribution of P2 receptors in non-mammalian RBCs is restricted to pharmacological studies, however study in this area is limited by the lack of specific P2 antibodies and antagonists, and published gene sequences.

It is well established that mammalian RBCs express functional P2X7 receptors. Parker and Snow (1972) and others (Elford 1975; Romualdez et al. 1976) originally demonstrated that ATP increased the permeability of canine erythrocytes to Na⁺ and
<table>
<thead>
<tr>
<th>P2 subunit</th>
<th>Speciesa</th>
<th>Expressionb</th>
<th>Functionb</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X1,4</td>
<td>Human reticulocytes</td>
<td>RT-PCR</td>
<td>-</td>
<td>Hoffman et al. 2004; Wang et al. 2005</td>
</tr>
<tr>
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<td>IB, IHC</td>
<td>-</td>
<td>Sluyter et al. 2004</td>
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<td></td>
<td>Mudpuppy</td>
<td>-</td>
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<td>-</td>
<td>Hoffman et al.2004</td>
</tr>
<tr>
<td></td>
<td>Canine</td>
<td>IB, IHC</td>
<td>Cation flux; haemolysis; pore formation</td>
<td>Parker and Snow 1972; Elford 1975; Romualdez et al. 1976; Sluyter et al.2007a; Stevenson et al.2009</td>
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<td>IB</td>
<td>PS exposure; MP release; cell death</td>
<td>Chatawala and Cantley 1984; Constantinescu 2008</td>
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<tr>
<td></td>
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<td>IB</td>
<td>Haemolysis</td>
<td>Skalsa et al. 2009</td>
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<td></td>
<td>Rat</td>
<td>-</td>
<td>EET release</td>
<td>Jiang et al. 2007</td>
</tr>
<tr>
<td>P2Y1</td>
<td>Human</td>
<td>IB, RT-PCR</td>
<td>Osmolyte permeability</td>
<td>Hoffman et al. 2004; Tanneur et al. 2006</td>
</tr>
<tr>
<td></td>
<td>Murine erythroblasts</td>
<td>RT-PCR</td>
<td>iCa(^{2+}) release</td>
<td>Paredes-Gamero et al. 2006</td>
</tr>
<tr>
<td></td>
<td>Turkey</td>
<td>-</td>
<td>PLC(\beta)/IP(_{3}); iCa(^{2+}) release</td>
<td>Berrie et al. 1989; Boyer et al. 1989; Sak et al. 2000</td>
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Table 1-1 continued

<table>
<thead>
<tr>
<th>P2Y2</th>
<th>Human</th>
<th>RT-PCR, IHC</th>
<th>-</th>
<th>Wang et al. 2005; Paredes-Gamero et al. 2006; Sluyter unpublished work</th>
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</thead>
<tbody>
<tr>
<td>P2Y4</td>
<td>Lizard</td>
<td>iCa(^{2+}) release</td>
<td></td>
<td>Beraldo et al. 2001, 2002; Sartorello and Garcia 2005</td>
</tr>
<tr>
<td>P2Y12</td>
<td>Murine erythroblasts</td>
<td>RT-PCR</td>
<td>-</td>
<td>Paredes-Gamero et al. 2006</td>
</tr>
<tr>
<td>P2Y13</td>
<td>Human</td>
<td>RT-PCR</td>
<td>ATP Release</td>
<td>Wang et al. 2005</td>
</tr>
</tbody>
</table>

a Erythrocytes whereas indicated
b Abbreviations: IB, immunoblotting; IHC, immunohistochemistry; cation flux, Na\(^+\) and K\(^+\) fluxes; PS, phosphatidylserine; MP, microparticle; EET: epoxyeicosatrienoic acids; PLC, phospholipase C; IP\(_3\), inositol trisphosphate.
K+. Similarly, Chatawala and Cantley (1984) observed that ATP induced Ca\(^{2+}\), Na\(^{+}\) and K\(^{+}\) fluxes in, and impaired the growth of MEL cells. In each of these studies ATP mediated its effects in a manner characteristic of P2X7; however a role for a P2 receptor in these processes was not considered at the time. Subsequently work from our group using immunohistochemistry and immunoblotting demonstrated that both human and canine erythrocytes express P2X7 (Sluyter et al. 2004; Sluyter et al. 2007a; Stevenson et al. 2009). Moreover, cation flux measurements demonstrated that these cells express functional P2X7, with the relative amount of functional P2X7 being 100-fold higher on canine compared with human erythrocytes; which paralleled differences in the amount of P2X7 observed by immunoblotting (Sluyter et al. 2007a; Stevenson et al. 2009). The physiological explanation for this difference however remains unknown. The presence of P2X7 in RBCs was confirmed by the detection of P2X7 mRNA transcripts in human reticulocytes and erythrocytes (Hoffman et al. 2004; Wang et al. 2005). Moreover, functional P2X7 is present in rat, murine and equine erythrocytes (Jiang et al. 2006; Skalsa et al. 2009), Finally, our group has confirmed using, immunohistochemistry, immunoblotting and ethidium\(^+\) uptake measurements that MEL cells also express functional P2X7 (Constantinescu, 2008). The mechanisms by which P2X7 induces RBC death and other downstream signalling events however remains unknown.

RBCs also express functional P2X1 receptors, and possibly P2X2 and P2X4. P2X1 and P2X4 mRNA transcripts are present in human reticulocytes and erythrocytes (Hoffmann et al. 2004; Wang et al. 2005), while the presence of P2X1 in murine and human erythrocytes has been confirmed by immunoblotting (Skals et al. 2009).
Direct evidence for functional P2X4 in these cells is lacking. In contrast, the use of specific P2X antagonists indicates a role for P2X1 activation, in additional to P2X7 activation, in the α-hemolysin-induced haemolysis of murine and human erythrocytes (Skals et al. 2009). Immunohistochemistry has also demonstrated the presence of P2X2 in human erythrocytes (Sluyter et al. 2004), but direct evidence for functional P2X2 in these cells is lacking. ATP, however, stimulates a regulatory volume decrease, which reverses the hypotonic swelling of Necturus (mudpuppy) but not human erythrocytes via a receptor characteristic of P2X2 (Light et al. 1999; Light et al. 2003).

RBCs express functional P2Y1 receptors. Boyer et al. (1989) and Berrie et al. (1989) originally describe that ATP, ADP and other P2Y agonists stimulated PLC activation and subsequent IP$_3$ formation in turkey erythrocytes. P2Y1 has been further identified the presence in turkey erythrocytes by statistical analysis of the available pharmacological parameters of several purinoceptor agonists and antagonists in the turkey erythrocytes (Sak 2000). The presence of P2Y1 on RBCs was confirmed by detection of P2Y1 mRNA and proteins in the murine erythroblasts and human erythrocytes, respectively (Paredes-Gamero et al. 2006; Tanneur et al. 2006). Moreover, P2Y1 activation induces iCa$^{2+}$ release in murine erythrocytes, and opening an organic osmolyte and anion permeability pathway in malaria-infected human erythrocytes (Tanneur et al. 2006).

RBCs express functional P2Y13 receptors. High amounts of P2X7 mRNA transcripts are present in human reticulocytes and erythrocytes (Wang et al. 2005). P2Y13
functions as a negative feedback pathway for ATP release from human erythrocytes (Wang et al. 2005) and plays a role in erythrocyte-associated vascular tone (Section 1.1.4). Wang and colleagues demonstrated that the activation of P2Y13 by ADP inhibits cAMP and subsequently decreases ATP release from human erythrocytes. Moreover, ATP and ADP are degraded by ectonucleotidases to adenosine, which is quickly taken up in the RBCs where it can be recycled to ATP by the glycolytic pathway (Wang et al. 2005). Thus, ADP may control ATP levels outside the RBC by inhibition of intracellular cAMP levels via activation of P2Y13 receptors. The described negative feedback pathway may be important to avoid high extracellular concentrations of ATP. In contrast, Locove et al. (2006) proposed that the co-expression of P2Y receptors and pannexin-1 channels could mediate ATP-induced ATP release from RBCs, however there is no direct experimental evidence nor did they identify the specific P2Y receptor involved to support this proposal.

Reptile (lizard) RBCs express a functional P2Y receptor characteristic of P2Y4 (Sartorello and Garcia 2005), P2Y4 however appears to be absent from mammalian RBCs, as mRNA for this receptor is absent from human RBCs (Wang et al. 2005) and murine erythroblasts (Paredes-Garmero et al. 2006). Although the physiological role of P2Y4-like receptor in lizard RBCs is unknown, ATP triggers a rapid and transient increase of intracellular Ca2+ by mobilization of the Ca2+ from internal stores (Beraldo et al. 2001; Sartorello and Garcia 2005). Lizard RBCs also express a functional P2X receptor (Beraldo and Garcia 2007), however the identity remains unknown.
RBCs may also express P2Y2 and P2Y12 receptors, however function evidence is lacking. Both human reticulocytes and murine erythroblasts express P2Y2 and P2Y12 mRNA transcripts (Paredes-Garmero et al. 2006; Wang et al. 2005). Moreover, immunohistochemistry has demonstrated the presence of P2Y2 in a sub-population of human erythrocytes (Sluyter, unpublished).

1.3 P2X7 receptor

1.3.1 Introduction to the P2X7 receptor

The P2X7 has attracted considerable interest because of its unique biological properties. In contrast to other P2X receptors, P2X7 has a large carboxyl terminus with an extra 200 amino acid residues compared with the other receptors. The carboxyl terminus of the P2X7 is important for modulating P2X7 expression and function (Costa-Junior et al. 2011). Similar to other P2X receptors, activation of P2X7 by ATP results in the opening of a non-selective cationic channel, allowing the rapid influx of Ca^{2+} and Na^{+}, and efflux of K^{+}. However, upon prolonged stimulation (>30s), the P2X7 forms an aqueous pore that allows the passage of organic cations up to 900 Da, such as choline^{+} and the fluorescent dyes ethidium^{+} and Yo-Pro-1^{2+} (North 2002). In mice, nicotinamide adenine dinucleotide (NAD) is an alternative agonist of P2X7 activation, a process which involves ADP ribosylation. This pathway was initially shown for murine T cells which express ADP-ribosyltransferase, ART2, that catalyzes ADP ribosylation of P2X7 using NAD as a substrate (Seman et al. 2003). However, research from our group showed that 300 µM NAD^{+} failed to induce ethidium uptake into MEL cells (Constantinescu and
Sluyter, unpublished) despite observing that NAD$^+$ induced ethidium$^+$ uptake into murine T cells (Stevenson et al. 2009).

P2X7 receptors are widely expressed in cells of haematopoietic lineages, as well as osteoblasts, fibroblasts, endothelial cells, epithelial cells and neural cells (Sluyter and Stokes 2011). P2X7 activation induces a number of downstream signaling events. The best characterized signaling downstream event of P2X7 activation is mediating the processing and release of mature, biologically active interleukin (IL)-1β and IL-18 (Di Virgilio 2007). P2X7 activation can also induce metalloprotease-mediated shedding of CD62L (L-selectin) and CD23 (low affinity IgE receptor) from the cell surface (Gu et al. 1998). P2X7 activation can induce the killing of intracellular pathogen (Coutinho-Silva et al. 2009). Finally, P2X7 activation can induce other signaling downstream events including, PS exposure, plasma membrane blebbing, release of microvesicle release as well as reactive oxygen and nitrogen species formation and cell death (Adinolfi et al. 2005; Hewinson and Mackenzie 2007; Qu and Dubyak 2009).

### 1.3.2 P2X7–induced reactive oxygen species formation

Reactive oxygen species (ROS) are oxygen-derived small molecules, including the superoxide anion radical (O$_{2}^-$), hydrogen peroxide (H$_2$O$_2$) and the highly reactive hydroxyl radical (•OH). The physiological generation of ROS can occur as by-products of cellular metabolism of mitochondrial electron transport chain, peroxisomes and oxidoreductase, or by the cell membrane complex NADPH oxidase (Turrens 2003). ROS maintain an integral role in host defense, regulation of
apoptosis and cell growth, differentiation and migration, and modulation of cell signaling. In contrast, excess ROS production can cause tissue damage by reacting with membrane lipids, DNA and proteins, each of which can have implications in various clinical conditions including anemia (Simon et al. 2000; Forman and Torres 2002; Waris and Ahsan 2006; Çimen 2008; West et al. 2011).

