The canine P2X7 receptor

Iman Jalilian

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The canine P2X7 receptor

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Iman Jalilian
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### Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>2MeSATP</td>
<td>2-methylthio adenosine triphosphate</td>
</tr>
<tr>
<td>7AAD</td>
<td>7-amino-actinomycin-D</td>
</tr>
<tr>
<td>A-438079</td>
<td>3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl] pyridine</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5´ diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine 5´ monophosphate</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
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<tr>
<td>ATP</td>
<td>adenosine 5´ triphosphate</td>
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<tr>
<td>αβMeATP</td>
<td>αβ-methylene-ATP</td>
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<tr>
<td>βγmeATP</td>
<td>βγ-methyleneadenosine 5´-triphosphate</td>
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<tr>
<td>ATPγS</td>
<td>adenosine 5´-O-(3-thio) triphosphate</td>
</tr>
<tr>
<td>BBG</td>
<td>brilliant Blue G</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BzATP</td>
<td>2(3´)-O-(4-benzoylbenzoyl) adenosine 5´ triphosphate</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CAPS</td>
<td>cryopyrin-associated periodic syndrome</td>
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<tr>
<td>CBX</td>
<td>carbenoxolone</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disorders</td>
</tr>
<tr>
<td>CPPD</td>
<td>calcium pyrophosphate dehydrate</td>
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<tr>
<td>CREB</td>
<td>cyclic-AMP response element-binding</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinases</td>
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<td>FCS</td>
<td>foetal calf serum</td>
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<td>FFA</td>
<td>flufenamic acid</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>forward scatter</td>
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<td>GPCR</td>
<td>g-protein coupled receptors</td>
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<td>h</td>
<td>hour(s)</td>
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<tr>
<td>HEK293</td>
<td>human embryonic kidney293 cells</td>
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<tr>
<td>HRP</td>
<td>horseredish peroxidase</td>
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xiii
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tr>
<td>IBD</td>
<td>inflammatory bowel diseases</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol triphosphate</td>
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<tr>
<td>KN-62</td>
<td>1-[N-O-bis-(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MDCK</td>
<td>madin-darby canine kidney cells</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>MFQ</td>
<td>melfoquine</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MSU</td>
<td>monosodium urate</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
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MWS  muckle-wells syndrome
NF-κB  nuclear factor-κB
NMDG  n-methyl-D-glucamine
oATP  2´, 3´ dialdehyde ATP
PARP  poly (ADP-ribose) polymerase
PBMC  peripheral blood mononuclear cells
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PE  phycoerythrin
PPADS  pyridoxal-phosphate-6-azophenyl-2´,4´-disulfonate
PRO  probenecid
PS  phosphatidylserine
RT-PCR  reverse Transcriptase polymerase chain reaction
s  second(s)
SAPK  stress activated protein kinase
SD  standard deviation
SNP  single nucleotide polymorphism
SSC  side scatter

TBST  tris-buffered saline tween-20

T_H  type 2 helper T cells

TLR  toll-like receptor

TM  transmembrane

TNF  tumour necrosis factor

TpIS  transcription initiation site

UDP  uridine 5′ diphosphate

UTP  uridine 5′ triphosphate

VEGF  vascular endothelial growth factor
Abstract

The P2X7 receptor is a trimeric ligand-gated ion channel, present in many mammalian species including the canine. P2X7 activation by its natural ligand, extracellular adenosine 5´-triphosphate (ATP), leads to the opening of a large non-selective pore, allowing the entry of large fluorescent dyes (such as ethidium+), and the activation of the NALP3 inflammasome and the subsequent release of pro-inflammatory cytokines interleukin (IL)-1β and IL-18. P2X7 is present on haematopoietic, bone and epithelial cells. Pannexin-1 may be involved in P2X7-induced uptake and IL-1β release, but its role remains unclear. This study aims to:

1. To determine if Madin-Darby canine epithelial kidney (MDCK) cells express functional P2X7;

2. To determine if pannexin-1 is involved in P2X7-induced ethidium+ uptake;

3. To clone P2X7 from an English Springer Spaniel;

4. To determine if P2X7 function differs between dogs or breeds.

Reverse transcriptase (RT)-PCR and immunoblotting demonstrated the existence of P2X7 mRNA and protein in MDCK cells, respectively. Incubation with ATP and 2(3´)-O-(4-benzoylbenzoyl) adenosine 5´triphosphate (BzATP) induced ethidium+ uptake into MDCK cells. Two specific P2X7 antagonists, KN-62 and A-438079, inhibited ATP-induced ethidium+ uptake in MDCK cells by 60% and 92%, respectively. Collectively, this demonstrated that MDCK cells express functional P2X7. RT-PCR also demonstrated that MDCK cells express the inflammasome components, NALP3 and caspase-1, and Toll-like receptor 4 (TLR4), but not
IL-1β and IL-18. Lipopolysaccharide (LPS) up-regulated the expression of caspase-1, but not P2X7 or NALP3, in a concentration-dependent manner. LPS failed to induce the expression of either IL-1β or IL-18. The up-regulation of caspase-1 suggests that MDCK cells express functional cell-surface TLR4. The presence of TLR4 on MDCK cells was confirmed using an anti-TLR4 monoclonal antibody and flow cytometry. Therefore, as MDCK cells express functional P2X7, NALP3, caspase-1 and TLR4, this cell line should provide future opportunities to study the role of P2X7 in canine epithelial cells.

RT-PCR confirmed the expression of P2X7 and pannexin-1 in murine J774 macrophages. The expression of P2X7 in J774 cells was confirmed by immunoblotting, and by flow cytometric and ELISA measurements of ATP-induced ethidium⁺ uptake and IL-1β release respectively in the absence or presence of KN-62 or A-438079. Pannexin-1 antagonists, carbenoxolone (CBX), melfoquine (MFQ), flufenamic acid (FFA) and probenecid (PRO), failed to inhibit ATP-induced ethidium⁺ uptake into J774 cells with the exception of CBX at 50 μM. In contrast, CBX, FFA and PRO impaired P2X7-induced IL-1β release from J774 cells. Thus, although the role of pannexin-1 in P2X7-induced responses remains questionable, pannexin-1 antagonists may be of value in future studies with monocytes from canines and other species.

P2X7 was cloned from the cDNA of an English Springer Spaniel, and tagged with green fluorescence protein (GFP). This resulted in the generation of two vectors with canine P2X7, without (pP2X7-Ac-N1) or with GFP (pP2X7-AcGFP-N1). Sequence comparisons of the cloned P2X7 (English Springer Spaniel) to previously sequenced or cloned canine P2RX7 genes identified two non-synonymous single nucleotide polymorphisms (SNPs). These SNPs produced amino acid changes at positions Pro462Ser and Cys507Gly. The functional significance of these
SNPs remains unknown. Flow cytometric analysis demonstrated that pcP2X7-AcGFP-N1 was expressed at low amounts in transfected human 1321N1 astrocytoma cells, but not in human embryonic kidney 293 (HEK293). It remains to be determined if this cloned P2X7 can form functional channel/pores in mammalian cells. Thus, despite successful cloning of canine P2X7, the expression and function of the cloned receptor needs further study.

Finally, a fixed-time flow cytometric assay to measure the function of P2X7 in canine peripheral blood monocytes was developed. This assay required the isolation of buffy coats from whole blood, followed by the isolation of peripheral blood mononuclear leukocytes cells by density centrifugation before assessment of ATP-induced YO-PRO-1^{2+} uptake into CD14^{+} monocytes by flow cytometry. ATP-induced YO-PRO-1^{2+} uptake into canine monocytes was blocked by KN-62 and A-438079 indicating that this process was mediated by P2X7. ATP-induced YO-PRO-1^{2+} uptake was higher in day 1 compared to day 0 monocytes, therefore P2X7 function was only compared in fresh (day 0) blood from different dogs. After standardization of the assay, P2X7 function in monocytes from different dogs was measured to determine the relative variation in P2X7 function between these animals. There was a 2.6-fold difference in P2X7 function between dogs with the lowest and highest ATP-induced YO-PRO-1^{2+} uptake. ATP-induced YO-PRO-1^{2+} uptake into monocytes, B cells and T cells was also compared among five different dog breeds. P2X7 function in T cells was significantly greater than that of monocytes and B-cells. Finally, dog genomic DNA was extracted from each dog sample and stored to establish a canine DNA bank for future sequencing of the P2RX7 gene.

This study forms part of a larger group project to understand the physiological and pathophysiological role of the canine P2X7 including the identification of SNPs which may alter
P2X7 function or be associated with canine disorders. Results from this thesis will help contribute to the future progress and direction of the larger study.
Chapter 1: Introduction

1.1 Adenosine 5’-triphosphate as an extracellular signaling molecule

Adenosine 5’-triphosphate (ATP) is a multifunctional molecule that acts as an intracellular energy source in all biological systems. Although the biological effects of extracellular ATP have been documented for over 81 years (Drury and Szent-Gyorgi 1929), and other cytoplasmic metabolites, such as glycine, glutamate and calcium can function as extracellular messengers, there has been some reluctance to fully accept the idea that this nucleotide has the capacity to act as an autocrine or paracrine chemical messenger.

1.1.1 ATP can be released in different ways

There are three main modes of ATP release from cells. First, ATP can passively leak from damaged cells due to sheer stress, stretch, osmotic swelling, ischemia, hypoxia, inflammation, apoptosis or necrosis (Boudreault and Grygorczyk 2002). Second, ATP can be released via vesicular exocytosis or via transport mechanisms, such as ATP-binding cassette transporters. Last, ATP can be released via channels, such as P2X7 receptor channels and plasmalemmal voltage dependent anion channels (Bulanova & Bulfone-Paus 2010; Oliveira et al. 2010; Romanov et al. 2008).

1.1.2 Physiological roles of extracellular ATP

Outside the cell, ATP and its metabolites elicits a broad spectrum of physiological responses through the activation of P1 and P2 receptors. These actions include the modulation of vascular tone, activation of leukocytes, cell death, synaptic transmission in brain, spinal cord and
peripheral nerve terminals, glial communication, neurite outgrowth, and proliferation (Gandelman et al. 2010; North and Verkhratsky 2006; Fields and Burnstock 2006; Davalos et al. 2005; McCloskey et al. 1999; Schulman et al. 1999; Burnstock 1990, 1976, 1972; Gordon 1986).

1.1.3 **Role of ATP as an extracellular messenger in purinergic signaling**

The action of extracellular ATP on a plasma membrane receptor is termed ‘purinergic signaling’. Purinergic signaling can also be mediated by uridine 5′ triphosphate (UTP), adenosine 5′ diphosphate (ADP), uridine 5′ diphosphate (UDP) and adenosine. The P1 receptors are much more responsive to adenosine than to ADP and ATP and are selectively and competitively antagonized by methylxanthines, such as theophylline and caffeine, while P2 receptors are more responsive to ATP and ADP than to adenosine 5′ monophosphate (AMP) and adenosine (Burnstock et al. 2010). During the last three decades, the subclassification of P1 and P2 receptors has been supported by numerous pharmacological, biochemical and molecular biological studies (Burnstock et al. 2010). Pharmacologically, P2 receptors have been placed into two distinct subclasses, P2Y and P2X receptors (Burnstock and Kennedy 1985).

1.2 **P1 receptors**

1.2.1 **General structure and function of P1 receptors**

Adenosine is an endogenous signaling molecule that is derived from ADP or ATP and manages physiological functions in many tissues and organs via activation of P1 (adenosine) receptors. It accumulates in the extracellular space in response to metabolic stress and cell damage (Hasko et al. 2008).
P1 receptors are G protein-coupled receptors (GPCR) (Brown et al. 2008). P1 receptors are divided into four subclasses, A₁, A₂A, A₂B and A₃ (Table 1.1) (Zhou et al. 1992; Meyerhof et al. 1991; Daly et al. 1983; Bruns et al. 1980, 1983). A₁ and A₃ receptors are predominantly coupled to the Gᵢ family of G proteins to decrease cyclic AMP (cAMP) levels, increase K⁺ conductance and decrease transient Ca²⁺ conductance. A₂A and A₂B receptors mainly signal via the Gₛ family of G proteins and raise levels of cAMP (Jenner et al. 2009). Binding studies show that the A₂B and A₃ receptors have lower affinities to adenosine compared with A₁ and A₂A receptors (Fredholm et al. 2001).

Table1.1: G-protein coupling and signal transduction mechanisms of P1 (adenosine) receptors

<table>
<thead>
<tr>
<th></th>
<th>A₁</th>
<th>A₂A</th>
<th>A₂B</th>
<th>A₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-protein</td>
<td>Gᵢ/Gₒ</td>
<td>Gₛ/Gₚₐf</td>
<td>Gᵢ/Gₚ₁₁</td>
<td>Gᵢ/Gₚ₁₁</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>↓ cAMP</td>
<td>↑ cAMP</td>
<td>↑ cAMP</td>
<td>↓ cAMP</td>
</tr>
<tr>
<td>Affinity for adenosine</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
</tbody>
</table>

Adapted from Brown et al. (2008). Abbreviations: cAMP, cyclic adenosine monophosphate; IP₃, inositol triphosphate; DAG, diacylglycerol.

### 1.2.2 Distribution and physiological roles of P1 receptors

P1 receptors are expressed on various cells types including monocytes, macrophages, dendritic cells, neutrophils, eosinophils, mast cells and lymphocytes (Brown et al. 2008). The role of adenosine and P1 receptors in immunity is well established. Activation of P1 receptor subtypes in immune cells results in many downstream signals that lead to the protection against infectious diseases and in some cases drive chronic diseases. The name of the immune cell, receptor and its effect are shown in Table 1.2.
It appears that adenosine receptors have tissue specific functions. For example, the A2A receptor is important in mediating vasodilatation in various vascular beds, including coronary blood flow. It also contributes to increased respiratory drive in systemic hypoxia and support synthesis of new blood vessels via the generation of vascular endothelial growth factor (VEGF) (Jenner et al. 2009). It has been determined that adenosine receptors have an important role in asthma and chronic obstructive pulmonary disorders (COPD), but little is known about the relative expression of P1 receptor subtypes; however, binding studies in healthy peripheral lung tissue have suggested that A2 receptor subtypes are much more abundant than the A1 and A3 receptor subtypes (Brown et al. 2008; Joad 1990). Important roles of P1 receptors have also been demonstrated in many others inflammatory and chronic diseases including, ischemia, arthritis, sepsis, inflammatory bowel diseases (IBD) and wound healing. Although P1 receptors play an important physiological role in many tissues and diseases, their interactions with other molecular signals and receptors needs to be further understood (Hasko et al. 2008).

Table 1.2: Downstream effects of P1 receptor activation in immune cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Responresive receptor</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>A2A</td>
<td>Inhibition of TNF-α and IL-10 production</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>A1 or A3</td>
<td>Mobilization of intracellular Ca2+, reorganization of the actin cytoskeleton, and reduced of IL-12, IL-6 and IFN-α production</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>A2A</td>
<td>Production of TNF-α, MIP-1α, MIP-1β, MIP-2α and MIP-3α</td>
</tr>
<tr>
<td>Mast cells</td>
<td>A2B</td>
<td>Secretion of Th2 cytokines such as IL-4 and IL-13, and IL-1β and IL-18</td>
</tr>
</tbody>
</table>

Adapted from Hasko et al. (2008). Abbreviations: IL, interleukin; TNF, tumour necrosis factor; IFN, interferon, MIP, macrophage inflammatory protein; Th, type 2 helper T cells.
1.3 P2Y receptors

1.3.1 General structure and function of P2Y receptors

P2Y receptors are G-protein coupled receptors (GPCRs). Like other GPCRs, P2Y receptors are composed of seven transmembrane domains with short extracellular N- and intracellular C-terminals (Boarder et al. 1995; Harden et al. 1995). P2Y receptors consist of 308-377 amino acids, with a mass of 41-53 kDa after glycosylation. The third transmembrane region displays a high degree of homology between the P2Y subtypes, while the C-terminus shows the greatest diversity (Burnstock et al. 2010; Burnstock 2001). Eight P2Y subtypes have been identified and cloned (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14) (Burnstock et al. 2010; Deli & Csernoch 2008). The missing numbers in the P2Y1-14 sequence represent GPCRs cloned from nonmammalian vertebrates or mammalian receptors for which a functional response to nucleotides has not yet been convincingly demonstrated (Abbracchio et al, 2006). All the P2Y subtypes are activated by ATP, however some are activated by other preferred agonists. For P2Y1, ADP is reported to be equipotent or even more potent than ATP, while UTP is inactive. For P2Y2, ATP and UTP are equipotent agonists. UTP is a more potent agonist of P2Y4 and P2Y6 than ATP (Bulanova and Bulfone-Paus 2010; Di Virgilio et al. 2001). P2Y14 differs from the other P2Y receptors, recognizing UDP-glucose, UDP-galactose, UDP-glucuronic acid and UDP-N-acetylglucosamine (Bulanova and Bulfone-Paus, 2010). P2Y1, P2Y2, P2Y4, P2Y6 receptors are coupled to G_q proteins and activate adenylate cyclase and the phospholipase C (PLC)-inositol 1,4,5-triphosphate (IP_3) signaling pathway to cause release of Ca^{2+} from the intracellular stores. The other four P2Y subtypes activate (P2Y11) or inactivate (P2Y12-14)
adenylate cyclase via Gs and Gi proteins, respectively (Marteau et al. 2003; Hollopeter et al. 2001; Ralevic and Burnstock 1998).

1.3.2 Distribution and physiological roles of P2Y receptors

P2Y receptors are widely distributed, and more than one subtype or combination can usually be found on a given cell. These receptors are also in most types of tumor cells, including cancers of the brain, ovary, breast, gastrointestinal tract and skin (Deli and Csernoch, 2008). P2Y receptors are also in most immune cells, including monocytes, macrophages and lymphocytes. In addition to cancer and immune cells, P2Y receptors can be detected in other cell types such as excitable cells, nerves, glial cells, muscles, endocrine and exocrine cells, gut, liver, kidney bladder, lung, bone, cartilage, skin and endothelial cells (Abbracchio et al. 2006). Based on the P2Y subtype and their expression on cells, these receptors have well established roles in different cancer and autoimmune diseases.

1.4 P2X receptors

P2X receptors are trimeric ligand-gated cation channels activated by extracellular ATP. They are selective for monovalent and divalent cations (Na\(^+\), K\(^+\) and Ca\(^{2+}\)) (Van Kolen and Slegers, 2006; Abbracchio et al. 2003). Seven P2X subtypes (P2X1-7) have been identified (Roberts et al. 2006; North 2002). Diversity of the P2X receptors phenotypes is determined by assembly of individual subunits; six homomeric (P2X1-P2X5 and P2X7) and seven heteromeric (P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, P2X4/6 and P2X4/7) channels have been identified (Abbracchio et al. 2009; Guo et al. 2007; Roberts et al. 2006). The protein sequences deduced from the cDNA sequences consist of 379 (P2X6) to 595 (P2X7) amino acid residues, and share 26-46% identity
with each other (North 2002). P2X4 and P2X7 subunits are among the most related pairs in regards to amino acid sequence. P2X subunits are characterized by intracellular N- and C-terminal, two transmembrane (TM) domains, and a large extracellular loop containing ATP-binding sites and 10 conserved cysteines (Burnstock, et al., 2010). These cysteines are paired (for zebra fish P2X4 for example) in the order: 1-6 (Cys$_{119}$-Cys$_{168}$), 2-4 (Cys$_{129}$-Cys$_{152}$), 3-5 (Cys$_{135}$-Cys$_{162}$), 7-8 (Cys$_{220}$-Cys$_{230}$), 9-10 (Cys$_{264}$-Cys$_{273}$) (Young 2010).

1.4.1 Trimeric structure of P2X receptors

The trimeric structure of the P2X receptor has been recently confirmed based on the crystallography studies of the P2X4 receptor in zebra fish (Kawate et al. 2009). Although, for these studies, the intracellular N- and C-termini were removed, the modified P2X subunits still formed a functional channel when expressed in human embryonic kidney cells. The symmetrical trimeric assembly of three interlocking subunits surrounding a central cavity was clearly seen in this model (Fig. 1.1). The high resolution structure will provide an opportunity to understand the way in which ATP binds to and activates the channel, and the inter-subunit molecular motion that takes place during channel opening (Browne et al. 2010; Burnstock 2010).
Fig. 1.1: The structure of zebrafish P2X4 subunit. (a) Trimeric structure and large extracellular loop of P2X receptor are shown. Receptor monomers are shown in different colors. (b) A sagittal section of a surface rendering of the transmembrane domain shows that closed pore formed by angled transmembrane two helices with vestibules on each side of the gate (taken from Li et al. 2010).

Cysteine mutagenesis and functional studies strongly suggest that the second transmembrane (TM2) in P2X receptors is responsible for ion transduction and that this channel narrows significantly toward the middle of the TM domains (Jelinkova et al. 2008; Li et al. 2008; Migita et al. 2001). This is supported by the crystal structure of P2X4. This structure indicated that the TM domains are highly tilted, with the three TM2 subunits framing the pore, supported from the outside by the three first transmembrane (TM1) subunits (Kawate et al. 2009). Moreover, a recent mutagenesis study of the P2X4 has revealed that the internal region of TM2 must move a large distance towards the central axis of the channel during opening (Li et al. 2010). Taken together, a model in which expansion of the gate region of the external channel and the
constriction of the internal channel are accomplished by straightening of the TM2 helix, from a steeply angled orientation, towards the normal of the bilayer, can be proposed (Fig. 1.2).

Fig. 1.2: Channel formation in P2X receptors. The straightening of the transmembrane 2 helix during channel opening process in P2X receptors is shown. The helices of transmembrane (TM1) and TM2 are illustrated from the extracellular side of the membrane (taken from Li et al. 2010).

1.4.2 The P2RX genes

Each of the seven P2X receptor subtypes are encoded by individual genes. The genes for human P2RX4 and P2RX7 are located close to the tip of the long arm of chromosome 12 (12q24.31). Human P2RX1 and P2RX5 genes are also located close to each other on the short arm of chromosome 13. The remaining human P2RX genes are on different chromosomes. Each P2RX genes consists of 11-13 exons, and all share a common structure with well-conserved intron/exon boundries (North 2002).

1.4.3 Glycosylation of P2X receptors

All the P2X family subunits have consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr), and some glycosylation sites are vital for trafficking of the P2X receptor to the cell surface (Vacca et al. 2011). The number of N-linked glycosylation sites varies among the P2X
subunits (P2X2, three sites; P2X3, four sites; P2X4, six sites; P2X5, two sites; P2X6, three sites; P2X7, three sites) (Vacca et al. 2011; Torres, et al. 1998). Absence of glycosylation at one of the glycosylated sites still allows the P2X receptor to appear at the cell surface and remain fully functional. In contrast receptors in which only one site is glycosylated show low levels of functionality, while receptors with no glycosylated sites are nonfunctional (Vacca et al. 2011).

1.4.4 The ATP binding site in P2X receptors

The ATP binding site in P2X receptors has been predicted based on the mutagenesis and binding studies, and is directly supported by the crystal structure of P2X4. So far, eight highly conserved residues have been demonstrated in ATP binding. These residues in zebrafish P2X4 are Lys$^{70}$, Lys$^{72}$, Phe$^{188}$, Thr$^{189}$, Asn$^{296}$, Phe$^{297}$, Arg$^{298}$ and Lys$^{316}$ (Young 2009). The exact nature of these interactions is still unknown. The positively charged residues Lys$^{70}$, Lys$^{72}$, Lys$^{316}$ might be involved in interactions with the negatively charged triphosphate moiety of ATP (Jiang et al. 2000). Moreover, Phe$^{188}$, Thr$^{189}$, Asn$^{296}$, Phe$^{297}$ and Arg$^{298}$ may be involved in binding to the adenine ring and ribose moiety (Roberts and Evans 2007).