Mitochondria are the major sources of cellular ROS formation. Several sites of mitochondrial source of ROS formation exist along the electron transport respiration chain including respiratory complexes I, II and III (Fig. 1-2) (Chen et al. 2003). These redox centres in the electron transport chain leak electrons to O₂, reducing this molecule to O₂⁻. Dismutation of O₂⁻ by superoxide dismutases produces hydrogen peroxide (H₂O₂), the main form of ROS (Turrens 2003).

Figure 1-2. Sites of ROS formation in the mitochondrial electron transport chain. Respiratory complexes I, II and III leak electrons to oxygen (O₂) producing the superoxide anion (O₂⁻). This species may be converted to hydrogen peroxide (H₂O₂) and O₂ (in both the matrix and the intermembrane space). Figure modified from (Turrens 2003).
Several factors induce ROS formation. Excess ROS are produced by exposure to ionizing and ultraviolet radiation, and during the metabolism of a wide spectrum of drugs and xenobiotics. Antioxidant enzyme systems exist to neutralize ROS, these include superoxide dismutases, glutathione peroxidases and catalase (Turrens 2003). Under normal conditions there is a critical balance between basal ROS formation and antioxidants. Oxidative stress occurs when this balance is disrupted because of excess ROS formation, defects in antioxidants, or both (Turrens 2003). Mitochondria function and intramitochondria Ca$^{2+}$ play an important role in ROS formation. (Brookes et al. 2004; Feissner et al. 2009). The underlying mechanism is still unclear, however, experimental evidence indicate that overloaded mitochondrial Ca$^{2+}$ leads ROS formation. Theoretically, increasing Ca$^{2+}$ would enhance the mitochondrial metabolic rate, resulting in increased respiratory chain electron leakage and subsequent ROS formation. Ca$^{2+}$ can also stimulate activity of nitric oxide synthase to generate NO$, which inhibits complex IV, and enhance ROS formation. Finally, Ca$^{2+}$ can enhance cytochrome c dislocation from the mitochondrial inner membrane, blocking respiratory chain at complex III, and enhancing ROS formation (Chen et al. 2003).

In addition to mitochondria, NADPH oxidases are also the main sites of ROS formation. NADPH oxidases are cell membrane enzyme complexes specifically dedicated to ROS production. The structure of NADPH oxidase consists of several subunits (Fig.1-3). Upon assembly of these subunits in the membrane, this enzyme via its subunit NOX2 (gp91$^{phox}$ ) generates a burst of O$_2^-$ on the extracellular side of the membrane by one-electron reduction of O$_2$ (Dusting et al. 2005). NADPH
oxidase proteins are present in a variety of cells and tissue and are thought to play specific roles of ROS production (Bedard and Krause 2007). As for mitochondrial ROS formation, NADPH oxidase-derived ROS formation requires the antioxidant enzyme systems to avoid excess ROS formation.

Figure 1-3. NADPH oxidase-derived ROS formation. NADPH oxidase complex consists of cytosolic subunits p47phox, p40phox and p67phox, two cell membrane bound subunits NOX2 (gp91phox) and p22phox, and a small G protein Rac. O$_2^-$ is produced on the extracellular side of the membrane by one-electron reduction of O$_2$ via the NOX2 subunit, using reduced NADPH as the electron donor. Figure modified from (Dusting et al. 2005)

P2X7 induces ROS formation in a variety of immune and non-immune cells to participate in P2X7-induced pro-inflammatory cytokine release and cell death (Hewinson and Mackenzie 2007). The involvement of P2X7 activation in ATP mediated ROS generation has been largely demonstrated by pharmacological
approaches in human neutrophils (Suh et al. 2001), murine macrophages (Moor et al. 2009, Noguchi et al. 2008), rat microglia (Parvathenani et al. 2003), human eosinophils (Ferrari et al. 2000), and rat and mouse submandibular cells (Seil et al. 2008; Fontanils et al. 2010). The role of P2X7 activation in ROS formation is further supported by comparison of ATP-induced responses in a murine macrophage cell line deficient in P2X7 with the P2X7-expressing RAW264.7 line (Pfeiffer et al. 2007).

The NADPH oxidase plays a dominant role in P2X7-induced ROS formation in macrophages and microglia (Hewinson and Mackenzie 2007). This conclusion is primarily based on the use of pharmacological inhibitors of the NADPH oxidase. However, Noguchi et al. (2008) and Moor et al. (2009) used NOX2 knockout mice to support the role of the NADPH oxidase in P2X7-induced ROS formation in macrophages. Moreover, stimulation of microglia with the most potent P2X7 agonist, BzATP triggers the translocation of p67\textsuperscript{phox} to the plasma membrane, which is a critical step in the activation of the NADPH oxidase (Parvathenani et al. 2003). In contrast to the NADPH oxidases, there is little evidence for a role for P2X7 in mitochondrial ROS formation. P2X7-induced ROS formation, however, is impaired by a mitochondria complex I inhibitor in murine bone marrow-derived macrophages, but not in J774 macrophages (Moore et al. 2009). Thus, NADPH oxidase appears to be the major mechanism for P2X7-induced ROS formation.

In addition to the NADPH oxidase, Ca\textsuperscript{2+} influx also may be important for P2X7-induced ROS formation. Removal of extracellular Ca\textsuperscript{2+} reduces P2X7-induced ROS
formation in murine macrophages (Guadalupe et al. 2010), murine microglia (Parvathenani et al. 2003) and rat submandibular cells (Fontanils et al. 2010). In contrast, P2X7-induced ROS in mouse submandibular cells is independent of extracellular Ca$^{2+}$ (Seil et al. 2008), indicating that the effect of Ca$^{2+}$ is cell and/or species specific, or that multiple pathways exist.

1.3.3 P2X7-induced cell death

Cell death plays an important role in the development and homeostasis of multicellular organisms (Krysko et al. 2008). According to the Nomenclature Committee on Cell Death (NCCD) (Kroemer et al. 2009), types of cell death can be classified as: apoptosis (type I), cell death associated with autophagy (type II), necrosis or oncosis (type III). Cells undergoing apoptosis show typical, well-defined morphological changes, including, cell shrinkage, plasma membrane blebbing, lost plasma integrity, chromatin condensation, DNA fragmentation and formation of apoptotic bodies (Krysko et al. 2008). Apoptosis has also been characterized by several biochemical criteria, including PS exposure on the outer leaflet of the plasma membrane, changes in mitochondrial membrane permeability, caspase activation and cytochrome c and bc1 release. In contrast, necrosis is characterized by rapid cytoplasmic swelling, cell lysis, breakdown and release of intracellular organelles, and a lack of caspase activation. The final fate of almost dying/dead cells regardless of death type is engulfment by phagocytes. In the absence of phagocytosis, apoptotic cells proceed to a stage called secondary necrosis, which shares many features of primary necrosis (Krysko et al. 2008).
Various methods have been developed to detect cell death. The most convenient technique to study cell death on a per-cell basis is the combined use of fluorochrome conjugated Annexin-V and with exclusive dye such as propidium iodide or 7-aminoactinomycin (7AAD) (Krysko et al. 2008).

Colourimetric assays, such as the lactate dehydrogenase (LDH) release and thiazolyl blue tetrazolium bromide (MTT) assays are relatively economical methods to measure cell death. The LDH release assay assesses the activity of LDH in culture supernatants released from cytoplasm following cells lysis. The MTT assay measures the mitochondrial metabolical activity as an indicator of the number of viable cells, and hence can measure cell death or proliferation (Kroemer et al. 2009).

DNA fragmentation is often used to distinguish apoptosis from necrosis. This is often detected by agarose gel electrophores. However, a more sensitive and quantitative way is by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling method and flow cytometry (Krysko et al. 2008).

DNA fragmentation, PS exposure, caspase activation and above morphological changes are considered to be features of apoptosis. LDH release is a feature of necrosis. The descriptions of apoptosis or necrosis in studies of cell death remain poor, which is attributed in part to limited use of experimental parameters. Therefore, the NCCD recommends “investigators studying cell death to quantify this process using more than one assay whenever possible” (Kroemer et al. 2009).
ATP-induced cell death was initially documented in murine lymphocytes (Di Virgilio et al. 1989; Zanovello et al. 1990) and then extended to a variety of immune cells, neural cells and epithelial cells as well as RBCs (Table 1-3). The involvement of P2X7 in ATP-induced cell death has largely been established through the use of P2X7 agonists and antagonists. However, a role for this receptor in this process has been confirmed by use of cells from P2X7 knockout mice (Le Feuvre et al. 2002) and subjects coding loss-of-function P2X7 polymorphisms (Gu et al. 2001; Fernando et al. 2005). P2X7-induced cell death can be initialized by either spontaneous or stimulated ATP release through an autocrine or paracrine loop (Chiozzi et al. 1997).

For example, basal ATP release induced the spontaneous death of murine macrophages which was significantly reduced by incubation with P2X7 antagonists or the ATP-hydrolyzing enzyme apyrase. NAD+ can also induce death of murine T cells at lower concentration, compared to ATP (Seman et al. 2003). However, whether P2X7 induces cell death differ in each study is mediated by apoptosis or necrosis remains controversial.

Di Virgilio et al. (1998) suggested that the type of cell death induced by P2X7 contribution depends upon the ATP incubation time or concentration, and/or cell type. Moreover, the mechanisms by which P2X7 activation induces either apoptosis or necrosis, and the cells involved varies (Table 1-3), with the pathways involved
Table 1-3 P2X7 activation induces cell death in different cell types

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Detection hallmarks</th>
<th>Signal transduction</th>
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<tbody>
<tr>
<td>P-815 mastocytoma</td>
<td>DNA fragmentation; Cr51 release</td>
<td>-</td>
<td>Di Virgilio et al. 1989</td>
</tr>
<tr>
<td>YAC lymphoma cells</td>
<td>MC; PS exposure/PI</td>
<td>Mitochondria; ceramide</td>
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<td>Thymocytes</td>
<td>7AAD</td>
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<td>Gu et al. 2001</td>
</tr>
<tr>
<td>T cells</td>
<td>MC; LDH release</td>
<td>CI ; ERK</td>
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<tr>
<td>Lymphocytes</td>
<td></td>
<td>-</td>
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<td>Monocytes/macrophages</td>
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<td>Fernando et al. 2005</td>
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<tr>
<td>Peritoneal macrophages</td>
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<td>Caspase-1; iPLA; LOX-5</td>
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<td>BMDMs</td>
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<td>Caspase-1</td>
<td>Moore et al. 2007</td>
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<td>J774A.1 macrophages</td>
<td></td>
<td>ROS; caspase-1; cathepsin</td>
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<td>BAC1.2F5 macrophages</td>
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<td>JNK; Caspase-1/3</td>
<td>Humphreys et al. 2000</td>
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<td>RAW 264.7 macrophages</td>
<td>LDH release; DNA fragmentation</td>
<td>PLD; ROS; ASK1,</td>
<td>Stuff et al. 2007, Noguchi et al. 2008</td>
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<td>Spleen dendritic cells</td>
<td>MC; PI; DNA fragmentation</td>
<td>p38; Caspase-3</td>
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<td>Caspase-3/9; Ca^{2+}</td>
<td>Wang et al. 2004</td>
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<tr>
<td>Neoplastic cells</td>
<td>DNA fragmentation</td>
<td>Caspase-3/9; Ca^{2+}</td>
<td>Fu et al. 2009</td>
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<tr>
<td>Cell Type</td>
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<td>Caspase Activities</td>
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<td>Ferrari et al. 2007</td>
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<td>Caspase-8/9/3; JNK; ERK</td>
<td>Kong et al. 2005</td>
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<td>RPMI-8226 myeloid cells</td>
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<td>Constantinescu 2008</td>
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<td>P2X7-transfected DT 40 lymphocytes cells</td>
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<td>Cl⁻</td>
<td>Tsukimoto et al. 2005</td>
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Abbreviations: 7AAD: 7-aminoactinomycin; BMDMs: Bone Marrow Derived macrophages; Hb: haemoglobin; iPLA₂: Calcium-independent-phospholipase; LDH: lactate dehydrogenase; LOX: lipoxygenase; A2: MTT: thiazoly blue tetrazolium bromide; MC: Morphological Changes, including, cell shrinkage, chromatin condensation, formation of apoptotic bodies; PI: propidium iodide; PS: phosphatidylserine
only partly defined. The best characterised pathways, each representing different cell types, are outlined below and summarised in Fig.1-4.