1.4.5 Channel formation in P2X receptors

Ion channels throughout the cell membrane contain a pore that opens and closes in response to special stimuli including binding of a ligand, changing of membrane voltage or stretching of the membrane (Li et al. 2010). The natural agonist of all P2X receptors is ATP. Upon agonist binding activation, some receptors desensitize rapidly such as P2X1 and P2X3, whilst others show little or no desensitization (Lalo et al. 2010; Markwardt 2007; North 2002). P2X activation also permits the passage of cations along their electrochemical gradients; in cells this leads to the
efflux of K⁺, and influx of Na⁺ and Ca²⁺. This in return leads to depolarization of the cell and downstream Ca²⁺ signaling (Young 2009).

1.4.6 Physiological roles of P2X receptors

P2X receptors are present in most cell types throughout the body, and initiate various downstream effects upon ATP binding and activation. P2X subunits play roles in many physiological processes such as afferent signaling in taste buds (P2X2, P2X3, P2X4 and P2X7), urinary bladder reflex (P2X3), inflammatory and neuropathic pain (P2X3, P2X4 and P2X7), vascular tone and remodeling (P2X4 and P2X7), collagen deposition, renal fibrosis, cytokine release, bone remodeling (P2X7) (Goncalves et al. 2006; Ke et al. 2003; Solle et al. 2001), exocrine hormone release (P2X4 and P2X7) and intestinal motility (P2X2 and P2X3). P2X receptors are also expressed in the central nervous system and have role in postsynaptic and presynaptic effects (P2X2, P2X4 and P2X6) (Surprenant and North 2008; Jarvis et al. 2002; Cockayne et al. 2000). As a result of the various physiological roles of P2X receptors, these receptors are therefore attractive as possible therapeutic targets.

1.5 The P2X7 receptor

Among the seven members of P2X subtypes, P2X7 is attracting much attention as a therapeutic target due to its many downstream properties, and involvement in inflammation, immunity and numerous disorders.

1.5.1 Channel/pore properties of the P2X7 receptor

Activation of the P2X7 with ATP or 3’-O-(4-benzoyl benzoyl) ATP (BzATP) opens a membrane channel permeable to small cations (Na⁺, Ca²⁺, K⁺), while sustained activation leads to pore
formation (4 nm), and the subsequent influx of larger molecules such as N-methyl-D-glutamine (NMDG; 195 Da), choline$^+$ (100 Da), methylglucamine (190 Da), ethidium$^+$ (314 Da), YO-PRO-1$^{2+}$ (376 Da), propidium$^{2+}$ (414 Da) and lucifer yellow (457 Da) (Duan and Neary 2006; Adinolfi et al. 2005; Ferrari et al. 1997). However, whether the P2X7 channel undergoes dilation to form a pore or involves a second molecule such as pannexin-1 is still a major debate (Pelegrin and Surprenant, 2009; Mayo et al. 2008; Pelegrin et al. 2008) and is discussed further below (Section 1.7).

1.5.2 Molecular significance of P2X7 activation

P2X7 receptor activation initiates various downstream signaling pathways. These include the stimulation of the NALP3 inflammasome complex (Surprenant and North 2008), phospholipase A$_2$ and D (North 2002), extracellular signal-regulated kinases (ERK1/2) and p38 mitogen-activated protein kinases (p38 MAPK) (Potucek et al. 2006; Aga et al. 2002), activation of transcription factors such as the cyclic-AMP response element-binding (CREB) protein (Lenertz et al. 2010), tumor necrosis factor TNFα-TRAIL, c-Jun N-terminal kinases 1 and 2/stress activated protein kinase (SAPK) and nuclear factor-κB (NF-κB) cascade (Gorodeski, 2009; Suzuki et al. 2004; Humphreys et al. 2000). Also, stimulation of P2X7 receptor increases protein tyrosine phosphorylation (Aga et al. 2004; Adinolfi et al. 2003), ultimately leading to MAPK activation.

1.5.3 P2X7 receptor structure

As previously mentioned (Section 1.4), the P2X7 receptor is comprised of intracellular N- and C- terminus, two plasma membrane segments and large extracellular loop. The intracellular N-terminus comprises (amino acids 1-25), while the first and second TM comprise 26-46 and
335-355 amino acids, respectively. The extracellular domain (amino acids 47-334) contains the ATP binding site. The long intracellular C-terminus (amino acids 356-595) is essential for pore formation, and also directs trafficking and stabilizes expression of the receptor in the plasma membrane (Gorodeski 2009).

1.5.4 Cloning of the P2X7 receptor

The P2X7 receptor has been cloned and characterized in many species including human, mouse, rat, dog and guinea pig (Table 1.3). Each of the cloned mammalian P2X7 receptors display > 70% sequence similarity to the human receptor.

Table 1.3: Cloned P2X7 from different species

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue source</th>
<th>Sequence similarity to human P2X7</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Brain</td>
<td>100%</td>
<td>Surprenant et al. (1996)</td>
</tr>
<tr>
<td>Human</td>
<td>Monocytes</td>
<td>-</td>
<td>Rassendren et al. (1997)</td>
</tr>
<tr>
<td>Mouse</td>
<td>NTW8 microglial cells</td>
<td>80%</td>
<td>Chessell et al. (1998)</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>Brain</td>
<td>77%</td>
<td>Fonfria et al. (2008)</td>
</tr>
<tr>
<td>Dog</td>
<td>Heart</td>
<td>85%</td>
<td>Roman et al. (2009)</td>
</tr>
</tbody>
</table>

1.5.5 P2X7 single nucleotide polymorphisms

The human P2RX7 gene is located within a 55 kb region of chromosome 12q24 and comprised of 13 exons. The P2X7 transcription initiation site (TpIS) is adenine (+1) at nucleotide 1683 of the human P2RX7 gene with a TTAAA sequence at nucleotides -32 to -28 and a native promoter region within nucleotides -158 to +32 (Gorodeski 2009). In contrast to the other P2X subtypes, P2X7 is highly polymorphic. More than 260 single nucleotide polymorphisms (SNPs) have been
described in the human *P2RX7* gene. So far, 32 non-synonymous (amino acid-altering) SNPs have been documented in the *P2RX7* gene. The first SNP described which lead to a loss-of-function of the receptor was 1513A>C, which changes glutamic acid to alanine at residue 496 (Glu^{496} to Ala) in the C-terminus of the receptor (Gu et al. 2001). Due to its early discovery and high frequency within the population, this SNP has been the most extensively studied (Table 1.4). The Glu^{496} to Ala substitution impairs ATP-induced IL-1β and IL-18 release from monocytes (Sluyter et al. 2004a; 2004c), L-selectin shedding from lymphocytes and monocytes (Sluyter et al. 2004c), CD23 shedding from dendritic cells (Sluyter and Wiley, 2002), phosphatidylserine (PS) exposure in erythrocytes (Sluyter et al. 2007), cell death of lymphocytes and macrophages (Saunders et al. 2003; Gu et al. 2001) and the killing of intracellular mycobacteria (Saunders et al. 2003). Moreover, 1513A>C SNP is associated with various disorders including tuberculosis (Fernando et al. 2007) and follicular papillary thyroid cancer (Dardano et al. 2009).
Table 1.4: Single nucleotide polymorphisms known to alter P2X7 function

<table>
<thead>
<tr>
<th>SNP</th>
<th>Amino acids</th>
<th>Minor allele frequency</th>
<th>P2X7 function</th>
<th>Cause of altered P2X7 function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>g (151 + 1g)</td>
<td>Intron 1</td>
<td>0.01</td>
<td>Decreased</td>
<td>Null allele</td>
<td>Skarrat et al. 2005</td>
</tr>
<tr>
<td>H155Y</td>
<td>His155Tyr</td>
<td>0.439</td>
<td>Increased</td>
<td>Unknown</td>
<td>Bradley et al. 2011</td>
</tr>
<tr>
<td>G489T</td>
<td>His155Tyr</td>
<td>0.50</td>
<td>Increased</td>
<td>Unknown</td>
<td>Cabrini et al. 2005</td>
</tr>
<tr>
<td>G946A</td>
<td>Arg307Gln</td>
<td>0.01</td>
<td>Decreased</td>
<td>Prevents ATP binding</td>
<td>Gu et al. 2004</td>
</tr>
<tr>
<td>A348T</td>
<td>Ala348Thr</td>
<td>0.400</td>
<td>Increased</td>
<td>Unknown</td>
<td>Bradley et al. 2011</td>
</tr>
<tr>
<td>C1096G</td>
<td>Thr357Ser</td>
<td>0.10</td>
<td>Decreased</td>
<td>Unknown</td>
<td>Shemon et al. 2006</td>
</tr>
<tr>
<td>A1513C</td>
<td>Glu496Ala</td>
<td>0.19</td>
<td>Decreased</td>
<td>Unknown</td>
<td>Gu et al. 2001</td>
</tr>
<tr>
<td>T1729A</td>
<td>He568Asn</td>
<td>0.02</td>
<td>Decreased</td>
<td>Trafficking defect</td>
<td>Wiley et al. 2003</td>
</tr>
</tbody>
</table>

SNPs have been found in mouse P2RX7. It has been demonstrated that Pro451Leu leads to reduce sensitivity to ATP-induced pore formation. The effect of this mutation is similar to that of the human Glu496Ala mutation in humans (Adriouch et al. 2002).

1.5.6 Regulation of P2X7 receptor expression

The amount of functional P2X7 expression is a critical factor for determination of receptor activity. P2X7 transcription is regulated by two groups of cis-regulatory enhancer elements.
located within nucleotide regions +222/+232 and +403/+573 downstream of the active promoter. P2X7 transcription is controlled by methylated cytosines at cytosine-phosphodiester-guanosines (CpG) sites that cluster or co-localized with the enhancer’s sites. Cytosines at CpG sites +211/+212, +330/+331 and +461/+464 are constitutively methylated in vivo, and hypermethylation of these sites inhibits P2X7 transcription (Gorodeski 2009). Moreover, the human P2X7 3’-untranslated region (3’UTR) consists of sequences that confer instability to the P2X7 transcript. Human poly (ADP-ribose) polymerase (PARP) interacts with transcribed P2X7 mRNA and de-stabilizes the (3’UTR)-P2X7 mRNA (Zhou et al. 2009). Inhibition of PARP could increase stability of the P2X7 mRNA and also augments P2X7-related apoptosis (Gorodeski 2009).