One of the best characterised P2X7-induced cell death pathways is described in myeloid cells. In murine RAW241.7 macrophages, P2X7-induced cell death pathway involves NADPH oxidase-induced ROS formation following by the activation of ASK1 activation and MAPK family p38 and the subsequent activation of caspase (Noguchi et al. 2008) (Fig. 1-4). The involvement of these compounds was established through the use of pharmacological blockage, as well as RAW264.7 cells deficient in NADPH oxidase subunit, NOX2 and ASK1. However, the role of JNK was excluded in this pathway. In contrast, Humphreys et al. (2000) revealed that P2X7-induced death of BAC1 macrophage involves pathways dependent on JNK and caspase-3 activation. In an early observation, Ferrari et al. (1999) demonstrated that P2X7-induced death of murine microglia cells involves the caspase-8 and caspase-3 (presumably downstream of caspase-8), and the subsequent processing of the caspase substrates Poly (ADP-ribose) polymerase and lamin B. Caspase-1 was also activated in these cells, however the authors assumed that this was related to IL-1β processing and not cell death. However, P2X7-induced necrosis (LDH) release is impaired in murine macrophages from caspase-1 knockout mice (Le Feuvre et al. 2002), thus a role for caspase-1 in P2X7-induced cell death remains unclear. Nevertheless, these results confirm that P2X7-induced cell death in various myeloid cells involves caspase-3.
P2X7-induced cell death in different cell types involves different signalling pathways. P2X7 activation induces various pathways which culminate in caspase-3 activation and cell death. (Left) P2X7-induced death of macrophage, and possibly microglia, depends on NADPH oxidase-generated ROS and ASK1-p38 pathway. (Middle) P2X7-induced death of epithelial cells involves the influx of Ca\(^{2+}\), the mitochondria and subsequent caspase-9. (Right) P2X7-induced death of neuronal cells depends on the ERK1/2 and JNK1 pathway and subsequent activation of caspase-8/9.

P2X7-induced cell death has also been described in non-myeloid cells. P2X7-induced death of epithelial cells is mediated by the mitochondrial apoptotic pathway (Fig. 1-4). P2X7-induced death of human cervical epithelial cells and murine keratinocytes requires an influx of Ca\(^{2+}\) and the subsequent activation of caspase-9 and caspase-3 (Wang et al. 2004; Fu et al. 2009). The activation of caspase-9 highly suggests that
P2X7-induced death of these cells is mediated predominantly by mitochondrial apoptotic pathway in these cells. Finally, P2X7-induced death of neuronal cells is mediated by ERK1/2 and JNK1, and the activation of caspase (Fig. 1-4). This pathway was demonstrated and involved the intrinsic caspase-8/9/3 activation pathway (Kong et al. 2005). Moreover, mitogen-activated protein kinases family numbers ERK1/2 and JNK1 up-regulate the P2X7-induced caspase activation in these cells.

1.4 Concluding remarks and aims

RBCs are essential for human health and defects in RBC development and cell death give rise to various disorders including anaemia and erythrocytosis. The presence of P2X7 on RBCs suggests a role for this receptor in RBC homeostasis and disorders. However, the physiological and pathophysiological roles of the P2X7 receptor on RBCs remain poorly defined. Chatawala and Cantley (1984) observed that extracellular ATP can induce Ca^{2+}, Na^{+} and K^{+} fluxes in, and impair the growth of MEL cells, a model of immature RBCs. Recently, our group demonstrated that this cell expresses the P2X7, and activation of this receptor induces ethidium^+ uptake and cell death in this cell line (Constantinescu 2008). Thus, the present study aims 1) to determine if activation of P2X7 induces ROS formation in MEL cells; 2) to determine the mechanism of P2X7-induced cell death of MEL cells.
2 MATERIALS AND METHODS

2.1 Materials

RPMI-1640 medium, fetal calf serum (heat inactivated before use), L-glutamine, gentamicin, trypsin, Yo-Pro-1^{2+}, the reactive oxygen species (ROS) detection probe 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) and thiazolyl blue tetrazolium bromide (MTT) were from Invitrogen (Grand Island, NY). Adenosine 5'-triphosphate (ATP), 3'-O-(4-benzoyl)benzoyl-adenosine 5'-triphosphate (BzATP), adenosine 5'-diphosphate (ADP), uridine 5'-triphosphate (UTP), rotenone, ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), PKH-26 Red Fluorescent Cell Linker Kit and 2-mercaptoethanol were from Sigma (St. Louis, MO). A-438079 was from Tocris Bioscience (Ellisville, MO). Bovine serum albumin (BSA) and ethidium bromide were from Amresco (Solon, OH). SB203580 was purchased from Jena Bioscience (Jena, Germany). N-acetyl-L-cysteine (NAC) and 7-aminoactinomycin D (7AAD) were from Alexis Biochemicals (Lausen, Switzerland). SB202190 and apocynin were from Calbiochem (Darmstadt, Germany). Z-VAD-FMK and CaspGLOW<sup>TM</sup> Fluorescein Active Caspase Staining Kit were from Biovision (San Diego, CA). Annexin-V-FLUOS and DNase I were from Roche Diagnostics (Pensberg, Germany). Diphenyleneiodonium (DPI) was from Cayman Chemical (Ann Arbor, MI). PhosphoPlus<sup>®</sup> p38 MAPK (Thr180/Tyr182) Antibody Kit was purchased from Cell Signaling Technology (Beverley, MA). Amersham Hyperfilm ECL was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Nitrocellulose membrane was from Bio-Rad (Hercules, CA). Other reagent grade chemicals were from Sigma or Amresco (Solon, OH).
2.2 Cell culture

RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA). Murine erythroleukemia (MEL) cells were kindly provided by Dr. Sally A. Eaton (The University of Sydney, Sydney, Australia). The murine macrophage cell line J774A.1 originally obtained from the American Type Culture Collection (Manassas, VA), was kindly provided by Ms. Jasmyn Dunn (The University of Queensland, Brisbane, Australia). All Cells were maintained in complete culture medium (RPMI-1640 medium containing 10% fetal calf serum, 5 mM L-glutamine and 5 mg/mL gentamicin) at 37 °C and 95% air/5% CO₂. RAW264.7 cells and J774A.1 cells were harvested by mechanical scraping.

2.3 Fluorescent cation dye uptake assay

P2X7-induced pore formation was measured to assess the P2X7 function using a fixed-time assay as described (Farrell et al. 2010). Cells (1×10^6 cells/mL), resuspended in NaCl medium (145 mM NaCl, 5 mM KCl, 5 mM glucose, 0.1% BSA and 10 mM HEPES, pH 7.5) or KCl medium (150 mM KCl, 5 mM glucose, 0.1% BSA and 10 mM HEPES, pH 7.5), were incubated with 25 μM ethidium⁺ (or 1 μM Yo-Pro-1²⁺ as indicated) in the absence or presence of nucleotides (as indicated) for 15 min at 37°C. Incubations were stopped by addition of an equal volume of ice cold NaCl medium containing 20 mM MgCl₂ (MgCl₂ medium) and centrifugation (300×g for 5 min). Cells were washed once with NaCl medium. In some experiments, cells were pre-incubated at 37°C in the absence or presence of 10 μM A-438079 for 15 min, or ROS, caspase and p38 MARK inhibitors (as indicated) before the addition of ethidium⁺. In some experiments, cells were resuspended in NaCl medium containing 1 mM CaCl₂ or 100
μM EGTA before the addition of ethidium\textsuperscript{+}. Since free Ca\textsuperscript{2+} lowers the concentration of ATP\textsuperscript{4−}, cells in the presence of 1 mM Ca\textsuperscript{2+} were incubated with 1.74 mM ATP, to provide equimolar ATP\textsuperscript{4−} concentrations (251 μM). The ATP\textsuperscript{4−} concentration in solution was calculated using the Bound and Determined Program (Marks and Maxfield 1991).

Data was collected by using a LSR II flow cytometer (BD Biosciences, San Diego, CA) and the mean fluorescence intensity (MFI) of ethidium\textsuperscript{+} or Yo-Pro-1\textsuperscript{2+} uptake determined using FlowJo software (Tree Star, Ashland, OR).

### 2.4 Reactive oxygen species formation assay

Nucleotide-induced ROS formation was assessed in MEL cells loaded with the ROS-sensitive compound, H\textsubscript{2}DCFDA, according to manufacturer’s instruction. Cells resuspended in NaCl medium (1×10\textsuperscript{6} cells/mL) were incubated with 5 μM H\textsubscript{2}DCFDA for 5 min at 37°C under limited light conditions. Incubations were stopped by centrifugation (300×g for 5 min). H\textsubscript{2}DCFDA-loaded cells, resuspended in NaCl or KCl medium without BSA were then incubated with in the absence or presence of nucleotides (as indicated) for 15 min at 37°C. Incubations were stopped by addition of an equal volume of ice cold MgCl\textsubscript{2} medium and centrifugation (300×g for 5 min). Cells were washed once with NaCl medium. In some experiments, cells were pre-incubated at 37°C in the absence or presence of 10 μM A-438079 for 15 min or various ROS inhibitors (as indicated) before the addition of nucleotide. In some experiments, cells were resuspended in NaCl medium containing 1 mM CaCl\textsubscript{2} or 100 μM EGTA before ATP addition. As above (Section 2.2), cells in the presence of 1 mM Ca\textsuperscript{2+} were incubated with 1.74 mM ATP, to provide equimolar ATP\textsuperscript{4−} concentrations (251 μM).
Data was collected by flow cytometry and the MFI of DCF converting from H$_2$DCFDA determined using FlowJo software.

2.5 Colormetric measurement of cell growth

MEL cell growth was measured using a MTT assay as described (Farrell et al. 2008). MEL cells ($5 \times 10^5$ cells/mL/well) in complete culture medium in 96-well plates were incubated in the absence or presence of ATP (as indicated) for 24 h at 37°C and 95% air/5% CO$_2$. During the final 4 h of ATP incubation, 0.5 mg/mL MTT was added (10 μL/well). Cells were then incubated with 10 mM HCl containing 10% SDS (solubilization solution) (100 μL/well) overnight at 37°C. Absorbances were read at a wavelength of 550 nm and a reference wavelength of 690 nm using a SpectaMax Plus384 microplate reader (Molecular Devices, Sunnyvale, CA).

2.6 Cytofluorometric measurement of cell death

MEL cells in complete culture medium in 24-well plates ($5 \times 10^5$ cells/mL/well) were incubated in the absence or presence of 1 mM ATP for 24 h at 37°C and 95% air/5% CO$_2$. In some experiments, cells were pre-incubated 37°C in the absence or presence of 10 μM A-438079 for 15 min or 20 μM Z-VAD-FMK, SB203580 or SB202190 for 30 min before the addition of ATP. Following 24 h incubation with ATP, cells were harvested and washed once with NaCl medium containing 5 mM CaCl$_2$. Cells in 100 μl NaCl medium containing 5 mM CaCl$_2$ were then incubated with Annexin-V-FLUOS and 7AAD for 15 min at room temperature, before addition of 400 μl NaCl medium containing 5 mM CaCl$_2$. Data was collected by flow cytometry. Cells were gated to exclude dust particles and small cellular debris (events of low forward and side scatter),
and the percentage of Annexin-V⁺/7AAD⁻, Annexin-V⁺/7AAD⁺ and Annexin-V⁺/7AAD⁺ cells within the gate determined using FlowJo software.

2.7 Phagocytosis assay

The phagocytosis of PKH-26-labelled MEL cells by J774A.1 macrophages was adapted from a previously described method for erythrocytes (Schrijvers et al. 2004), except MEL cells were labelled with PKH-26 using the manufacturer’s reagents as described (Healey et al. 2007). Cells (2×10⁷) were washed twice with serum-free RPMI-1640 medium and incubated in 2 mL of 2 μM PKH-26 in Diluent C (as provided) for 5 min at room temperature with gentle mixing. The reactions were stopped by adding an equal volume (2 mL) of serum, incubating for 1 min at room temperature, diluting to 10 mL with complete culture medium and centrifugation (400×g for 10 min). The cells were washed twice with complete culture medium as above. The labelled cells were then suspended in complete culture medium, and incubated in 24-well plates in the absence or presence of 1 mM ATP for 24 h at 37°C and 95% air/5% CO₂. The following day, PKH-26-labeled MEL cells were washed three times and suspended in complete culture medium. Cells (2×10⁶ cells/mL/well) were then incubated with adherent J774A.1 cells in a 24-well plate (2×10⁵/mL/well) for 1 h at 37°C and 95% air/5% CO₂. The wells were washed five times with cold PBS (137.9 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄ and 8.1 mM Na₂HPO₄, PH7.2) and incubated with 0.05% trypsin for 5 min to remove semi-adherent/non-phagocytosed MEL cells. J774A.1 cells were collected by scraping. Data was collected by flow cytometry and the cells gated to distinguish the J774A.1 cells and any remaining MEL cells and the percentages of phagocytosed MEL cells were determined using FlowJo software.
2.8  Caspase activation assay

The caspase activation assay was performed using the CaspGLOW\textsuperscript{TM} Fluorescein Active Caspase Staining Kit according to the manufacturer’s instructions. MEL cells (5×10\textsuperscript{5} cells/mL/well) in complete culture medium in 24-well plates were incubated in the absence or presence of 1 mM ATP for 6 h at 37°C and 95% air/5% CO\textsubscript{2}. In some experiments, cells were pre-incubated in the absence or presence of 10 μM A-438079 for 15 min at 37°C before the addition of ATP. Following incubation with ATP, 300 μl of cells were incubated with 1 μl fluorescein isothiocyanate (FITC)-VAD-FMK for 1 h at 37°C and 95% air/5% CO\textsubscript{2}. Cells then were harvested, washed twice and suspended in Wash Buffer (as provided). Data was collected by flow cytometry and the percentage of caspase activated cells was determined using FlowJo software.

2.9  Immunoblotting analysis

Immunoblotting analysis of the of p38 MAPK and phospho-p38 MAPK (Thy180/Tyr182) was performed using phosphoPlus p38 MAPK (Thr180/Tyr182) Antibody Kit according to the manufacturer’s instructions as described (Yu et al. 2010). The cells (5×10\textsuperscript{5} cells/mL/well) in complete cell culture in 24-well plates were incubated in the absence or presence of 1 mM ATP for 5 min at 37°C and 95% air/5% CO\textsubscript{2}. In some experiments, cells were pre-incubated in the absence or presence of 10 μM A-438079 for 15 min at 37°C before the addition of ATP. Following incubation with ATP, cells were harvested and placed on ice until centrifuged (16000×g for 5 min at 4°C). Cells were resuspended in 100 μl ice-cold SDS sample buffer containing 5% 2-mercaptoethanol, and then the cells were sonicated in the presence of DNase I (10μg/mL) for 5 min. The cell lysates were separated by SDS-PAGE (4% stacking gel,
10% resolving gel) and transferred to nitrocellulose membranes. Nitrocellulose membranes were blocked overnight at 4°C with Tris-buffered saline (250 mM NaCl, 0.2% Tween-20 and 50 mM Tris, pH 7.5) containing 5% skim milk powder, and then incubated for 1 h at room temperature with rabbit anti-p38 MAPK or anti-phospho-p38 MAPK antibodies diluted in Tris-buffered saline containing 5% BSA. Membranes were washed three times over 30 min with Tris buffered saline and then incubated with HRP-conjugated anti-rabbit IgG antibody diluted in Tris-buffered saline containing 5% BSA for 1 h. Membranes were washed as above, incubated with LumiGLO chemiluminescent (as provided), and visualised using Amersham Hyperfilm ECL.

2.10 Presentation of data and statistics

Results are represented as means ± SD. Data was compared using either the unpaired Student's t-test for single comparisons or one-way analysis of variance (using Tukey's post test) for multiple comparisons using Prism 5 for Windows Version 5.01 (GraphPad Software, San Diego, CA) with \( P < 0.05 \) considered significant.
3 P2X7 ACTIVATION INDUCES MITOCHONDIAL REACTIVE OXYGEN SPECIES FORMATION IN MURINE ERYTHROLEUKEMIA CELLS

3.1 Results

3.1.1 P2X7 mRNA is expressed in murine erythroleukemia cells

Previous works from our group (Constantinescu 2008) using immunoblotting and immunofluorescence staining demonstrated that murine erythroleukemia (MEL) cell line expresses P2X7 receptor. Thus, to further confirm that MEL cells express P2X7, mRNA was isolated and amplified by RT-PCR. mRNA from RAW264.7 cells, which express P2X7 (Cankurtaran-Sayar et al. 2009), were included as a positive control. P2X7 mRNA was expressed in both cell types (Fig. 3-1).

![Figure 3-1 P2X7 mRNA is expressed in MEL cells. RNA was isolated from RAW264.7 and MEL cells, and analysed by RT-PCR using primers to P2X7. RNA substituted with H2O was used as a negative control. PCR products were visualised using ethidium bromide staining. Results are representative of 3 experiments.](image)

3.1.2 A P2X7 antagonist impairs ATP-induced ethidium\(^+\) and Yo-Pro-1\(^2+\) uptake

Constantinescu (2008) reported that adenosine 5’-triphosphate (ATP) induces ethidium\(^+\) uptake into MEL cells and this uptake was impaired by incubation of cells with the
P2X7 antagonist A-438079 (Nelson et al. 2006). To confirm these observations, cells were pre-incubated in the absence (control) or presence of A-438079 (10 μM), before incubating in the absence (basal) or presence of ATP. As observed previously (Constantinescu 2008), ATP induced ethidium+ uptake into MEL cells, and this process was significantly impaired by A-438079 (81% ± 7%, Fig. 3-2A). To determine if P2X7 activation could induce the uptake of a second cation, Yo-Pro-12+ (Cankurtaran-Sayar et al. 2009), cells were pre-incubated in the absence or presence of A-438079, and ATP-induced Yo-Pro-12+ uptake examined as above. As for ethidium+, ATP induced Yo-Pro-12+ uptake into MEL cells, and pre-incubation with A-438079 significantly impaired this uptake by 79 ± 9% (Fig. 3-2B).

### 3.1.3 ATP induces reactive species formation in a time- and concentration-dependent fashion

P2X7 activation induces reactive oxygen species (ROS) formation in various myeloid cell types (Hewinson and Mackenzie 2007). To determine whether extracellular ATP can induce ROS formation in MEL cells, cells were loaded with 2’,7’-dichlorodihydrofluorescein diacetate (H2DCFDA) which is converted to the highly fluorescent product 2’,7’-dichlorofluorescein (DCF) by intracellular ROS formation. Cells were then incubated in the absence (basal) and presence of ATP for up to 30 mins. ATP (1 mM) induced ROS formation in MEL cells in a time-dependent fashion (Fig. 3-3A). ATP also induced ROS formation in a concentration-dependent fashion with a maximal response at 2 mM ATP (Fig. 3-3B). This ATP concentration response gave an
EC$_{50}$ of $153 \pm 10 \mu M$, which is similar to the previously determined for ATP-induced ethidium$^+$ uptake in MEL cells ($EC_{50} 154 \pm 13 \mu M$) (Constantinescu 2008).

**Figure 3-2** A P2X7 antagonist impairs ATP-induced ethidium$^+$ and Yo-Pro-1$^{2+}$ uptake into MEL cells. MEL cells in NaCl medium were pre-incubated in the absence (control) or presence of 10 μM A-438079 at 37°C for 15 min, and then with (A) 25 μM ethidium$^+$ or (B) 1 μM Yo-Pro-1$^{2+}$ were incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 15 min. (A, B) Incubations were stopped by addition of NaCl medium containing 20 mM MgCl$_2$ (MgCl$_2$ medium) and centrifugation, and the cells analysed by flow cytometry. Results are mean ± SD ($n = 3$); **P $< 0.01$ compared to corresponding basal; †† P $< 0.01$ compared to ATP alone.
Figure 3-3 ATP induces ROS formation in MEL cells. MEL cells were loaded with 5 μM H₂DCFDA for 5 min at 37°C in NaCl medium under limited light conditions. After washing, cells in NaCl medium were incubated in the absence (basal) or presence of (A) 1 mM ATP at 37°C for up to 30 min, or (B) varying ATP concentrations for 15 min. (A, B) Incubations were stopped by addition of MgCl₂ medium and centrifugation, and the mean fluorescence intensity (MFI) of DCF was determined by flow cytometry. ROS formation is expressed as (A) MFI or (B) percent maximum response compared to 2 mM ATP. Results are mean ± SD (n = 3); where SDs are not visible, SDs are ≤1.1.
3.1.4 *P2X7 agonists induces reactive oxygen species formation*

To determine whether P2X7 activation induces ROS formation in MEL cells, the cells were incubated in the absence (basal) or presence of the most potent P2X7 agonist 3'-O-(4-benzoyl)benzoyl-adenosine 5'-triphosphate (BzATP), but not adenosine 5'-diphosphate (ADP) or uridine 5'-triphosphate (UTP) that do not activate P2X7 (Donnelly-Roberts *et. al.* 2009). ATP was included as a positive control. ATP and BzATP, but not ADP or UTP, induced ROS formation in MEL cells (Fig.3–4).

![Figure](image)

**Figure 3–4** P2X7 agonists induce ROS formation in MEL cells. H$_2$DCFDA -loaded MEL cells were incubated in NaCl medium in the absence (basal) or presence of 1 mM agonist (as indicated, except BzATP, 200 μM) at 37°C for 15 min. Incubations were stopped by addition of MgCl$_2$ medium and centrifugation, and the cells analysed by flow cytometry. Results are mean ± SD (n = 3); **P < 0.01 compared to basal.
3.1.5 A P2X7 antagonist impairs ATP-induced reactive oxygen species formation

To confirm that the ATP-induced ROS formation in MEL cells was mediated by P2X7 activation, cells were pre-incubated in the absence (control) or presence of the P2X7 antagonist A-438079 (10 µM), and then incubated in the absence (basal) or presence of ATP. As above, ATP induced ROS formation in MEL cells. Pre-incubation with A-438079 significantly impaired ATP-induced ROS formation in MEL cells by 87 ± 1% (Fig.3-5).

Figure 3-5 The P2X7 antagonist impairs ATP-induced ROS formation in MEL cells. H$_2$DCFDA-loaded MEL cells in NaCl medium were pre-incubated in the absence or presence of 10 µM A-438079 at 37°C for 15 min, and then incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 15 min. Incubations were stopped by addition of MgCl$_2$ medium and centrifugation, and the cells analysed by flow cytometry. Results are mean ± SD (n = 3); ** $P < 0.01$ compared to corresponding basal; †† $P < 0.01$ compared to ATP alone.
3.1.6 Mitogen-activated protein kinase p38 inhibitors increased basal P2X7-induced reactive oxygen species formation

A previous study reported that a p38 MAPK inhibitor, SB203580, impaired P2X7-induced ROS formation in primary rat microglia (Parvathenani et al. 2003). To determine whether the p38 MAPK pathway involves in P2X7-induced ROS formation in MEL cells. H$_2$DCFDA-loaded cells were pre-incubation in the presence of DMSO control or p38 MAPK inhibitors, SB202190 and SB203580, before incubation in the absence (basal) or presence of ATP. Pre-incubation of cells with SB202190 or SB203580 in the absence of ATP induced significant increases in ROS formation, compared to basal (Fig.3-6A, B). Pre-incubation of SB202190 and SB203580 failed to induce a significant decrease of ATP-induced ROS formation. Thus, the effect of the p38 MAPK inhibitors on ROS formation following ATP could not be assessed due to the high amount of ROS formation induced by these inhibitors alone (Fig.3-6A, B).
Figure 3-6 p38 MAPK inhibitors increase basal P2X7-induced ROS formation in MEL cells. H$_2$DCFDA-loaded MEL cells in NaCl medium were pre-incubation in the presence of DMSO control and p38 MAPK inhibitors, SB202190 and SB203580 at 37°C for 30 min, and then incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 15 min. Incubations were stopped by addition of MgCl$_2$ medium and centrifugation, and the cells analysed by flow cytometry. Results are mean ± SD (n = 3); ** $P < 0.01$ compared to corresponding basal; †† $P < 0.01$ compared to ATP alone. ‡‡ $P < 0.01$ compared to basal alone.
3.1.7 Broad spectrum and mitochondrial complex reactive oxygen species inhibitors impaired P2X7-induced ROS formation