1.5.7 P2X7 receptor agonists

In contrast to the other P2X receptors, P2X7 receptors are activated by relatively high concentrations of ATP (EC50 ~100 µM). BzATP is the most potent P2X7 agonist (EC50 ~20 µM) however, like ATP it is not specific for P2X7 receptor (Jacobson et al. 2002; Donnelly-Roberts & Jarvis 2007; Bianchi et al. 1999). Other nucleotides are either weak P2X7 agonists 2-methylthio adenosine 5’ triphosphate (2MeSATP), adenosine 5’-O-(3-thio) triphosphate (ATPγS) and ADP or ineffective as P2X7 agonists αβ-methylene-ATP (αβMeATP), βγ-methyleneadenosine 5’-triphosphate (βγmeATP) and UTP (North and Surprenant 2000). Thus, the rank order of P2X7 agonists is BzATP>ATP>2MeSATP>ATPγS>ADP>>αβMeATP=βγmeATP=UTP (Anderson and Nedergaard 2006; Bianchi et al. 1999).
1.5.8 P2X7 receptor antagonists

P2X7 receptor function can be impaired by extracellular ions, as well as non-specific antagonists. Extracellular ions such as Ca\(^{2+}\) (3000 μM), Mg\(^{2+}\) (500 μM), Zn\(^{2+}\) (11 μM), Cu\(^{2+}\) (0.5 μM) and H\(^{+}\) (1 μM, pH 6) at the indicated concentrations can block P2X7-evoked currents (Virginio et al. 1997). The inhibitory action of Mg\(^{2+}\) is commonly used to stop P2X7-induced events including pore formation and CD23 shedding (Sluyter and Wiley 2002). A number of non-selective P2X7 antagonists have been identified including suramin (IC\(_{50}\) ~300 μM), pyridoxal-phosphate-6-azophenyl-2’,4’-disulfonate (PPADS; IC\(_{50}\) ~50 μM), 2’, 3’ dialdehyde ATP (oxidased ATP; IC\(_{50}\) ~0.9 μM) and Brilliant Blue G (BBG; IC\(_{50}\) ~0.2 μM) (Jiang et al. 2000; Chessell et al. 1997; Lammas et al. 1997; Surprenant 1996). The non-selective action of these compounds is well known (Burnstock 2007; Jacobson et al. 2002). Among these antagonists, BBG is the most potent inhibitor of human and rat P2X7 receptors (Jiang et al. 2000), however this compound is a known protein dye and thus is likely to bind to various molecules to potentially altering their activity. Calmidazolium and KN-62 are also effective antagonists of rat (IC\(_{50}\) ~15 nM) and human (IC\(_{50}\) ~50 nM) P2X7 respectively (Gargett and Wiley 1997; Virginio et al. 1997), however these compounds also impair calmodulin-related kinases. Finally, P2X7 can also be inhibited by 17β estradiol (Cario-Toumaniantz et al. 1998), however this compound is not used as regular P2X7 antagonist. Progress in the field of P2X7 has been greatly enhanced by the development and recent availability of a number of selective P2X7 antagonists. These include A-438079 (IC\(_{50}\) ~300 nM; Nelson et al. 2006), A-740003 (IC\(_{50}\) ~40 nM; Honore et al. 2006), and AZ 11645373 (IC\(_{50}\) ~10-90 nM; Stokes et al. 2006). Of note, A-438079 and A-740003 can block P2X7 activity in vivo in dose-dependent manner as observed in neuropathic and inflammatory pain models (Donnelly-Roberts et al. 2007), supporting the
potential therapeutic use of P2X7 antagonists. A number of other selective P2X7 antagonists have also been developed (Friedle et al. 2010), but these are not commercially available. In addition, a monoclonal antibody can block human P2X7 (Buell et al. 1998), while a monoclonal antibody raised against the rat P2X7 potentiates receptor activity (Kim et al. 2001).

Each of the above non-selective and selective P2X7 antagonists demonstrate a variable degree of species selectively (Table 1.5). Such differences between species have proved useful in identifying residues imported for antagonist residues. For example, the species selectivity of AZ11645373, but not a second P2X7 antagonist (compound-22), is related to the amino acid at position 95 of the P2X7 receptor (Michel et al. 2009). Similar future studies like that of Michel et al. (2009) will be of value to further identify P2X7 antagonist binding sites.

Table 1.5: Relative potency of agonists and antagonists at cloned mammalian P2X7

<table>
<thead>
<tr>
<th>Cloned P2X7</th>
<th>EC\textsubscript{50} ATP</th>
<th>Effective antagonists</th>
<th>Non-effective antagonists</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>3.06 ± 0.04</td>
<td>A-740003 &gt; PPNDS &gt; KN-62 &gt; BBG</td>
<td>NF023 &lt; Reactive Blue</td>
</tr>
<tr>
<td>Rat</td>
<td>3.66 ± 0.02</td>
<td>A-740003 &gt; A-438079 &gt; PPNDS &gt; BBG</td>
<td>KN-62 &lt; Cibracon Blue</td>
</tr>
<tr>
<td>Mouse</td>
<td>2.92 ± 0.05</td>
<td>KN-62 &gt; BBG &gt; A-438079</td>
<td>Reactive Blue &lt; PPADS</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>3.22 ± 0.10</td>
<td>KN-62 &gt; PPADS &gt; suramin*</td>
<td>_</td>
</tr>
<tr>
<td>Dog</td>
<td>2.5 ± 0.05</td>
<td>KN-62 &gt; GSK361390*</td>
<td>_</td>
</tr>
</tbody>
</table>

Table adapted from Donnelly-Roberts et al. 2009. * Data from Fonfria et al. (2008) and Roman et al. (2009), respectively.
1.5.9 Distribution of the P2X7 receptor

The P2X7 receptor is expressed by many cell types throughout the body. It is mainly expressed on cells of haematopoietic lineage, including erythrocytes, T and B lymphocytes, mast cells, monocytes, macrophages, dendritic cells, and epidermal Langerhans cells (North 2002; Surprenant et al. 1996). As a result of this many cell lines such as murine RAW 246.7 and J774 macrophages have been used as valuable models of P2X7 expression and function (Sperlagh et al. 1998; Chiozzi et al. 1997; Dubyak et al. 1996). P2X7 can also be found in microglia, Schwann cells and astrocytes (Skaper et al. 2010). Due to the poor selectivity of antibodies however, the existence of P2X7 on peripheral and central neurons remains controversial (Anderson and Nedergaard 2006). P2X7 is also expressed in many epithelial cells from many tissues including skin, bladder, kidney and salivary glands (Turner et al. 2007; Goncalves et al. 2006; Adinolfi et al. 2005).

The up-regulation of P2X7 receptor expression has been detected in various inflammatory diseases including chronic renal injury. For instance, epithelial P2X7 is up-regulated in polycystic kidney disease (Turner et al. 2009; Xu et al. 2009) and in patients with autoimmune-related glomerulonephritis (Goncalves et al. 2006; Turner et al. 2006). Further, models using P2X7 knockout mice or wild type rats models have confirmed a role for P2X7 in unilateral ureteral obstruction.

1.5.10 Physiological role of the P2X7 receptor

The physiological role of the P2X7 remains to be fully elucidated. However, due to the many events downstream of P2X7 activation (as listed below), P2X7 is thought to play important roles in inflammation and immunity, renal disease, bone formation, cancer and neurodegenerative
disorders (Di Virgilio 2007; Hillman et al. 2004; Gartland, et al. 2003; Ke et al. 2003; Adinolfi et al. 2002; Di Virgilio 1995). P2X7 activation can induce a number of downstream events including the release of cytokines (IL-1β and IL-18), intracellular enzymes (cathepsins and matrix metalloproteinase (MMP-9)), cell surface molecules (CD23 and CD62L) and microparticles. P2X7 activation can also induce reactive oxygen species formation and cell death (Hewinson and Mackenzie 2007; Ferrari et al. 2006). Of the above, the maturation and release of IL-1β and IL-18 is the most well characterized event downstream of P2X7 activation. This process, as well as the function of these cytokines will be discussed in greater detail below.

1.6 P2X7 activation, and the release of IL-1β and IL-18

1.6.1 IL-1β

IL-1β is one of the most well studied pro-inflammatory cytokines due to its important role in inflammation, injury and immunity (Dinarello 2009; Ferrari et al. 2006). IL-1β is synthesized as an inactive and leaderless 34 kDa precursor. Upon cellular activation by a number of mechanisms including P2X7 activation, this precursor is released as a biologically active 17 kDa form following cleavage by the cysteine protease caspase-1 (Ferrari et al. 2006, Solle et al. 2001; Ferrari et al. 1997). It has been demonstrated that pro-IL-1β in P2X7 knock-out mice cannot be activated by caspase-1 and secreted in the presence of ATP (Solle et al. 2001). IL-1β is produced predominantly by monocytes, macrophages and dendritic cells, but can also be produced by other cell types such as epithelium (Dinarello 1998). The release of IL-1β has many effects on different cells and tissues. IL-1β can act on lymphocytes in several ways including upregulating IL-2 receptor expression, prolonging survival of T cells, activating macrophages and neutrophils, inducing Th1 and Th2 enhancing antibody production by B cells, and increasing B cell
proliferation (Ben-Sasson et al. 2009; Maliszewski et al. 1990). It also induces fever and bone resorption by modulation of muscle metabolism and activation of osteoclasts, respectively (Ferrari et al. 2006). IL-1β is elevated during infections and in several chronic inflammatory diseases such as arthritis, scleroderma, systemic lupus erythematosus, vasculitis, sepsis, septic shock, and in the presence of atherosclerotic lesions leading to myocardial infections (Ferrari et al. 2006). IL-1β also plays a significant role in driving the differentiation and amplification of Th17 cells, via up-regulating Th17 transcription factors IRF4 and RORγt (Chung et al. 2009; Kryczek et al. 2007; Sutton et al. 2006). IL-1β release also leads to the up-regulation of gene products that contribute to the inflammatory state, including matrix metalloproteases, cyclooxygenase-2, sustaining nitric oxide production, interleukins, and cell adhesion molecules (Guan et al. 1998a and b).

The processing of IL-1β by caspase-1 is largely mediated by the NALP3 inflammasome (as discussed below). It should be noted however that, IL-1β processing can be mediated by NALP3 inflammasome-independent pathways. During acute inflammatory conditions the serine proteinases cathepsin G, elastase and proteinase 3 are able to proetelytically cleave precursor IL-1β (Dinarello 2010; Stehlik 2009). Further, matrix metalloproteinases (MMP) such as stromelysin 1, gelatinases A and B can act on Precursor IL-1β to form mature IL-1β. It has been observed that PR3, which predominantly exists in activated neutrophils, is one of the most potent preoteases that can process IL-β. Thus due to these caspase-1-independent pathways of IL-1β maturation, the application of caspase-1 antagonists in controlling of IL-1β secretion and subsequently inflammation may be limited in some diseases.
1.6.2 IL-18

IL-18 was originally discovered as an interferon (IFN)-γ inducing cytokine in endotoxemic mice (Manhart et al. 2002). Similar to IL-1β, IL-18 is initially synthesized as an inactive and leaderless 24 kDa precursor, which then is converted to its mature 18 kDa form by caspase-1 (Dinarello 2009; Ferrari et al. 2006). IL-18 is released from many cell types including monocytes, macrophages, dendritic cells, epithelial cells, keratinocytes and synovial fibroblasts. IL-18 binds to an αβ heterodimeric receptor inducing the synthesis of other pro-inflammatory cytokines such as IL-6, IL-8, TNF-α, IL-1β, and IFN-γ, CD95 ligand and several chemokines. IL-18 also enhances expression of cell adhesion molecules ICAM-1 and VCAM-1 in endothelial cells (Ferrari et al. 2006; Dinarello 2002). Similar to IL-1β, IL-18 can also amplify T and B cell responses, including the amplification and differentiation of Th17 responses (Yoshimoto et al. 1998). In contrast to IL-1β however, IL-18 is not an endogenous pyrogen. Over-production of IL-18 and subsequent IFN-γ production are related to many human diseases such as systemic lupus erythematosus, macrophage activation syndrome, rheumatoid arthritis, type 1 diabetes, Crohn’s disease, psoriasis and graft-versus-host disease (Dinarello 1996).

1.7 The NALP3 inflammasome

Inflammasomes are large macro-molecules complexes involved in the maturation of IL-1β and IL-18. These include three types: NALP1, NALP3 and IPAF inflammasomes. The NALP3 inflammasome has been studied extensively due to its prominent function in sterile inflammatory and antimicrobial responses, adjuvanticity and hereditary autoinflammatory syndrome (Bauernfeind et al. 2010). NALP3 is also known as NLRP3, CIAS1, cryopyrin and PYPAF1 (Shigeoka et al. 2010; Hoffman et al. 2010). NALP3 contains an N-terminal PYD domain, a
central NBD domain, and a C-terminal LRR domain. Upon activation, NALP3 recruits the bridging molecule apoptosis-associated speck-like protein containing a CARD (ASC) via homotypic PYD-PYD interactions. Recruited ASC binds to pro-caspase-1 via a CARD-CARD interaction and activates caspase-1. Activation of the inflammasome and caspase-1, along with expressed NF-κB, leads to maturation and releasing of IL-1β and IL-18 pro-inflammatory cytokines (Chen et al. 2011; Fernandes-Alnemri et al. 2007; Agostini et al. 2004). The oligomerization and assembling of the NALP3 inflammasome after stimulation requires intracellular ATP binding to the central nucleotide-binding element and also CARD8 as a facilitator (Duncan et al. 2007). Recent studies have revealed that excessive inflammasome activation can cause autoinflammatory disorders such as the hereditary periodic fevers and also other diseases, including type 2 diabetes, Muckle-Wells syndrome (MWS), chronic infantile neurological cutaneous, articular syndrome, inflammatory bowel diseases (IBD) and atherosclerosis (Shaw et al. 2011; Liu-Bryan 2010). Mutations in NALP3 have been described in the cryopyrin-associated periodic syndrome (CAPS), while specific NALP3 polymorphisms have been associated with Crohn’s disease (Villani et al. 2009). The NALP3 inflammasome has also been linked with polygenic inflammatory disorders such as gout and pseudogout (Liu-Bryan 2010).