Several cellular sources contribute to the production of ROS, predominantly the mitochondrial respiratory chain and NADPH oxidase (Turrens 2003). To determine which cellular source(s) contribute to ROS formation following P2X7 activation, MEL cells were pre-incubated in the absence or presence of pharmacological inhibitors of ROS formation before ATP addition. Pre-incubation of MEL cells with broad spectrum inhibitors of ROS formation, 10 mM N-acetyl-L-cysteine (NAC) and 20 µM diphenyleneiodonium (DPI), impaired ATP-induced ROS formation by 70 ± 5% and 51 ± 5%, respectively (Fig.3-7A, B). Pre-incubation of MEL cells with 5 µM rotenone, an inhibitor of mitochondrial complex I (Chen et al. 2003), impaired ATP-induced ROS formation by 60 ± 30% (Fig.3-7C). In contrast, pre-incubation of cells with 100 µM apocynin, a specific NADPH oxidase inhibitor (Chen et al. 2003) did not inhibit ATP-induced ROS formation (Fig.3-7D). To determine if the above inhibitors of ROS formation directly impaired P2X7 function, ATP-induced ethidium⁺ uptake was measured in the absence and presence of each inhibitor. None of the above inhibitors directly affected ATP-induced ethidium⁺ uptake into MEL cells (Fig.3-8). This indicates that the observed inhibition of P2X7-induced ROS formation was not due to a direct action on the receptor.
**Figure 3-7** Broad spectrum and mitochondrial complex ROS inhibitors impaired P2X7-induced ROS formation. H2DCFDA-loaded MEL cells in NaCl medium were pre-incubated at 37°C in the presence of (A) H2O control or NAC (10 mM, 30 mins), (B) DMSO control or DPI (20 μM, 30 mins), (C) DMSO control or apocynin (100 μM, 60 mins), (D) DMSO control or rotenone (5 μM, 60 mins), then incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 15 min. (A-D) Incubations were stopped by addition of MgCl₂ medium and centrifugation, and the cells analysed by flow cytometry. Results are mean ± SD (A, B n = 3; C, D n = 6); *P < 0.05 and ** P < 0.01 compared to corresponding basal; †† P < 0.01 compared to ATP alone.
Figure 3-8 ROS formation inhibitors do not impair ATP-induced ethidium\(^+\) uptake into MEL cells. MEL cells were pre-incubated in the absence or presence of ROS inhibitors as Fig. 3-7. Cells then were incubated with 25 μM ethidium\(^+\) in the absence (basal) or presence of 1 mM ATP at 37°C for 15 min. (A-D) Incubations were stopped by addition of MgCl\(_2\) medium and centrifugation, and the cells analysed by flow cytometry. Results are mean ± SD (n = 3); **P < 0.01 compared to basal.
3.1.8  *P2X7-induced reactive oxygen species formation is not dependent on Ca\(^{2+}\) influx*

A previous study showed that P2X7-induced ROS formation in submandibular cells is dependent on extracellular Ca\(^{2+}\) (Fontails *et al.* 2010). To determine whether P2X7-induced ROS formation is dependent on extracellular Ca\(^{2+}\) in MEL cells, cells were incubated in the absence (control) or presence of 1 mM Ca\(^{2+}\) and ATP. Since free Ca\(^{2+}\) lowers the concentration of ATP\(^4-\), the form responsible for P2X7 activation (North 2002), cells in the absence or presence of 1 mM Ca\(^{2+}\) were incubated with 1 or 1.74 mM ATP, respectively, to provide equimolar ATP\(^4-\) concentrations (251 \(\mu\)M). ATP induced significant ROS formation in MEL cells incubated in the absence or presence of additional Ca\(^{2+}\) compared to corresponding MEL cells incubated in the absence of ATP (Fig. 3-9A). Of note, ATP induced ROS formation was slightly but significantly lower in the presence of Ca\(^{2+}\) (Fig. 3-9A).

To exclude a role for extracellular Ca\(^{2+}\) in P2X7-induced ROS formation, MEL cells were resuspended in the absence (control) or presence 100 \(\mu\)M EGTA (a Ca\(^{2+}\) chelator), and then incubated in the absence (basal) and presence of 1 mM ATP. ATP induced a significant and similar amount of ROS formation in cells incubated in the absence (control) or presence of EGTA, compared to cells incubated in the absence of ATP (Fig. 3-9B).

To confirm that the failure to find a role for extracellular Ca\(^{2+}\) in the P2X7-induced ROS formation in MEL cells was not due to altered P2X7 function, ATP-induced ethidium\(^+\) uptake was assessed in the absence or presence of Ca\(^{2+}\) or EGTA. ATP-induced ethidium\(^+\) uptake was similar in the absence or presence of Ca\(^{2+}\) or EGTA (Fig. 3-10).
However, as for ROS formation, the presence of additional Ca\textsuperscript{2+} slightly but significantly impaired ATP induced ethidium\textsuperscript{+} uptake (Fig. 3-9A).

**Figure 3-9** P2X7-induced reactive oxygen species formation is not dependent on Ca\textsuperscript{2+} influx. H\textsubscript{2}DCFDA-loaded MEL cells (A) in the absence (control) or presence of 1 mM Ca\textsuperscript{2+} in NaCl medium were incubated in the absence (basal) or presence of 1 mM ATP (251 μM ATP\textsuperscript{4−}) or 1.74 mM ATP (251 μM ATP\textsuperscript{4−}) were incubated at 37°C for 15 min. (B) H\textsubscript{2}DCFDA-loaded MEL cells were incubated in NaCl medium in the absence or presence of 100 μM EGTA in the absence (basal) or presence of 1 ATP mM at 37°C for 15 min. (A,B) Incubations were stopped by addition of MgCl\textsubscript{2} medium and centrifugation, and the cells analysed by flow cytometry. Results are mean ± SD (A, \(n = 6\), B, \(n = 3\)); **\(P < 0.01\) compared to corresponding basal; ††\(P < 0.01\) compared to ATP alone.
Figure 3-10 Effect of extracellular Ca$^{2+}$ and EGTA on P2X7-induced ethidium$^+$ uptake into MEL cells. (A) Cells in the absence (control) or presence of 1 mM Ca$^{2+}$ in NaCl medium containing 25 μM ethidium$^+$ were incubated in the absence (basal) or presence of 1 mM ATP (251 μM ATP$^4$) or 1.74 mM ATP (251 μM ATP$^4$), respectively. (B) Cells in the absence or presence of 100 μM EGTA in NaCl medium containing 25 μM ethidium$^+$ were incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 15 mins. Incubations were stopped by addition of MgCl₂ medium and centrifugation, and the cells analysed by flow cytometry. Results are mean ± SD (n = 3); **P < 0.01 compared to basal; ††P < 0.01 compared to ATP alone.
3.1.9  *P2X7-induced reactive oxygen species formation is not dependent on K\(^+\) efflux*

Previous studies have shown that ROS formation is linked to interleukin (IL)-1\(\beta\) release (Hewinson *et al.* 2008; Martinon *et al.* 2009). Moreover, high extracellular K\(^+\) was shown to prevent IL-1\(\beta\) release (Ferrari *et al.* 1997; Sanz and Virgilio 2000) indicating that K\(^+\) efflux involves in IL-1\(\beta\) release and implicating a role for K\(^+\) efflux in ROS formation as well. Therefore, to determine if P2X7-induced ROS formation is also dependent on K\(^+\) efflux, ATP-induced ROS formation was compared in NaCl medium and KCl medium. The amounts of ATP-induced ROS formation in MEL cells in KCl medium were similar to that of cells in NaCl medium (Fig.3-11A). ATP-induced ethidium\(^+\) uptake was similar in cells incubated in either medium (Fig.3-11B). Collectively, this indicates that P2X7-induced ROS formation is not dependent on K\(^+\) efflux from MEL cells.

3.2  *Discussion*

Our group recently demonstrated that MEL cell line, a model of immature red blood cells (RBCs), expresses P2X7 receptor, and that activation of this receptor induces rapid phosphatidylserine (PS) exposure, microparticle release and cell death in this cell line (Constantinescu 2008). The present study provides the first evidence that P2X7 activation also induces ROS formation in this cell line. In addition, pharmacological approaches indicated P2X7 activation induced mitochondrial, but not NADPH oxidase ROS formation, and this process is not dependent on the Ca\(^{2+}\) influx or K\(^+\) efflux. Collectively, this data indicates that P2X7 activation induces ROS formation in RBCs via a mechanism different to myeloid and other cell types.
Figure 3-11 P2X7-induced reactive oxygen species formation is not dependent on K\(^+\) efflux. (A) H\(_2\)DCFDA-loaded MEL cells were incubated in NaCl medium or KCl medium in the absence or presence of 1 mM ATP at 37°C for 15 mins. (B) Cells in NaCl medium or KCl medium containing 25 \(\mu\)M ethidium\(^+\) were incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 15 mins. (A, B) Incubations were stopped by addition of MgCl\(_2\) medium and centrifugation, and the cells analysed by flow cytometry. Results are mean ± SD \((n = 3)\); \(*P < 0.05\), \(**P < 0.01\) compared to basal.
P2X7 induces ROS formation in MEL cells. The conclusion is based on several lines of evidences. First, immunoblotting, immunofluorescence staining (Constantinescu 2008) and RT-PCR (Fig.3-1) demonstrated P2X7 is present on MEL cells. Secondly, cytometric measurement demonstrated that ATP-induced ethidium\(^+\) and Yo-PRO-1\(^{2+}\) uptake (Constantinescu 2008) (Fig.3-2) in MEL cells. Thirdly, Cytofluorometric measurements of H\(_2\)DCFDA loaded MEL cells revealed that ATP induces the oxidation of H\(_2\)DCFDA to DCF, which directly corresponds to the amounts of ROS formation. This effect of ATP was time- and concentration-dependent with a maximal response at 2 mM ATP and an EC\(_{50}\) value of ~150 \(\mu\)M (Fig.3-3), which is similar to that of P2X7-induced ethidium\(^+\) uptake in MEL cells (Constantinescu 2008) and typical type of recombinant P2X7 (Donnelly-Roberts et al. 2009). Finally, BzATP, but not ADP nor UTP induced ROS formation into MEL cells (Fig.3-4), again typical of recombinant P2X7 (Donnelly-Roberts et al. 2009). Finally, ATP-induced ROS formation was completely impaired by the specific P2X7 antagonist, A-438079 (Nelson et al. 2006) (Fig.3-5), confirming that the ATP-induced ROS formation in MEL cells is due to P2X7 activation.

P2X7 activation induces ROS formation via a mitochondrial pathway. Mitochondria are a major site of cellular ROS formation. In contrast, previous studies have reported that the cell membrane enzyme complex NADPH oxidase, play a dominant role in P2X7-induced ROS formation in microglia cells and macrophages (Parvathenani et al. 2003; Pfeiffer et al. 2007; Noguchi et al. 2008; Moore and MacKenzie 2009). Several mitochondrial sources of ROS are present along the electron transport chain, including respiratory complexes I and III (Chen et al. 2003). In the present study, rotenone, a
specific mitochondrial complex I inhibitor (Chen et al. 2003) significantly impaired P2X7-induced ROS formation (Fig.4-6C). Moreover, the broad spectrum ROS formation inhibitor DPI, that inhibits ROS formation of mitochondrial complex I in murine monocytes/macrophages (Li and Trush 1998), also impaired P2X7-induced ROS formation (Fig.4-6B). In contrast, apocynin, a specific NADPH oxidase inhibitor, had no effect on ATP-induced ROS formation (Fig.4-6D), ruling out the involvement of the NADPH oxidase. Indeed, rotenone also decreased the P2X7-induced ROS production in murine bone marrow-derived macrophages, but not in J774A.1 cell and RAW264.7 (Noguchi et al. 2008; Moore and MacKenzie 2009). However in these two studies, P2X7-induced ROS formation was predominantly inhibited by apocynin, thus it was concluded that ROS was mainly generated by NADPH oxidase. In contrast, the present study indicates P2X7-induced ROS formation in murine RBCs is mediated by mitochondria. Thus, P2X7-induced ROS formation may be mediated by different pathways in different cell types.

P2X7-induced ROS formation in MEL cells is not dependent on an influx of Ca^{2+} or efflux of K^{+}. P2X7 activation is coupled to major changes in the ionic composition of the cytosol (North 2002; Surprenant and North 2009). Upon activation, P2X7 forms a non-selective cation channel to cause the influx of Ca^{2+} and Na^{+} and an efflux of K^{+}, leading to various cellular responses (Surprenant and North 2009). P2X7-induced ROS formation in murine macrophages and rat submandibular cells is dependent on extracellular Ca^{2+} (Fontanils et al. 2010; Guadalupe Martel-Gallegos et al. 2010). In contrast, the present study indicates that P2X7-induced ROS formation in MEL cells was not significantly altered in the presence of EGTA or Ca^{2+} (Fig.4-8), excluding a role
for extracellular Ca\(^{2+}\) in this process. Similarly, P2X7-induced ROS formation in murine (but not rat) submandibular cells is independent of extracellular Ca\(^{2+}\) (Seil et al. 2008). Thus, the different results indicate that the role of Ca\(^{2+}\) in P2X7-induced ROS formation is cell type and/or species specific.