1.7.1 Inflammasome activation

In addition to P2X7, NALP3 inflammasome can be activated by many pathogens such as Sendai virus, influenza A virus, adenoviruses, *Stephlococcus aureus, Listeria monocytogenes*, *Escherichia coli, Mycobacterium marinum, Shigella flexneri, Neisseria gonorrhoe* and *Candida albicans*. Activation of the NALP3 inflammasome by viruses involves the virus-encoded M2 ion
channel protein and specific pore-forming toxins. Some specific bacterial pore forming agents include Listerolysin O, nigericin (Mariathasan et al. 2006), and hemolysin (Munoz-Planillo et al. 2009), as well as the detergent saponin (Li et al. 2008) and the marine toxin, maitotoxin (Gurcel et al. 2006). In addition to the aforementioned compounds and particles, single-stranded RNA, double-stranded RNA, peptidoglycans, and CpG DNA, have been reported to activate the NALP3 inflammasome (Kanneganti et al 2006, 2007). It is still unclear however for many pathogens whether inflammasome activation is caused by single or multiple pathogenic factors.

Apart from microbial pore-forming toxins, phagosomal materials such as crystals and other crystalline materials, particles and protein aggregates can also activate NALP3 inflammasome (Martinon et al. 2006). The first crystal-induced inflammatory diseases linked to the NALP3 inflammasome were gout and pseudogout, where monosodium urate (MSU) and calcium pyrophosphate dehydrate (CPPD) crystals respectively can activate the NALP3 inflammasome and cause IL-1β release (Martinon et al. 2006). Subsequent studies have also shown that several environmental pollutants consisting of material and endogeneous aggregates can also trigger NALP3 activation. For example, macrophages deficient in components of the NALP3 inflammasome were incapable of secreting the IL-1β and IL-18 in response to inorganic crystalline silica and asbestos fibres (Cassel et al. 2008; Dostert et al. 2008; Hornung et al. 2008). Moreover, silica and asbestos can cause pneumonitis and the progressive pulmonary fibrotic disorders silicosis and asbestosis, respectively in a NALP3-dependent manner.

In addition to the above activators of the NALP3 inflammasome, the level of NALP3 expression is crucial for its activation. The baseline expression of NALP3 is not sufficient for caspase-1 activation in unprimed monocytes and macrophages, but can be elevated by priming these cells
with Toll-like receptor (TLR) ligands. This priming results in increased transcription of NALP3 via the transcription factor NF-κB (Bauernfeind et al. 2009). Thus, like IL-1β synthesis, NALP3 also requires TLR ligand priming. Moreover, NALP3 and pro-IL-1β expression are not necessarily linked, as NALP3 activation exerts biological effects in the absence of caspase-1 and pro-IL-1β (Mariathasan et al. 2006).

Molecular mechanisms involved in NALP3 inflammasome activation (Fig. 1.3) are still poorly understood. K⁺ efflux is required for NALP3 activation (Petrilli et al. 2007; Perregaux and Gabel 1994), however it is still unclear whether this efflux alone acts as a NALP3-activating stimulus itself or whether low intracellular K⁺ acts as additional stimuli for inflammasome activation. It is possible that inflammasome assembly is favored by low intracellular K⁺ concentrations but it may not be sufficient for activation (Bauernfeind et al. 2010). In addition to cellular K⁺ efflux, the production of reactive oxygen species (ROS), may also activate NALP3 inflammasome (Dostert et al. 2008). On the other hand, it has been demonstrated that lysosomal disintegration, which leads to leakage of lysosomal enzymes into the cytosol, can also trigger NALP3 activation. The lysosomal protease cathepsin B plays a major role in this pathway. Moreover, the direct link between cathepsins and NALP3 activation has not been explained yet. However, the source of ROS and their interaction with the inflammasome remains unknown.
Fig. 1.3: NALP3 inflammasome activation. Stimulation of Toll-like receptors (TLRs) results in the up-regulation of NALP3 (NLRP3) and pro-interleukin (IL)-1β. It has been suggested that NALP3 inflammasome can be activated by three distinct pathways: (i) efflux of potassium (via P2X7 activation) or pore forming, (ii) lysosomal damage and (iii) reactive oxygen species (ROS) production (Bauerfeind et al. 2010).

1.8 Pannexin-1

1.8.1 Pannexin hemichannels

Intercellular communications are mediated by gap junctions, which are formed by hemichannels between adjoining cells. Hemichannels are formed by hexamers of connexins. However, it is observed that pannexins, which are structurally similar to connexins, are also expressed in vertabrates and have functional properties. The vertebrate pannexins comprise of three members in mammals: pannexin-1 (426 amino acids, 47.6 kDa), pannexin-2 (664 amino acids, 73.3 kDa), and pannexin-3 (392 amino acids, 44.7 kDa) (Bruzone et al. 2003). Pannexins are expressed in
many different cell types particularly in the central nervous system (Penuela et al. 2008). In addition to their localization in plasma membrane, pannexins are also present in intracellular organelles and Golgi apparatus (Huang et al. 2007; Lai et al. 2007). Channel gating by pannexins is a highly regulated and finely tuned process. Voltage patch clamp studies have demonstrated that pannexin-1 has at least five open states: the fully open state and states with no less than 5, 25, 30 or 90% conductance of the maximal conductance (Bao et al. 2004; Bruzzone et al. 2003). Pannexins channels reside mainly in the subconductance states and rarely remain in the fully open or closed states (Weber et al. 2004).

1.8.2 Pannexin-1 and its physiological properties

Among all the hemichannels, pannexin-1 shows unique properties in terms of activation and function. It can be activated by different physiological stimuli, including mechanical stress during osmotic shock (D’Hondt et al. 2009), strong depolarization (>+20 mV), and by extracellular ATP and other nucleotides via activation of purinergic receptors such as P2Y1, P2Y2, and P2X7 (D’Hondt et al. 2009).

Although extracellular calcium has no affect on pannexin-1 activation, intracellular \( \text{Ca}^{2+} \) regulates function of pannexin-1. Pannexin-1 expressed in \textit{Xenopus} oocytes displays a linear dependence on \( \text{Ca}^{2+} \), with higher intracellular \( \text{Ca}^{2+} \) concentrations leading to larger pannexin-1 currents (Locovei et al. 2006). \( \text{Ca}^{2+} \) concentrations above resting levels (>100 nM) are normally sufficient to activate pannexin-1 opening. However, increasing \( \text{Ca}^{2+} \) is not a requisite for pannexin-1 activation especially in hippocampal neurons. (Thompson et al. 2008). It is likely that pannexin-1 hemichannels are opened during agonist-induced \( \text{Ca}^{2+} \) signaling, thereby playing an important role in mediating ATP release and intracellular communications.
1.8.3 Physiological role of pannexin-1

Pannexin-1 appears to have a number of physiological roles. Pannexin-1 is involved in the recognition of bacterial molecules (Kanneganti et al. 2007), apoptosis (Reigada et al. 2008), ischemic death of neurons (D’hondt et al. 2009) and tumor suppression (Lai et al. 2007). Moreover, pannexin-1 mediates the regulated release of “find-me” signals during apoptosis (Chekeni et al. 2010). Further, pannexin-1 is involved in the release of muramyl dipeptide from acidified vesicles into the cytosol, and in NF-κB and MAPK activation (Marina-Garcia, 2008). Finally, pannexin-1 expression is elevated upon exposure to various pro-inflammatory stimuli such as TNF-α, IFN-γ, IFN-α and LPS supporting a role for this hemichannel molecule in inflammation and other disorders (Shestopalov and Panchin, 2008).

1.8.4 Pannexin-1 and P2X7

In addition to the above physiological roles, pannexin-1 may also be involved in P2X7 function. Pelegrin and Surprenant (2006) originally demonstrated that pannexin-1 is the pore forming unit mediating P2X7-induced dye uptake and IL-1β release. This observation that pannexin-1 may mediate P2X7-mediated dye uptake was subsequently supported by others (Locovei et al. 2007). Moreover, the same group suggested a role for pannexin-1 in P2X7-induced cell death. Nevertheless, these results have largely been based on the over-expression of pannexin-1 and P2X7 in transfected cells, and the use of non-specific gap junction inhibitors as pannexin-1 antagonists. Moreover, reports verifying pannexin-1 as the pore forming unit of P2X7 are scant. Finally, the observation that binding of ATP to an extracellular region of pannexin-1 results in inhibition of pannexin-1 mediated currents complicates the understanding of pannexin-1 as the P2X7 pore (Qiu and Dahl 2009).
1.9 Caspase-1

The majority of caspases are involved in apoptosis, however a few are called ‘inflammatory caspases’. These few caspases are named inflammatory caspases as the main caspase-1 is involved in maturation of IL-1β and IL-18 pro-inflammatory cytokines. Inflammatory caspases (or group I caspases) are encoded by four genes in humans (caspase-1, caspase-4, caspase-5 and caspase-12) and three genes in mouse (caspase-1, caspase-11 and caspase-12) (Guarda and So 2010; Martinon and Tschopp 2004; Lamkanfi et al. 2002). In mammals, these caspases are characterized by the presence of a CARD domain at the N-terminus. Based on the evolutionary relations, caspases are linked to the clan CD cysteine peptidases that include legumain, sterptopain, separin, metacaspases and paracaspases (Barrett et al. 2001). Caspases are produced in the cells as inactive zymogens and undergo proteolytic processing during activation (Matinon and Tschopp 2007). Following proteolytic processing, caspase-1 consists of 10 (p10) and 20 (p20) kDa subunits. Activated caspase-1 converts pro-IL-1β (31 kDa) and pro-IL-18 (24 kDa) into its active 17 kDa and 18 kDa forms respectively. Recent studies suggest that another IL-1β related cytokine, IL-33 is also a possible caspase-1 substrate. IL-33 can act on its ST2 receptor after processing by caspase-1 (Schmitz et al. 2005). IL-33 however is also an intracellular cytokine and can function as a DNA-binding nuclear factor (Dinarello 2009). Although secondary to caspase-1 in the catalytic activity, caspase-5, and its murine homologue caspase-11 interact with caspase-1 to increase its proteolytic activity (Li et al. 1995; Wang et al. 1998). Besides IL-1 cytokines, caspase-1 can cleave the apoptotic caspase-7 (Lamkanfi et al. 2008; Ghayur et al. 1997; Gu et al. 1997). Thus, it has been suggested that cleavage of caspase-7
downstream of caspase-1 might be involved in pyroptosis, a type of caspase-1-dependent inflammatory cell death (Bergsbaken et al. 2009).

**1.10 Aims**

Our group is currently investigating the canine P2X7 receptor including its roles in inflammation and immunity. As part of this large project, this thesis aims:

1. To determine if Madin-Darby canine epithelial kidney (MDCK) cells express functional P2X7 receptors;

2. To confirm if pannexin-1 antagonists impair P2X7-induced ethidium\(^+\) uptake and IL-1\(\beta\) release from murine macrophages;

3. To clone the P2X7 receptor from an English Springer Spaniel;

4. To determine if P2X7 receptor function differs between dogs or breeds, and to collect corresponding DNA samples for future genetic analysis.

These aims will produce the following outcomes:

Aim 1 will determine if MDCK cells express functional P2X7. If absent, this cell line may provide a suitable cell line to study the cloned canine P2X7 (aims 3). If present, this cell line may provide a future model to study P2X7 in canine kidney epithelial cells.

Aim 2 will confirm the role for pannexin-1 in P2X7-mediated cells in a murine macrophage line before attempting this line of investigation in canine monocytes and macrophages, which are less readily available.
Aim 3 will provide a cloned canine P2X7 receptor (English Springer Spaniel) for future comparison to previous published work relating to native P2X7 in cells of the same breed (Stevenson et al. 2009; Sluyter et al. 2007) and comparison to the other cloned P2X7 receptor (Beagle) (Roman et al. 2009).

Aim 4 will begin to assess if P2X7 function varies between days or different breeds as a possible future means to determine if polymorphic variants exist between dogs or breeds. This future work would also include use of the cloned canine P2X7 receptor (from aim 3) to verify the identity of potential polymorphisms through site directed mutagenesis studies.
Chapter 2: Madin-Darby Canine Kidney Cells Express P2X7, TLR4 and NALP3 Inflammasome

2.1 Materials and Methods

2.1.1 Materials

RPMI-1640 medium (containing 25 mM HEPES), foetal calf serum (FCS), 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA), Dulbecco’s phosphate-buffered saline (PBS) and L-glutamine were obtained from Invitrogen Corporation (Auckland, New Zealand). Adenosine 5’ triphosphate (ATP), 2 (3’)-O-(4-benzoylbenzoyl) ATP (BzATP), lipopolysaccharide (LPS) from *Escherichia Coli* serotype 055:B5, ethidium+ bromide, N-methyl-D-glucamine (NMDG) and non-essential amino acids were from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulphoxide (DMSO), n-dodecyl β-D-maltoside, phenylmethylsulfonyl fluoride, 2-mercaptoethanol, Tween-20, Triton-X-100, sodium dodecyl sulphate (SDS) and other general reagents grade chemicals were from Amresco (Solon, OH). 1-[N-O-bis-(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) was from Alexis Biochemicals (Lausen, Switzerland). 3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine (A-438079) was from Tocris Bioscience (Park Ellisville, MO). Precision Plus Protein Standards (Dual colour), nitrocellulose membrane and Precision StrepTactin–horseradish peroxidase (HRP) conjugate were from Bio-Rad (Hercules, CA). Human AB+ serum was from the Australian Red Cross Blood Bank Service (Penrith, Australia). Mini-Complete inhibitor cocktail tablets (used according to manufacturer’s instructions) were from Roche Applied Science (Mannheim, Germany). Diploma skim milk powder was from Fonterra™ Foodservices
(Mount Waverley, Australia). SuperSignal® West Pico Chemiluminescent Substrate was from Thermo Scientific (Rockford, IL). Developer and fixer were from Kodak (Rochester, NY). RNeasy® Mini Kit was from QIAGEN (Hilden, Germany). SuperScript® III One-Step RT-PCR with Platinum® Taq was provided from Invitrogen Corporation (Carlsbad, CA). Ficoll-Paque™ Plus and Amersham Hyperfilm were from GE Healthcare (Uppsala, Sweden). RNaseZap was from Ambion (Austin, TX). Agarose powder and HyperLadder™ I were from BIOLINE (Sydney, Australia). CB-X™ Protein Assay was from kit (G-Biosciences, St. Louis, MO).

2.1.2 Antibodies

Rabbit anti-rat and anti-mouse P2X7 polyclonal antibody (Ab) (against a C-terminal and extracellular epitope respectively) were from Alomone Labs (Jerusalem, Israel). HRP-conjugated goat anti-rabbit IgG Ab was from Rockland Immunochemicals (Gilbertsville, PA). Phycoerythrin (PE)-conjugated anti-human Toll-like receptor 4 (TLR4; CD284) and PE-conjugated mouse IgG2a, κ isotype control monoclonal antibody (mAb) were from eBioscience (San Diego, CA).

2.1.3 Cell lines

Madin-Darby Canine Kidney (MDCK) epithelial cells were obtained from the European Collection of Cell Cultures (Porton Down, UK). Murine RAW264.7 macrophage-like cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI-1640 medium supplemented with 10% FCS and 5 mM L-glutamine (complete culture medium); MDCK complete culture medium also contained 1% non-essential amino acids. Cells were incubated at 37°C/5% CO₂ and were passaged twice a week, as required. MDCK and RAW264.7 cells were harvested using trypsin-EDTA and mechanical scraping, respectively. In
some experiments, MDCK cells were incubated with LPS (0.1, 1 or 10 µg/mL) for 24 h as indicated.

### 2.1.4 Canine peripheral blood monocytes

Blood was taken from pure and mixed pedigree dogs with informed, signed consent of the owners, and with approval by the University of Wollongong Ethics committee. Peripheral blood was collected into heparin vacutainer tubes (Greiner Bio-One, Frickenheisen, Germany). Blood was centrifuged at 580 g for 15 mins (with brake off). The buffy coat was transferred to a 50 mL centrifuge tube (Greiner Bio-One) and sterile PBS added to the 35 mL graduation mark. The diluted blood was then underlaid with 15 mL Ficoll-Paque™ and the tubes centrifuged at 580 g for 20 min (with brake off). The mononuclear cells were removed and transferred to another 50 mL centrifuge tube, resuspended up to 50 mL with sterile PBS and centrifuged at 450 g for 10 mins. Cells were washed twice in 25 mL PBS at 300 g for 5 mins. Cells were then resuspended in 20 mL complete culture medium, and cultured in a T75 flask (Greiner Bio-One) for 2 h at 37°C/5% CO₂. Non-adherent mononuclear cells were removed by gently swirling the flask six times, then standing the flask upright for 1 min before removing medium containing non-adherent cells. Then 10 mL sterile PBS was added to the flask and the process repeated to remove any remaining non-adherent cells. Finally, 20 mL of complete culture medium containing LPS (0.1 µg/mL) was added and the cells incubated for 4 h at 37°C/5% CO₂.

### 2.1.5 Detection of P2X7 and other molecules by reverse transcription-PCR (RT-PCR)

Total RNA was prepared from 90% confluent MDCK cells or from LPS-primed monocytes using RNeasy® Mini Kit according to the manufacturer’s protocol. Extracted RNA was translated to cDNA using SuperScript® III One-Step reverse transcription (RT)-PCR with
Platinum® Taq. The primers are shown in Table 2.1. Also, RNA substituted with H2O was used as a negative control. Before PCR cycles, a reverse transcription step was performed by incubation of each sample at 50°C for 25 min. The reactions were run at 94° for 2 min, 30 cycles of 94° for 30 s, 59° for 30 s, 72° for 3.5 min for P2X7, 94° for 2 min, 40 cycles of 94° for 15 s, 58° for 20 s, 65° for 45 s for NALP3, 94° for 2 min, 40 cycles of 94° for 15 s, 58° for 30 s, 66° for 45 s for Caspase-1, 94° for 2 min, 40 cycles of 94° for 30 s, 47° for 30 s, 72° for 2 min for interleukin (IL)-1β, 94° for 3 min, 40 cycles of 94° for 10 s, 50° for 10 s, 72° for 20 s for IL-18 and 94° for 5 min, 35 cycles of 94° for 1 min, 51° for 1 min, 72° for 1 min for TLR4. Reactions were analyzed with 2% agarose gel electrophoresis (Appendix) and DNA was visualized using ethidium bromide staining.

Table 2.1: Primers used for reverse transcription (RT)-PCR.

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<th>Reverse 5'-3'</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>P2X7</td>
<td>TGCCTCCCATCCCAGCTCCC</td>
<td>GTCCTGGGAGCCAAAGGC</td>
<td>XM 534669*</td>
</tr>
<tr>
<td>NALP3</td>
<td>CACTGTCAGCCTTTGGCAGGTT</td>
<td>GTCTCCCAGGGCGTTGTC</td>
<td>NC 006621*</td>
</tr>
<tr>
<td>Caspase-1</td>
<td>ACAGACGCTGGGGCTCTCCT</td>
<td>CCCAGGCCCTCCAGCACT</td>
<td>NC 006587*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGCAAAAACAGATGCGGATAA</td>
<td>GTAACCTTGAGGCCAGGATT</td>
<td>Maccoux et al. 2007</td>
</tr>
<tr>
<td>IL-18</td>
<td>CCTGGAATCAGATTACTTGGGC</td>
<td>GACTCCTTTTTCCGCTTTCCTTAG</td>
<td>Chamizo et al. 2001</td>
</tr>
<tr>
<td>TLR4</td>
<td>GTCTGGCTGGCTTAAAGATC</td>
<td>CTGCAATCTAGGATCTGGAG</td>
<td>Asahina et al. 2003</td>
</tr>
<tr>
<td>β-actin</td>
<td>TGACCCAGATCATGTGTTGAGACC</td>
<td>TCCTGCTTGCTGATCCACATC</td>
<td>Swerdlow et al. 2006</td>
</tr>
</tbody>
</table>

2.1.6 Detection of Toll-like receptor 4 (TLR4) by flow cytometry

MDCK cells were washed once in NaCl medium (145 mM NaCl, 5 mM KCl, 5 mM D-glucose, 0.1 % BSA, 10 mM HEPES, pH 7.5) at 300 g for 5 min. Cells (1 x 10^6) were then incubated at room temperature for 20 min with PE-conjugated anti-human TLR4 or mouse IgG2a κ isotype control mAb in NaCl medium containing 10% human AB+ serum. Cells were washed once in NaCl medium as above. Data was collected using a LSR II flow cytometer and FacsDiva software (both BD Bioscience, San Diego, CA) with a total of 3 x 10^4 events collected using an emission window of 575 ± 10 nm. The mean fluorescence intensity (MFI) of relative TLR expression was determined using FlowJo software (TreeStar Inc., Ashland, CA).

2.1.7 Detection of P2X7 by immunoblotting

Cell lysates were produced as previously described (Constanteniscu et al. 2010). MDCK and RAW264.7 cells were washed three times with ice-cold PBS at 300 g for 5 min and then incubated (1 x 10^7/mL) on ice for 60 min in lysis buffer (50 mM BisTris, 750 mM 6-aminohexanoic acid, 1 mM phenylmethylsulfonyl fluoride, 1% n-dodecyl β-D-maltoside and Mini-Complete protease inhibitor cocktail, pH 7.0). Cells were then sheared 10 times using a 21 gauge needle and centrifuged at 16,000 g for 10 mins at 4°C. Protein concentration of supernatants was determined using CB-X™ Protein assay according to the manufacturer’s instructions. The supernatants were then separated under reducing conditions (5% 2-mercaptoethanol) by SDS-PAGE using 10% separating gel with a 4% stacking gel (Appendix), and the proteins transferred to nitrocellulose membrane. Immunoblotting was performed as previously described (Sluyter et al. 2007). The membrane was blocked overnight in Tris-Buffered Saline Tween-20 (TBST) (20 mM Tris, 500 mM NaCl, 0.2% Tween 20, pH 7.5)
containing 5% skim milk powder. The membrane was washed three times over 30 mins with
TBST and incubated with rabbit anti-rat or anti-mouse P2X7 polyclonal Ab in TBST containing
5% skim milk powder at room temperature for 2 hours. The membrane was washed as above and
incubated with HRP-conjugated goat anti-rabbit IgG Ab and StrepTactin-HRP conjugated in
TBST containing 5% skim milk powder for 1 hour at room temperature. The membranes were
washed again as above and visualised using chemiluminescence substrate and Hyperfilm.
Hyperfilm was developed manually using developer and fixer according to the manufacturer’s
instructions.