The present study also addressed whether K\(^{+}\) efflux is required for P2X7-induced ROS formation. Previous studies have shown that ROS formation is linked to P2X7-mediated IL-1\(\beta\) release (Hewinson et al. 2008; Martinon et al. 2009) and high extracellular K\(^{+}\) prevents P2X7-induced IL-1\(\beta\) release (Ferrari et al. 1997; Sanz and Virgilio 2000) indicating that K\(^{+}\) efflux is involved in IL-1\(\beta\) release. However, the role of K\(^{+}\) effect in ROS formation during IL-1\(\beta\) release is unknown. In the present study, the amount of P2X7-induced ROS formation was similar for MEL cells in KCl medium and in the NaCl medium (Fig.4-10), indicating that P2X7-induced ROS formation is not dependent on K\(^{+}\) efflux in these cells.

P2X7 mediated-ROS formation has been mainly examined on immune and microglial cells, where it thought to play a role in microbial killing, inflammation response and cell death (Guerra et al. 2007). Cellular ROS formation is tightly regulated by various antioxidant systems, and deficiencies in these systems results in deleterious effects including tissue damaging (Turrens 2003). RBCs are exposed to high amount of oxidative-stress during circulation and deficiencies in ROS scavengers indicate that these cells are particularly vulnerable to excess ROS production (Turrens 2003). Accumulation of ROS in erythroid cells often results in shortened RBC life span leading to various anaemias (Çimen 2008; Ghaffari 2008). Thus, given that ROS may
coordinate RBCs cycle and differentiation, mechanisms of ROS production may regulate RBC differentiation and life span, P2X7-induced ROS formation in MEL cells may contribute to these processes in RBC. Although the role of P2X7 in RBC differentiation and life span has not directly elucidated, our study provides a possible mechanism by which ROS production is regulated in RBC.
4 P2X7 INDUCED DEATH OF MURINE ERYTHROLEUKEMIA CELLS IS DEPENDENT ON CASPASE AND P38 ACTIVATION

4.1 Results

4.1.1 ATP reduces cell numbers of murine erythroleukemia cells

Chatawala and Cantley (1984) originally demonstrated that extracellular ATP impaired the growth of MEL cells; however the mechanism was not determined. Subsequently work from our group using cytofluorometric assay reported that ATP induced cell death of MEL cells, and that this process could be impaired by pre-incubation with the P2X7 antagonist A-438079 (Constantinescu 2008). Therefore, to confirm these observations, cells were incubated with increasing concentrations of ATP for 24 h and the relative cell numbers (expressed as absorbances) were measured by thiazolyl blue tetrazolium bromide (MTT) assay. ATP reduced cell numbers in a concentration-dependent fashion, with a maximal reduction occurring at 2 mM ATP (Fig. 4-1). ATP at 1 mM reduced of the cell number by approximately half, compared to cells incubated in the absence of ATP (basal) (Fig. 4-1).
Figure 4-1 ATP reduces the cell numbers of MEL cells. MEL cells in complete culture medium were incubated in the absence (basal) or presence of ATP (as indicated) at 37°C for 24 h. MTT was added during the final 4 h, before overnight incubation with solubilization solution at 37°C. Absorbances were then read at 550 and 690 nm. Results are mean absorbance ($A_{550 \text{ nm}} - A_{690 \text{ nm}}$) ± SD ($n = 3$); ** $P < 0.01$ compared to corresponding basal.

4.1.2 A P2X7 antagonist prevents the ATP-induced reduction numbers of murine erythroleukemia cell

To determine if the ATP-reduced number of MEL cells was mediated by P2X7 activation, cells were pre-incubated in the absences (control) or presence of the specific P2X7 antagonist, A-438079 (Nelson et al. 2006), before overnight incubation in the absences (control) or presence of 1 mM ATP. As above, incubation with ATP reduced numbers of MEL cells (Fig. 4-2). Pre-incubation of 10 µM A-438079 impaired the ATP-induced reduction of cell number by 94 ± 3%, compared to cells incubated with ATP along (Fig.4-2)
A P2X7 antagonist prevents the reduction of numbers of MEL cells by ATP. MEL cells in complete culture medium were pre-incubated in the absence (control) or presence of 10 µM A-438079 at 37°C for 15 min. Cells were then incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 24 h. MTT was added during the final 4 h, before overnight incubation with solubilization solution at 37°C. Absorbances were then read at 550 and 690 nm. Results are mean absorbance (A$_{550\text{ nm}}$−A$_{690\text{ nm}}$) ± SD (n = 3); ** $P < 0.01$ compared to corresponding basal; †† $P < 0.01$ compared to ATP alone.

4.1.3 ATP induces the death of murine erythroleukemia cells

To further confirm the study of Constantinescu (2008), MEL cells were incubated with in the absence or presence of 1 mM ATP for 24 h, and the amount of cell death was determined by flow cytometry using Annexin-V binding (Phosphatidylserine or PS exposure) and 7-aminoactinomycin D (7AAD) uptake (loss of plasma membrane integrity) (Fig. 4-3A). Incubation with ATP induced an approximately 5.7-fold increase in Annexin-V$^+$ and 7AAD$^+$ cells, compared to cells incubated in the absence of ATP (Fig. 4-3B). These double positive cells represent either necrotic or late apoptotic cells,
or a combination of both (Kroemer et al. 2005). Incubation with ATP resulted in an approximately 3.4-fold and 6.2-fold increase in Annexin-V⁺/7AAD⁻ (early apoptotic) cells and Annexin-V⁻/7AAD⁺ (necrotic) cells respectively, compared to cells incubated in the absence of ATP (Fig. 4-3B).

4.1.4 A P2X7 antagonist impairs ATP-induced cell death

To confirm that the ATP-induced MEL cell death was via P2X7 activation as reported previously (Constantinescu 2008), cells were pre-incubated in the absence (control) or presence of A-438079, before overnight incubation in the absence (basal) or presence of 1 mM ATP. ATP failed to induce a significant increase in Annexin-V⁻/7AAD⁺ cells (Fig. 4-4B). This inconsistent ability of ATP to induce an increase in Annexin-V⁻/7AAD⁺ cells MEL cells has also been previously observed (Constantinescu 2008). The ability of ATP to induce necrotic cells (in Annexin-V⁻/7AAD⁺) was also variable in subsequent experiments (below) (data not shown). Therefore, the effect of ATP and other treatments on this dead cell population is not reported further. As above (Fig. 4-3), however, incubation with ATP induced a significant increase in Annexin-V⁺/7AAD and Annexin-V⁺/7AAD⁺ cells, compared to cells incubated in the absence of ATP (Fig. 4-4A, C). Pre-incubation with A-438079 significantly impaired the ATP-induced increase of Annexin-V⁺/7AAD⁻ and Annexin-V⁺/7AAD⁺ cells by 88 ± 6% and 78 ± 15%, respectively (Fig. 4-4B, C). Pre-incubation with A-438079 did not alter the basal and ATP-induced cell death.
Figure 4-3 ATP induces the death of MEL cells. (A, B) MEL cells in complete culture medium were incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 24 h. Cells were harvested, incubated with Annexin-V-FLUOS and 7AAD, and analysed by flow cytometry. (A) (Top panels) Representative dot plots showing the forward and side scatter of cells; (bottom panels) representative dot plots showing (lower left quadrant) viable Annexin-V^-7AAD^- cells, (lower right quadrant) Annexin-V^-7AAD^+ cells representing early apoptotic cells, (upper left quadrant) Annexin-V^-7AAD^+ cells representing necrotic cells and (upper right quadrant) Annexin-V^+7AAD^- cells representing either necrotic or late apoptotic cells or a combination of both. (B) Results expressed as mean percentage of cells ± SD (n = 3); **P < 0.01 compared to corresponding basal.
Figure 4-4 A P2X7 antagonist impairs ATP-induced death of MEL cells. MEL cells in complete culture medium were pre-incubated in the absence (control) or presence of 10 µM A-438079 at 37°C for 15 min. Cells then were incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 24 h. Cells were harvested, incubated with Annexin-V-FLUOS and 7AAD, and analysed by flow cytometry. Cells were defined as (A) necrotic (Annexin-V<sup>-</sup>/7AAD<sup>+</sup>) (B) early apoptotic (Annexin-V<sup>+</sup>/7AAD<sup>-</sup>) or (C) late apoptotic/necrotic (Annexin-V<sup>+</sup>/7AAD<sup>+</sup>). Results expressed as mean ± SD (n = 3); **P < 0.01 compared to corresponding basal. ††P < 0.01 compared to ATP alone.
4.1.5 Murine erythroleukemia cells following P2X7 activation are phagocytosed by J774A.1 macrophages

Dead RBCs are removed from the circulation by phagocytic cells of the spleen and liver. (Foeller et al. 2008). However, it is currently unknown if dead cells, as a result of P2X7 activation, can be phagocytosed by macrophages. Therefore, MEL cells were labelled with membrane dye, PKH-26, as described (Healey et al. 2007). Then, PKH-26 labelled MEL cells were incubated in the absence or presence of 1 mM ATP for 24 h and co-cultured with the macrophage-like cell line J774A.1 cells for 1 h and the amount of phagocytosis determined by flow cytometry as described (Schrijvers et al. 2004). J774A.1 cells incubated without MEL cells were used to determine the background fluorescence of these cells (Fig.4-5A, D). Co-incubation of macrophages with MEL cells (pre-incubated in the absence of ATP; control) resulted in phagocytosis with 26 ± 1% of macrophages containing PKH-26 labelled MEL cells (Fig.4-5B, E). Co-incubation of macrophages with MEL cells (pre-incubated with ATP) showed a slightly but significantly higher amount of phagocytosis with 31 ± 1% of macrophages containing PKH-26 labelled MEL cells (Fig.4-5C, F), compared to control.

To determine if this slightly but significantly higher increase in phagocytosis of MEL cells following ATP incubation was due to P2X7 activation, MEL cells were pre-incubated with 10 µM A-438079 prior to the addition of ATP and the amount of phagocytosis determined as described above. As above, pre-incubation of ATP induced a slight but significant amount of phagocytosis, compare to control MEL cells (Fig.4-6). Pre-incubation of MEL cells with A-438079 did not affect the phagocytosis of MEL cells incubated without ATP (Fig.4-6). In contrast, pre-incubation of MEL cells with 10
μM A-438079 prior to the addition of ATP prevented the amount of phagocytosis of ATP treated cells by 82 ± 2%, compared to phagocytosis of ATP-treated MEL cells not pre-incubated with A-438079 (Fig.4-6).

**Figure 4-5** ATP-treated MEL cells are phagocytosed by J774A.1 macrophages. PKH-26 labelled MEL cells in complete culture medium were incubated in the absence (control) or presence of 1 mM ATP at 37°C for 24 h. MEL cells were then incubated with J774A.1 cells at 37°C for 1 h. Followed incubation, cells were washed with cold PBS and treated with 0.05% trypsin for 5 min to remove non-phagocytosed MEL cells. Finally, adherent cells were harvested by scraping and analysed by flow cytometry. (A-C) Representative forward and side scatter dot plots of J774A.1 cells incubated with (A) complete culture medium alone, (B) control MEL cells or (C) ATP treated MEL cells. (A-C) The gating strategy shown was used to gate J774 cells only (gate 1), and to exclude MEL cells (gate 2) and debris. (D-F) Representative dot plot representing the proportion of macrophages positive for PHK-26 staining. Gate 3 was used to determine the percentage of PKH-26 positive J774A.1 macrophages. Percentages are expressed as mean ± SD (n = 3) and representative dot plots of each are shown.
Pre-treatment of MEL cells with a P2X7 antagonist prevented phagocytosis of ATP-treated MEL cells by J774A.1 macrophages. PKH-26 labelled MEL cells in complete culture medium were pre-incubated in the absence (control) or presence of 10 µM A-438079 at 37°C for 15 min. Cells then were incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 24 h. MEL cells were then incubated with J774A.1 cells at 37°C for 1 h. Followed incubation, cells were washed with cold PBS and treated with 0.05% trypsin for 5 min to remove non-phagocytosed MEL cells. Finally, adherent cells were harvested by scraping and analysed by flow cytometry. Results expressed as mean percentage ± SD (n=3); * P < 0.05 compared to corresponding basal. † P < 0.05 compared to ATP alone.