2.1.8 Detection of ethidium\(^+\) uptake into MDCK cells by fixed-time flow cytometry assay

Nucleotide-induced ethidium\(^+\) uptake into MDCK cells was measured using a fixed-time flow
cytometry assay as previously described (Farrell et al. 2010). Cells (1 x 10\(^6\)/mL) were
resuspended in NaCl medium or sucrose medium (5 mM KCl, 5 mM glucose, 10 mM HEPES,
10 mM NMDG, 280 mM sucrose, pH 7.4) containing 25 μM ethidium\(^+\), and incubated in the
absence and presence of nucleotide at 37°C for up to 30 min as indicated. For P2X7-antagonist
related experiments, cells were pre-incubated (before nucleotide addition) for 15 mins at 37°C
with 1 μM KN-62 or 10 μM A438079 or their respective diluents, DMSO or H\(_2\)O. Incubations
with nucleotide were terminated by addition an equal volume of ice-cold MgCl\(_2\) medium (20
mM MgCl\(_2\), 145 mM NaCl, 5 mM KCl and 10 mM HEPES, pH 7.5) and samples centrifuged at
300 g for 5 min. Cells were washed once with NaCl or sucrose medium, and the data was
collected using a LSR II flow cytometer and FacsDiva software. A total of 3 x 10\(^4\) events were
collected using an emission window of 575 ± 10 nm and the MFI of ethidium\(^+\) uptake was
determined using FlowJo software.
2.1.9 Data presentation and statistical analyses

Results are expressed as means ± standard deviation (SD). All graphs were compiled using GraphPad Prism 5 software for Windows (GraphPad Software, Inc; La Jolla, CA). Differences between each treatment were compared using either the unpaired Student’s *t*-test for single comparisons to control samples or ANOVA for multiple comparisons (with Bonferroni post hoc test) using GraphPad Prism 5 software with *P* < 0.05 considered significant.

2.2 Results

2.2.1 MDCK cells express P2X7 mRNA

To determine if MDCK cells express P2X7, total RNA was extracted from MDCK cells and the expression of P2X7 examined using RT-PCR (Fig. 2.1). RNA from canine monocytes, which express functional P2X7 (Stevenson et al. 2009; Sluyter et al. 2007), were used as a positive control. The MDCK cells, as well as canine monocytes were found to express P2X7 mRNA (Fig. 2.1). H₂O in place of RNA was used as a negative control and failed to yield any products (Fig. 2.1).
Fig. 2.1: MDCK cells express P2X7 mRNA. Total RNA extracted from confluent cultured MDCK cells or canine monocytes and P2X7 expression examined using RT-PCR. H2O in place of RNA used as a negative control. Reactions were analyzed with 2% agarose gel electrophoresis and DNA was visualized after staining with ethidium bromide. Results are representative of three independent experiments.

2.2.2 MDCK cells contain P2X7 protein

Immunoblotting was performed of whole cell lysates of MDCK cells to determine if MDCK cells contain P2X7 protein. RAW264.7 macrophages, which have been previously shown to contain P2X7 protein (Tran et al. 2010), were used as a positive control. Immunoblotting of MDCK and RAW246.7 cell lysates with two different anti-P2X7 polyclonal antibody (Ab) demonstrated the presence of a major band at 75 kDa (Fig. 2.2), the predicted size of glycosylated P2X7.
Fig. 2.2: MDCK cells contain P2X7 protein. Whole cell lysates of MDCK and RAW246.7 (RAW) cells (containing an equal amount of protein) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes probed with anti-P2X7 polyclonal Ab (extracellular or intracellular epitopes), then with HRP-conjugated goat anti-rabbit IgG Ab and visualised with chemiluminescence substrate. HRP-conjugated StreptActin was used for visualization of the molecular weight markers. Results are representative of four independent experiments.
2.2.3 P2X7 agonists do not induce ethidium\(^+\) uptake into MDCK cells in NaCl medium over 5 min

To determine if MDCK cells express functional P2X7, cells in NaCl medium were incubated in the absence (basal) or presence of 1 mM ATP or 0.2 mM BzATP for 5 min. RAW246.7, which express functional P2X7 (Tran et al. 2010), were used as a positive control. As expected, ATP and the most potent P2X7 agonist BzATP induced significantly large amounts of ethidium\(^+\) uptake into RAW246.7 cells compared to basal (Fig. 2.3). In contrast, neither ATP nor BzATP induced ethidium\(^+\) uptake into MDCK cells with values similar to basal values (Fig. 2.3).

![Graph showing ethidium\(^+\) uptake](image)

**Fig. 2.3: P2X7 agonists do not induce ethidium\(^+\) uptake into MDCK cells in NaCl medium over 5 min.** MDCK and RAW264.7 cells in NaCl medium containing 25 µM ethidium\(^+\) were incubated in the absence (basal) or presence of 1 mM ATP or 0.2 mM BzATP at 37°C for 5 min. Incubation were stopped by addition of cold MgCl\(_2\) medium and centrifugation. Ethidium\(^+\) uptake (MFI) was measured by flow cytometry. Results are expressed as the mean ± SD (\(n = 3\) replicates from one experiment). *** \(P < 0.001\) compared to corresponding control.
2.2.4 P2X7 agonists induce ethidium$^+$ uptake into MDCK cells in sucrose media in a time-dependant manner

The ability of ATP or BzATP to activate P2X7 is several-fold higher in cells incubated in sucrose medium compared to NaCl medium (Jursik et al. 2007). Therefore, to further determine if MDCK cells express functional P2X7, cells were suspended in sucrose medium, and incubated in the absence or presence of ATP or BzATP for up to 30 min. Both ATP and BzATP induced a small, but significant uptake of ethidium$^+$ into MDCK cells compared to control levels (Fig. 2.4).

![Ethidium$^+$ Uptake](image)

**Fig. 2.4: P2X7 agonists induce ethidium$^+$ uptake into MDCK cells in sucrose medium in a time-dependent manner.** MDCK cells in sucrose medium containing 25 µM ethidium$^+$ were incubated in the absence (basal) or presence of 1 mM ATP, 0.2 mM BzATP at 37°C for up to 30 min as indicated. The incubations were stopped by addition of cold MgCl$_2$ medium and centrifugation. Ethidium$^+$ uptake (MFI) was measured by flow cytometry. Results are expressed as the mean ± SD ($n = 3$ replicates from one experiment). * $P < 0.05$ compared to corresponding no ATP treatment.
2.2.5 P2X7 antagonists inhibit ATP-induced ethidium\(^+\) uptake into MDCK cells

KN-62 and A-438079 are well studied antagonists of the P2X7 receptor (McGaraughty et al. 2007; Gargett and Wiley 1997). To confirm that the P2X7 receptor was responsible for the ATP-induced ethidium\(^+\) influx into MDCK cells, cells in sucrose medium were pre-incubated with 1 mM KN-62 or DMSO (diluent control) or 10 mM A-438079 or H\(_2\)O (diluent control) for 15 mins at 37°C before measurement of ethidium\(^+\) uptake in the absence or presence of 1 mM ATP. As above (Fig. 2.4), ATP induced significantly higher amounts of ethidium\(^+\) uptake into MDCK cells compared to basal (Fig. 2.5). Pre-incubation of cells with KN-62 and A-438079 impaired ATP-induced ethidium\(^+\) uptake by 60% and 92%, respectively (Fig. 2.5).

2.2.6 MDCK cells express TLR4, NALP3 and caspase-1 but not IL-1\(\beta\) and IL-18

The above results indicate that MDCK cells express functional P2X7. Thus, although these cells are unsuitable for transfection with recombinant canine P2X7 due to the presence of endogenous P2X7, these cells may be useful to study events downstream of P2X7 activation in canine epithelial cells. Activation of the NALP3 inflammasome, and subsequent maturation and release of IL-1\(\beta\) and IL-18 is the best characterized downstream of P2X7 activation (Di Virgilio et al. 2007). Therefore, RT-PCR was used to determine if MDCK cells express the inflammasome components NALP3 and caspase-1, and the pro-inflammatory cytokines IL-1\(\beta\) and IL-18. The expression of TLR4, which induces the synthesis of these molecules was also examined. Due to the expression of these molecules in LPS-primed monocytes from human and mice (Guha and Mackman 2001; Visintin et al. 2001), LPS-primed canine monocytes were used as a positive control. In addition, RT-PCR of P2X7 was used as an additional positive control for both cell
types. As expected, LPS-primed canine monocytes expressed each of the examined molecules (Fig. 2.6). As above (Fig. 2.1), MDCK cells were found to express P2X7 (Fig. 2.6). In addition, MDCK expressed TLR4, as well as NALP3 and caspase-1 but not IL-1β and IL-18. H2O in place of RNA was used as a negative control and failed to yield any products in either cell type (Fig. 2.6).

![Graph](image)

**Fig. 2.5:** P2X7 antagonists impair ATP-induced ethidium\(^{+}\) uptake into MDCK cells. MDCK cells in sucrose medium were pre-incubated with (A) 1 μM KN-62 at 37°C or an equal volume of DMSO or (B) 10 mM A-438079 or an equal volume of H\(_2\)O for 15 mins. Cells were then incubated with 25 μM ethidium\(^{+}\) in the absence or presence of 1 mM ATP for 20 mins, and the incubations stopped by addition of cold MgCl\(_2\) medium and centrifugation. Results are expressed as mean ± SD (n = 3 replicates from one experiment). * P < 0.05 compared to corresponding basal; † P < 0.05 compared to ATP without antagonist.
Fig. 2.6: MDCK cells express NALP3, caspase-1 and TLR4 but not IL-1β and IL-18. Total RNA was extracted from (A) MDCK cells or (B) LPS-primed canine monocytes and the expression of P2X7, NALP3, caspase-1, IL-1β, IL-18 and TLR4 examined using RT-PCR. H₂O in place of RNA used as a negative control. Reactions were analyzed with 2% agarose gel electrophoresis and DNA was visualized after staining with ethidium bromide. Results are representative of three independent experiments.
2.2.7 LPS induces the synthesis of caspase-1 but not P2X7, NALP3, IL-1β or IL-18 in MDCK cells

To determine if LPS could upregulate P2X7, NALP3, caspase-1, IL-1β and IL-18 in MDCK cells, and to test the functionality of the LPS receptor, TLR4, in these cells, MDCK cells were incubated overnight in the absence (0 μg) or presence of 0.1, 1 or 10 μg/mL LPS, and RT-PCR performed. β-actin was used as a “housekeeping gene” to ensure equal amount of RNA was used in reactions for the different treatment groups. LPS upregulated the expression of caspase-1, but not P2X7 or NALP3, in a concentration-dependent manner (Fig. 2.7). LPS failed to induce the expression of either IL-1β or IL-18 (Fig. 2.7).

2.2.8 MDCK cells express cell surface TLR4

The indication of caspase-1 transcription by LPS (Fig. 2.7) suggest that MDCK cells express functional cell-surface TLR4. To confirm that these cells express cell-surface TLR4, MDCK cells were labelled with an anti-human TLR4 monoclonal antibody (mAb), which has been previously shown to recognise canine TLR4 (Burgener and Jungi 2007), and examined by flow cytometry. MDCK cells were found to express TLR4 on the cell-surface with a MFI of 11.1 ± 0.9, (n = 3; Fig. 2.8).
Fig. 2.7: LPS induces the synthesis of caspase-1 but not P2X7, NALP3, IL-1β or IL-18 in MDCK cells. MDCK cells were incubated overnight in the absence of LPS (0 µg/mL) or in the presence of 0.1, 1 or 10 µg/mL LPS. Total RNA was extracted and the relative expression of P2X7, NALP3, caspase-1, IL-1β and IL-18 examined using RT-PCR. Equal amounts of RNA from each treatment were used for RT-PCR and was verified by RT-PCR of β-actin. RT-PCR products were analyzed with 2% agarose gel electrophoresis and DNA was visualized after staining with ethidium bromide. Results are representative of three independent experiments.
Fig. 2.8: MDCK cells express cell surface TLR4. MDCK cells were labelled with PE-conjugated anti-human TLR4 (blue line) or isotype control (red line) mAb and analysed by flow cytometry. The gating of viable MDCK cells by forward (FSC-A) and side scatter (SSC-A) is shown in the left panel. Relative TLR4 expression is shown in the right panel. Data is representative of three replicates from one experiment.