4.1.6 P2X7-induced death of murine erythroleukemia cells requires the activity of caspase

Activation of caspases plays a central role in apoptosis (Riedl and Shi 2004). Moreover, P2X7-induced macrophages apoptosis involves caspase activation (Ferrari et al. 1999; Wang et al. 2004; Kong et al. 2005; Noguchi et al. 2008; Fu et al. 2009). Therefore, to determine the role of caspase activation in P2X7-induced MEL cell death, cells were incubated in the presence of DMSO control or broad spectrum caspase inhibitor Z-
VAD-FMK (20 µM) (Nogchi et al. 2008) prior to addition of ATP. As shown (Section 4.1.4), incubation of ATP induced a significant increase in Annexin-V$^+$/7AAD$^-$ and Annexin-V$^+$/7AAD$^+$ cells (Fig. 4-7A, B). Pre-incubation of cells with Z-VAD-FMK failed to impair the ATP-induced increase of Annexin-V$^+$/7AAD$^-$ cells (Fig. 4-7A). In contrast, Z-VAD-FMK significantly impaired ATP-induced increase of Annexin-V$^+$/7AAD$^+$ cells by 36 ± 2% (Fig. 4-8B). Pre-incubation of cells in presence or DMSO and Z-VAD-FMK also did not alter basal cell death (Fig. 4-8A, B). This suggests blockage of caspase activity impairs the ability of MEL cells to undergo late apoptosis or necrosis following P2X7 activation, and indicates a role for this enzymes family in P2X7-induced death of MEL cells.

A previous study reported that pre-incubation with Z-VAD-FMK blocked P2X7-mediated pore formation in human THP-1 cells (Donnelly-Roberts et al. 2004). To exclude the possibility that Z-VAD-FMK impaired ATP-induced death of MEL cells by directly inhibiting P2X7 function rather than casapase activity, cells were pre-incubated in the presence of DMSO control or Z-VAD-FMK, and the ATP-induced ethidium$^+$ uptake measured. ATP-induced ethidium$^+$ uptake into MEL cells was similar following pre-incubation with DMSO or Z-VAD-FMK (Fig. 4-8).

4.1.7 P2X7 stimulates caspase activation in MEL cells

To examine if ATP stimulates caspase activation in MEL cells, a cytofluorometric caspase activation assay was performed. This assay utilizes the caspase family inhibitor VAD-FMK conjugated to fluoresceinisothiocyanate (FITC) as a measure of activated caspases in cells. Following incubation in the absence (basal) or presence of 1 mM ATP
for 6 h, MEL cells were incubated with FITC-VAD-FMK for 1 h and caspase activation was measured by flow cytometry. Incubation of cells with ATP resulted in approximately a 2.5-fold increase in the relative percentage of cells with activated caspases, compared to cells incubated in the absence of ATP (23 ± 3% and 9 ± 1%, respectively, n = 3, p < 0.01; Fig. 4-9A).

**Figure 4-7** A broad spectrum caspase inhibitor impairs P2X7-induced MEL cell death. MEL cells in complete culture medium were pre-incubated in the presence of DMSO or 20 µM Z-VAD-FMK at 37°C for 30 min, and then incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 24 h. Cells were harvested, and incubated with Annexin-V-FLUOS and 7AAD, and analysed by flow cytometry. Cells were defined as (A) early apoptotic (Annexin-V⁺/7AAD⁻) or (B) late apoptotic/necrotic (Annexin-V⁺/7AAD⁺). Results are mean ± SD (n = 3); **P < 0.01 compared to corresponding basal; ††P < 0.01 compared to ATP with DMSO.
A broad spectrum caspase inhibitor does not affect P2X7-induced ethidium\(^+\) uptake into MEL cells. MEL cells in NaCl medium were pre-incubated in the presence of DMSO or 20 \(\mu\)M Z-VAD-FMK at 37°C for 30 min, then 25 \(\mu\)M ethidium\(^+\) was added, and cells were incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 15 min. Incubations were stopped by addition of MgCl\(_2\) medium and centrifugation, and the cells analysed by flow cytometry. Results are mean ± SD (\(n = 3\)); **\(P < 0.01\) compared to basal.

To determine if ATP-induced caspase activation in MEL cells was the result of P2X7 activation. Cells were pre-incubated in the absence (control) and presence of 10 \(\mu\)M A-438079. As above, ATP induced caspase activity in MEL cells (Fig. 4-9B). In contrast, pre-incubation with A-438079 impaired the ATP-induced increase in the percentage of caspase-activated cells by 99 ± 13% (Fig. 4-9B). The percentage of caspase-activated cells in the absence or presence of A-438079 without ATP was similar (Fig. 4-9B).
**Figure 4-9** P2X7 induces caspase activation in MEL cells. (A, B) MEL cells in complete culture media were incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 6 h. (B) MEL cells were pre-incubated in the absence or presence of 10 µM A-438079 prior to the addition of ATP. Cells were harvested, and incubated with FITC-VAD-FMK at 37°C for 1 h, and analysed by flow cytometry. (A) Representative histogram showing relative caspase activity in basal cells (shaded) and ATP treated cells (solid line). A marker region (as shown) was used to determine the percentage of cells with activated caspase. (B) results expressed as mean percentage ± SD (n = 3); ** P < 0.01 compared to corresponding basal. †† P < 0.01 compared to ATP alone.
4.1.8 P2X7-induced MEL cell death requires the activity of mitogen-activated protein kinase p38

A previous study has reported that P2X7-induced macrophage death is dependent on mitogen-activated protein kinase (MAPK) p38 activation (Noguchi et al. 2008). Therefore, to determine if P2X7-induced MEL cell death requires the activation of p38 MAPK, cells were incubated in the presence of DMSO control or the p38 inhibitors, SB202190 or SB203580 (Noguchi et al. 2008) prior to incubation in the absence (basal) or presence of ATP. As shown above (Section 4.1.4 and 4.1.6), incubation of ATP induced a significant increase in Annexin-V+/7AAD− and Annexin-V+/7AAD+ cells (Fig. 4-10A, B). Pre-incubation of cells with 20 µM SB202190 significantly impaired the ATP-induced increase of Annexin-V+/7AAD− cells and Annexin-V+/7AAD+ cells by 56 ± 5% and 56 ± 9%, respectively (Fig. 4-10A, B). Similarly, pre-incubation of MEL cells with SB203580 impaired the ATP-induced increase of Annexin-V+/7AAD− cells and Annexin-V+/7AAD+ by 52 ± 6% and 48 ± 17%, respectively (Fig. 4-10C, D). Incubation in the absence or presence of each p38 MAPK inhibitors did not alter basal cell death (Fig. 4-10).

As for Z-VAD-FMK, both SB202190 or SB203580 (20 µM) can block P2X7-mediated pore formation in human THP-1 cells (Donnelly-Roberts et al. 2004). Thus, to determine if either of these p38 MAPK inhibitors directly impaired P2X7 function in MEL cells, cell was pre-incubated in the presence of DMSO, or SB202190 or SB203580, and the P2X7-induced ethidium+ uptake accessed. Neither of these inhibitors affected ATP-induced ethidium+ uptake into MELs (Fig. 4-11A, B).
**Figure 4-10** p38 MAPK inhibitors impair P2X7-induced MEL cell death. MEL cells in complete culture medium were pre-incubated in the presence of (A-D) DMSO, (A, B) 20 µM SB202190 or (C, D) 20 µM SB203580 at 37°C for 30 min. (A-D) Cells then were incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 24 h. Cells were harvested, and incubated with Annexin-V-FLUOS and 7AAD, and analysed by flow cytometry. Cells were defined as (A) early apoptotic (Annexin-V+/7AAD-) or (B) late apoptotic/necrotic (Annexin-V+/7AAD+). Results are mean ± SD (n = 3); *<0.05, **<0.01 compared to corresponding basal; †<0.05, ††<0.01 compared to ATP alone.
**Figure 4-11** p38 MAPK inhibitors did not alter ATP-induced ethidium\(^+\) uptake into MEL cells. MEL cells in NaCl medium were pre-incubated in the (A, B) presence of DMSO, (A) 20 µM SB202190 or (B) 20 µM SB203580 at 37°C for 30 min. (A, B) Then 25 µM ethidium\(^+\) was added and cell were incubated at 37°C for 15 min in the absence (basal) or presence of 1 mM ATP. Incubations were stopped by addition of MgCl\(_2\) medium and centrifugation, and the cells analysed by flow cytometry. Results are mean ± SD (n = 3); **P < 0.01 compared to corresponding basal.
4.1.9  *P2X7 stimulates p38 activation in murine erythroleukemia cells*

To further support the role of p38 MAPK activation in the P2X7-induced cell death of MEL, immunoblotting phospho-p38 MAPK was performed as a measure of p38 MAPK activation. Immunoblotting of total p38 was used as a loading control. Immunoblotting with an anti-phospho-p38 MAPK antibody revealed that ATP at 1 mM induced considerable p38 MAPK phospho-p38, compared to cells incubated in the absence of ATP (5 min) (Fig. 4-12). Pre-incubation of cells with 10 µM A-438079 before the incubation with ATP significantly impaired ATP-induced p38 phospho-p38 compared to ATP alone (Fig. 4-12). Pre-incubation of cells with A-438079 alone induced a small amount of p38 MAPK phospho-p38, compared to cells incubated in the absence of A-438079 and ATP (Fig. 4-12).

![Image](image.png)

**Figure 4-12** P2X7 induces activation of p38 MAPK. MEL cells in complete medium were pre-incubated in the absence and presence of 10 µM A-438079 at 37°C for 15 min, and then incubated in the absence and presence of 1 mM ATP at 37°C for 5 min. Incubation was stopped by addition of complete medium and centrifugation. Whole cell lysates of MEL cells were separated by electrophoresis, transferred to nitrocellulose membrane and probed with phospho-p38 antibody and anti-p38 antibody.
Figure 4-13 Effects of ROS inhibitors on P2X7-induced cell death in MEL cells. MEL cells in complete culture medium were pre-incubated in the presence of (A, B) H$_2$O control or 10 mM NAC, or (B) in the presence of DMSO control and 10µM DPI at 37°C for 30 min. (A-D) cells were then incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 24 h. Cells were harvested, and incubated with Annexin-V-FLUOS and 7AAD, and analysed by flow cytometry. Results are mean ± SD ($n = 3$); * $P<0.05$, ** $P < 0.01$ compared to corresponding basal, †† $P < 0.01$ compared to ATP alone, ‡‡ $P < 0.01$ compared to basal alone.
4.1.10 P2X7-induced cell death is independent on reactive oxygen species formation

Several groups have reported that P2X7-induced macrophages death is dependent on ROS formation via the activity of NADPH oxidase (Harada et al. 2003; Noguchi et al. 2008; Moore and MacKenzie 2009). To explore the role of ROS formation in P2X7-induced cell death in MEL cells, cells were pre-incubated in the absence and presence of the broad spectrum ROS inhibitors N-acetyl-L-cysteine (NAC) and diphenyleneiodonium (DPI) before incubation cells in the absence (basal) and presence of ATP. As above, ATP induced a significant increase in Annexin\(^+\)/7AAD\(^-\) and Annexin\(^+\)/7AAD\(^+\) cells. Pre-incubation of cells with NAC did not impair P2X7 induced MEL cell death (Fig. 4-13A, B). Pre-incubation of cells in the absence or presence of NAC alone did not significantly alter basal cell death (Fig. 4-13A, B). In contrast, the effect of DPI on P2X7-induced cells death could not be fully assessed due to the high amount of cell death induced by DPI alone and in the presence of ATP (Fig. 4-13C, D). In fact, incubation of cells with DPI in the presence of ATP induced nearly 100% cell death (Fig. 4-13C, D).

4.2 Discussion

Chatawala and Cantley (1984) originally showed that extracellular ATP impaired the growth of MEL cells. Subsequently work from our group using a cytofluorometric assay involving Annexin-V binding and 7AAD staining demonstrated that P2X7 activation induced MEL cell death (Constantinescu 2008). The aims of this study were to confirm that P2X7 activation induces MEL cell death and to determine the signalling pathways involved in this process. Using an MTT assay, as well as the same cytofluorometric assay as Constantinescu (2008), ATP was shown to induce cell death and these
processes were blocked by P2X7 antagonist A-438079 (Nelson et al. 2005), confirming
the role of P2X7 in this process. Moreover, following P2X7 activation, MEL cells were
phagocytosed by J774A.1 macrophages, providing another indicator of cell death
(Krysko et al. 2008). Finally, the current study demonstrates that P2X7-induced cell
death involves the activation of the p38 MAPK and caspase, but not ROS formation.

MTT assay indirectly demonstrated that P2X7 activation induces the death of MEL
cells (Fig. 4-1, 2). The MTT assay is a relatively inexpensive and convenient method to
measure cell proliferation or cell death. However, this assay measures the cellular
metabolic activity rather than cell numbers directly, and does not distinguish between
increased cell death or impaired cell number. Nevertheless, this assay provides indirect
evidences that P2X7 activation induces MEL cell death and parallels the earlier
observations where ATP impaired MEL cell growth (Chatawala and Cantley 1984).
Moreover, this assay determined that ATP induced-cell death is in a concentration-
dependent fashion, with cell death maximal at 2 mM, and ATP at 1 mM induced about
50% cell death, compared to basal (Fig. 4-1). Thus, 1 mM ATP was selected for
subsequent studies to investigate the signaling pathway involving in P2X7-induced
MEL cell death.

Cytofluorometric analysis of PS exposure cells using Annexin-V and lost in membrane
integrity using 7AAD revealed that P2X7 activation results in MEL cell apoptosis.
However, whether this cell death represents apoptosis or necrosis, or both cannot be
exclusively determined by using only these two markers of cell death (Kroemer et al.
2005). Nevertheless, the consistent increase in Annexin-V⁺/7AAD⁻ (early apoptosis)
cells and Annexin-V+/7AAD+ (late apoptosis/necrosis) cells (Fig. 4-3, 4) is consisted with study of Constantinescu (2008), and favours the idea that P2X7 activation induces apoptosis rather than necrosis of MEL cells.

The phagocytosis of MEL cells by J774A.1 macrophages is enhanced by following P2X7 activation in MEL cells. Healey et al. (2007) have used a flow cytometric assay to measure the phagocytosis of aged sheep erythrocytes by THP-1 monocytes in vitro. While Schrijvers et al. (2009) have also used a flow cytometric assay to measure the phagocytosis of fluorescent beads or apoptotic monocytes by J774A.1 macrophages in vitro. Using a combination of these conditions, PKH-26-labelled MEL cells were incubated in the presence or absence of ATP for 24 h, co-cultured with J774A.1 macrophages for 1 h and the relative phagocytosis of MEL cells were assessed. The results in this study demonstrated that ~25% J774A.1 macrophages phagocytosed untreated MEL cell, and that the amount of phagocytosis of P2X7 activated MEL cells was only slightly greater (Fig. 4-5, 6). The relatively high amount of phagocytosis of untreated MEL cells by J774A.1 macrophages was unexpected and not consistent with other studies using fluorescent beads or apoptotic monocytes (Schrijvers et al. 2004). Moreover, the small but significant difference between the phagocytosis of untreated and P2X7 activated-MEL cells is disproportionate to the amount of cell death observed above. One possible explanation is that the PBS washing and 0.05% trypsin treatment to remove non-phagocytosed MEL cells adhering to macrophages was not totally effective. Hypotonic lysis of non-phagocytosed MEL cells may minimize this effect. Alternatively, the relative amount of P2X7 activation and subsequent cell death may have been too low to see an effect. Collectively, the present study suggests that the
The process by which senescent erythrocytes, after their 120 day lifespan, are recognised and removed by macrophages of the spleen and liver is poorly understood but is important for balancing the total number of circulating RBCs. PS exposure is one of the signals that allow macrophages to bind and ingest dying cells, including senescent RBCs (Foeller et al. 2008; Lang et al. 2008). P2X7 induces cell death in a variety of cell types, however, the physiological role of this process in RBCs and other cell types is barely known. Studies of P2X7-deficient mice suggest that the P2X7 is not involved in regulating cell numbers under normal, physiological conditions (Labasi et al. 2002; Chessell et al. 2005), except in bone homeostasis (Ke et al. 2003), while impaired P2X7-induced cell death may promote epithelial malignancies, suggesting a role for P2X7 activation regulating epithelial cell growth under some circumstances (Gorodeski 2009). Thus, given the role of P2X7 in mediating MEL cells cell death and their subsequent phagocytosis revealed by the present study, it is reasonable to hypothesise that P2X7 activation may play a role in removing senescent, damaged or diseased RBCs from the circulation.

P2X7-induced cell death of MEL cells is mediated by caspase activation. Pre-incubation of MEL cells with the broad spectrum caspase inhibitor Z-VAD-FMK impaired Annexin-V⁺/7AAD⁺ cells (late apoptotic / necrotic cells), but not Annexin-V⁻/7AAD⁻ cells (early apoptotic cells), following P2X7 activation (Fig. 4-6). This observation
however suggests that inhibition of caspases in MEL cells does not completely impair cell death, but only delays this process. To further determine the role of caspase in P2X7 induced early apoptotic events in MEL cells, studies should include examination of the effect of Z-VAD-FMK on ATP induced DNA fragmentation, which is typical event in early apoptosis. Nevertheless, the detection of P2X7-induced caspase activation in MEL cells (Fig. 4-9) support a role for caspase in P2X7-induced MEL cell death. Caspase activation plays a central role in the execution of apoptosis by causing nuclear condensation, DNA fragmentation and other apoptotic changes (Riedl and Shi 2004). Previous studies reported that caspase activation in macrophages, microglia and neural cells plays an important role in P2X7 induced cell death (Ferrari et al. 1999; Humphreys et al. 2000; Kong et al. 2005; Noguchi et al. 2008). Although the specific caspase was (were) not identified, this result is consistent with others.

P2X7 induced cell death can be mediated by p38 MAPK pathway in MEL cells. Pre-incubation of MEL cells with p38 inhibitors impair P2X7-induced cell death (Fig. 4-10), and that P2X7-induced p38 activation was subsequently indentified (Fig. 4-12), supporting the role of p38 activation in MEL cell death. MAPK p38 activation was shown to mediated P2X7-induced death of macrophages(Noguchi et al. 2008). The present results are consistent with previous study.

Finally, the present study indicates that ROS formation does not mediate P2X7-induced MEL cell death. Incubation of MEL cells with the ROS inhibitors NAC did not prevent the P2X7-induced MEL death (Fig. 4-13), despite these inhibitors impairing P2X7-induced ROS formation in MEL cells (Chapter 3). Previous studies have indicated that ROS formation generating from NADPH oxidase plays an important role in P2X7-
induced death of macrophages (Noguchi et al. 2008) (Moore and MacKenzie 2007). In contrast, my study showed that P2X7 induced mitochondrial ROS formation in MEL cells rather than NADPH oxidase. This result is different from that the role of ROS in other myeloid cells. The relation of P2X7-induced ROS formation and cell death pathways will be further discussed in the General Discussion (Chapter 5).
P2X7 activation induces a variety of downstream effects including reactive oxygen species formation (ROS) and cell death (Sluyter and Stokes 2011). The P2X7-induced ROS formation is mainly documented in macrophages and microglia cells, where it plays a role in microbial killing, inflammation response and cell death (Hewinson and Mackenzie 2007). One topic of this thesis was to determine whether P2X7 induces ROS formation in an immature red blood cell (RBC) model, murine erythroleukemia (MEL) cell line. The present study confirmed that MEL cells express functional P2X7. Various species RBCs express P2X7 receptor, however, the role of P2X7 on RBCs is barely known. The presence of functional P2X7 in MEL cells substantiates the presence of this receptor in erythroid cells and provides a model to further elucidate the role of P2X7 in RBCs. The present study also demonstrates that P2X7 activation induces ROS formation via the mitochondrial pathway in MEL cells (Fig. 5-1). Previous studies have identified NADPH oxidase as the source of P2X7-induced ROS formation in macrophages and microglia cells (Parvathenani et al. 2003; Noguchi et al. 2008). However, to date there has been little evidence for a role for P2X7 in mitochondrial ROS formation or for P2X7-induced ROS formation in RBCs.

The other major topic of this thesis was to determine the mechanism of P2X7-induced cell death. Chatawala and Cantley (1984) originally showed that extracellular ATP impaired the growth of MEL cells; however the mechanism was not determined. Work from our group demonstrated that P2X7 activation induces death of MEL cells (Constantinescu 2008). The present study demonstrates that P2X7-induced death of MEL cell involves the activation of mitogen-activated protein kinase (MAPK) family
member p38 and caspase but not ROS formation (Fig. 5-1). Moreover, MEL cells following P2X7 activation can be phagocytosed by J774A.1 macrophages.

The mechanism of P2X7-induced ROS formation and cell death revealed in MEL cells is different from that of macrophages. Several groups have reported that P2X7-induced ROS formation is via the activation of NADPH oxidase in macrophages and microglia cells (Harada et al. 2003; Noguchi et al. 2008; Moore and MacKenzie 2009). In one of

Figure 5-1 Proposed mechanisms of P2X7-induced ROS formation and death in MEL cells. (Left) Activation of P2X7 by extracellular ATP induces mitochondrial ROS formation, possibly by enhancing the intramitochondrial Ca\(^{2+}\) load. (Right) P2X7-induced cell death involves activation of p38 MAPK and caspase, but not ROS formation. Whether p38 MAPK is upstream of caspase activation, or belong to a separated pathway from caspase remains unknown.
these studies (Noguchi et al. 2008), P2X7-induced ROS formation was found to mediate P2X7-induced cell death. However, the current study indicates that P2X7-induced death in MEL cells is independent on ROS formation. The different results also indirectly support that P2X7 induces ROS formation in MEL cells uses a different mechanism from that in macrophages, namely the mitochondria versus NADPH oxidase respectively.

Future study is needed to confirm the role of mitochondria in the P2X7-induced ROS formation in MEL cells. Mitochondrial ROS-sensitive probes, such as Mitosox red superoxide indicator, could be used to directly confirm mitochondrial ROS formation following P2X7 activation. Although the current study exclude a role of extracellular Ca\(^{2+}\) and an influx of Ca\(^{2+}\) across the plasma membrane in P2X7-induced ROS formation in MEL cells, this pathway still may involve intracellular Ca\(^{2+}\). Study of P2X7-transfected cells showed that intramitochondrial Ca\(^{2+}\) is at least twice higher than control cells (Adinolfi et al. 2005; Di Virgilio et al. 2009). Given that P2X7 activation can enhance the flux of Ca\(^{2+}\) into mitochondria, and this increased mitochondrial Ca\(^{2+}\) can lead to increased ROS formation (Brookes et al. 2004; Feissner et al. 2009), it will be of interest to further investigate whether intracellular Ca\(^{2+}\) plays a role in the P2X7 induced mitochondria pathway of ROS formation in MEL cells. BAPTA-AM, an intracellular Ca\(^{2+}\) chelator, could be used to assess the role of intracellular Ca\(^{2+}\) in this process.

The present study also showed inhibitors of p38 MAPK and caspase only partially impaired P2X7-induced cell death. Thus, it is possible that P2X7 induced MEL cell
death may involve other signalling molecules or pathways. Moreover, as described (Fig. 5-1), p38 MAPK activation may up-regulates the caspase activity, which could be assessed by directly examining the effect of p38 MAPK inhibitors on ATP-induced caspase activation in MEL cells. Future experiments could also include the use of caspase specific inhibitors, as well as caspase specific cytofluorometric or immunoblotting assays determine which caspase family member are responsible for P2X7 induced MEL cell death. The most likely candidates would be caspase-1, -3, -8 and/or 9.

Finally, to confirm the process of MEL cells phagocytosed by J774A.1 macrophages following P2X7 activation, it appears necessary to incubate MEL cells with a maximal concentration of ATP (2 mM) to induce maximal cell death, and increase the difference in phagocytosis between untreated and ATP-treated MEL cells. Recently, a role for ATP and purinergic receptor activation on macrophages has been shown to enhance the phagocytosis of dying cells (Marques-da-Silva et al.). However, it appears unlikely that the exogenous ATP added to MEL cells contributed to their enhanced phagocytises, as MEL cells were extensively washed prior to incubation with J774A.1 macrophages. However, a role for ATP released from dying MEL cells contributing to enhanced phagocytosis cannot be excluded. Thus, future phagocytosis assays with MEL and J774A.1 cells should be conducted in the presence of P2X antagonists and/or apyrase.

The role of mitochondria in P2X7-induced ROS formation revealed by the present study raises questions about the role of P2X7 in mature RBCs, which lack of mitochondria and nucleus. Whether P2X7 activation can mediate ROS formation in mature RBCs
remain unknown. In contrast, the absence for mitochondrial ROS formation in P2X7-induced death of MEL cells suggests that the same pathway, involving p38 MAPK and caspase, may be also operated in mature RBCs. Future studies on primary erythrocytes are required to address and confirm the role of P2X7 in mature RBCs.

In conclusion, the present study provides first evidence that P2X7 induces ROS formation in MEL cells, and that this process occurs via mitochondrial pathway. This study also demonstrates that MEL cells following P2X7 activation can be phagocytosed by macrophages. Finally, P2X7 induced cell death is dependent on p38 and caspase activation, but not ROS formation. Future studies of MEL cells and primary RBC cells will help to further understand the roles of the P2X7 receptor in RBC development and lifespan, and disease.
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