2.3 Discussion

This study aimed to determine if MDCK cells express functional P2X7 receptors. Absence of this receptor in these cells would enable MDCK cells to be potentially used for transfection studies (as conducted in chapter 4). Conversely, the presence of P2X7 in MDCK cells, would provide a future model cell line in which to study the role of P2X7 in kidney epithelial cells. This study demonstrated that MDCK cells express functional P2X7. First, RT-PCR demonstrated the existence of P2X7 mRNA in MDCK cells (Fig. 2.1). Second, immunoblotting with an anti-P2X7
polyclonal Ab demonstrated the presence of P2X7 protein in MDCK cell lysates (Fig. 2.2). Third, incubation with ATP and the most potent P2X7 agonist, BzATP, induced ethidium$^+$ uptake into MDCK cells (Fig. 2.4). Finally, the P2X7 antagonists, KN-62 and A-438079, inhibited ATP-induced ethidium$^+$ uptake into MDCK cells (Fig. 2.5). The expression of this P2 receptor in these cells however is not unique to P2X7, as others have previously shown the expression of P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 in MDCK cells (Turner, et al. 2006; Insel et al. 2001; Zambon et al. 2000). Stimulation of P2Y receptors in these cells leads to elevation of intracellular cyclic adenosine monophosphate (cAMP), and the release of arachidonic acid and its metabolites.

The current study demonstrates the presence of functional P2X7 in MDCKs cells. Although the presence of functional P2X7 in epithelial cells from various tissues including skin, lung and bladder is well established (Gorodeski 2009; Garcia-Marcos et al. 2006), less is known about P2X7 in kidney epithelium. P2X7 is absent or expressed at very low amounts in the kidney epithelium (Turner et al. 2007). However it is up-regulated during a number of disease states including diabetes and hypertension (Vonend et al. 2004), experimental and human glomerulonephritis (Taylor et al. 2009; Turner et al. 2007), unilateral ureteral obstruction (Gonclaves et al. 2006), autosomal recessive and dominant polycystic kidney disease (Xu et al. 2009; Hillman et al. 2004, 2002). Each of these studies implicate a pro-inflammatory or pro-apoptotic role for P2X7 activation in kidney epithelium and related disorders, however direct evidence is lacking with the exception of two studies (Taylor et al. 2009; Gonclaves et al. 2006), which utilized P2X7 knockout mice or the P2X7 antagonist, A-438079. The near-complete inhibition of P2X7 by A-438079 in MDCK cells (Fig. 2.5) is consistent with this latter
observation. This inhibition of P2X7 receptor function in kidney epithelial cells might be pathologically useful for treatment of kidney diseases (Turner et al. 2009).

Incubation with ATP and the most potent P2X7 agonist BzATP induced similar amounts of ethidium\(^+\) uptake into MDCK cells (Fig 2.3). This is consistent with Sluyter et al. (2007) in which ATP and BzATP induced similar amounts of \(^{86}\text{Rb}\(^+\) efflux from canine erythrocyte. However it should be noted that in both these studies, BzATP was used at a five-fold lower concentration than ATP. This difference is supported by studies of recombinant and native canine P2X7 (Roman et al. 2009; Stevenson et al. 2009; Sluyter et al. 2007). Finally it should be noted that the ethidium\(^+\) uptake assay with MDCK cells was performed in sucrose medium to increase the receptor functionality. This is consistent with results by Michel et al. (1999) and Jursik et al. (2007) which demonstrated P2X7 function increases in sucrose medium compared to NaCl medium.

The current study also demonstrated the presence of functional TLR4 in MDCK cells (Fig. 2.6 and 2.8). Both RT-PCR and immunolabelling demonstrated the presence of TLR4 mRNA and protein in these cells. Moreover, the TLR4 was functional as incubation of MDCKs with the TLR4 ligand, LPS, upregulated caspase-1 mRNA synthesis (Fig. 2.7). LPS has been previously shown to upregulate caspase-1 in monocytes (Kahlenberg et al. 2005), but its modulation in kidney epithelial cells has not been reported. MDCK cells were expected to express TLR4 because this TLR is present in many tissues including epithelial cells in the convoluted and straight renal tubules (Wassef et al. 2004). TLR4 in the kidney is a potential mediator of innate activation and inflammation. For example it has been shown that TLR4 expression increases in tubular epithelial cells and infiltrating leukocytes within the kidney following ischemia. Further,
TLR4 signaling through the MyD88-dependent pathway is required for the full development of kidney ischemia/reperfusion injury (Wu et al. 2007).

The current study further shows that components of the NALP3 inflammasome are expressed in MDCK cells (Fig. 2.6). NALP3 and caspase-1 have been observed in renal tubular epithelial cells (Shigoeka et al. 2010). Moreover, pharmacological evidence using the specific caspase-1 inhibitor, YVAD, suggests the presence of functional caspase-1 in MDCK cells (Feldenberg et al. 1999). Although the presence of NALP3 and caspase-1 in MDCK cells needs to be confirmed at the protein level, the presence of these molecules indicates a potential role for P2X7 in NALP3 inflammasome activation, and the subsequent processing and maturation of caspase-1 in MDCK cells. This role of P2X7 in NALP3 inflammasome activation, however appears to be independent of IL-1β and IL-18 as RT-PCR failed to detect mRNA transcripts of these cytokines in untreated and LPS-primed MDCK cells despite their detection in LPS-primed canine monocytes (Fig. 2.6 and 2.8). Although epithelial cells do not secret large amounts of IL-1β (Gorcel et al. 2006), it has been demonstrated that IL-1β and IL-18 are constitutively expressed by tubular epithelial cells in normal rat kidney (Tesch et al. 1997), and renal and lung epithelial cells (Krásná et al. 2005; Fraust et al. 2002). Thus, it remains possible that IL-1β and IL-18 mRNA in MDCK cells is expressed at very low quantities but below the detection limits of the RT-PCR method used in the current study. Nevertheless, NALP3 and caspase-1 may play a role in epithelial cell death. It is also shown that activation of caspase-1 leads to pyroptosis in macrophages, dendritic cells and neurons during bacterial infection (Lamkanfi 2011; Miao et al. 2010). Thus, NALP3 and caspase-1 activation might function as a defense mechanism in various cell types including kidney epithelium to prevent infection.
Finally, the current study demonstrates for the first time the presence of NALP3 and caspase-1 in canine monocytes, and confirms the presence of P2X7 (Stevenson et al. 2009), TLR4 (House et al. 2008), IL-1β and IL-18 (Fujiwara et al. 2003) in canine monocytes. TLR4, IL-1β and IL-18 however were only detected at the mRNA, thus these results still need to be confirmed at the protein level. Nevertheless, these results indirectly indicate that canine monocytes have the capacity to release IL-1β and IL-18 via the P2X7/NALP3 inflammasome axis.

In conclusion, MDCK epithelial cells express functional P2X7 and TLR4, as well as NALP3 and caspase-1, but not IL-1β and IL-18. These cells may produce a useful model to study P2X7-induced processes in canine epithelial cells, but the presence of this receptor limits their use to study recombinant P2X7 following transfection.
Chapter 3: Effect of Pannexin-1 Antagonists on P2X7-Induced Ethidium⁺ Uptake and Interleukin-1β Release from Murine J774 Macrophages

3.1 Materials and Methods

3.1.1 Materials

Materials are described in Chapter 2. Additional materials are listed below. Interleukin (IL)-1β ELISA MAX™ Deluxe Set was from BioLegend (San Diego, CA). Carbenoxolone (CBX), melfoquine (MFQ), flufenamic acid (FFA) and probenecid (PRO) were from Sigma Chemical Co. (St. Louis, MO).

3.1.2 Cell lines

Murine J774 macrophage-like cells, originally obtained from the American Type Culture Collection (Manassas, VA), were kindly provided by Ms. Jasmyn Dunn (The University of Queensland, Brisbane, Australia). RAW264.7 macrophage-like cells were described in Section 2.1.3. Cells were maintained in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS) and 5 mM L-glutamine (complete culture medium). Cells were incubated at 37°C/5% CO₂ and were passaged twice a week, as required. Cells were harvested using mechanical scrapping.

3.1.3 Detection of P2X7 and pannexin-1 by reverse transcription-PCR (RT-PCR)

Total RNA was prepared from 90% confluent J774 cells using RNeasy® Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. Extracted RNA was reversed
transcribed into cDNA using SuperScript® III One-Step reverse transcription (RT)-PCR with Platinum® Taq. The sequence of the primers used are shown in Table 3.1. RNA substituted with H2O was used as a negative control. Before PCR cycles, a reverse transcription step was performed by incubation of each sample at 50°C for 25 min. The reactions were run at 94° for 2 min, 30 cycles of 94° for 30 s, 59° for 30 s, 72° for 3:30 min for P2X7, and 95° for 3 min, 50 cycles of 94° for 15 s, 55° for 30 s, 72° for 30 s for pannexin-1.

Table 3.1: Primers used for reverse transcription (RT)-PCR

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X7</td>
<td>ATATCCACTTTCCCAGCCAC</td>
<td>TCGGCAGTGATGGGACCAG</td>
<td>Hillman et al. 2004</td>
</tr>
<tr>
<td>Pannexin-1</td>
<td>CGAGATTGGACCTAAGAGCGG</td>
<td>GTGGGCAGATTTCATACATTG</td>
<td>Pelegrin et al. 2006</td>
</tr>
</tbody>
</table>

3.1.4 Detection of ethidium+ uptake by fixed-time flow cytometry

Nucleotide-induced ethidium+ uptake into cells was measured using a fixed-time flow cytometry assay (Farrell et al. 2010). Cells (1 x 10⁶/mL) were resuspended in NaCl medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 5 mM glucose, 0.1% BSA, pH 7.4) or physiological medium (147 mM NaCl, 10 mM HEPES, 13 mm glucose, 2 mM CaCl₂, 1 mM MgCl₂, and 2 mM KCl pH 7.5) containing 25 μM ethidium+, and incubated in the absence and presence of nucleotide at 37°C for 5 min as indicated. For P2X7 antagonist-related experiments, cells were pre-incubated (before nucleotide addition) for 15 mins at 37°C with 1 μM KN-62 or 10 μM A438079 or their respective diluents, DMSO or H2O. For pannexin-1-antagonist related experiments, cells were pre-incubated (before nucleotide addition) for 15 mins at 37°C with 50, 20 and 10 μM CBX,
100, 10 and 1 nM MFQ, 50, 20 and 10 mM PRO and 50, 20 and 10 mM FFA or their respective diluents, DMSO, NaOH or H₂O. Incubations with nucleotide were terminated by addition an equal volume of ice-cold MgCl₂ medium (20 mM MgCl₂, 145 mM NaCl, 5 mM KCl and 10 mM HEPES, pH 7.5) and samples centrifuged at 300 g for 5 min. Cells were washed once with their respective assay medium. Data was collected using LSR II flow cytometer and FacsDiva software (both BD Bioscience, San Diego, CA) with a total of 3 x 10⁴ events collected using an emission window of 575 ± 10 nm. Relative nucleotide-induced ethidium⁺ uptake is presented as the difference in MFI between nucleotide and control treatments (unless otherwise stated).

3.1.5 Detection of interleukin (IL)-1β secretion by ELISA

ATP-induced IL-1β secretion from J774 cells was examined as previously described (Pelegrin et al. 2006). J774 cells were plated in 24-well plates (Greiner Bio-One, Frickenhausen, Germany) at 1 x 10⁶/mL/well and incubated overnight at 37°C/5% CO₂. Cells were washed twice with complete culture medium and stimulated with LPS (1 µg/mL) in complete culture medium at 37°C/5% CO₂ for 4 h to induce pro-IL-1β expression. Cells were then washed twice with physiological medium, and incubated in the absence or presence of 5 mM ATP in physiological medium at 37°C/5% CO₂ for 20 min. For P2X7 antagonist-related experiments, cells were pre-incubated (before nucleotide addition) for 15 mins at 37°C with 1 µM KN-62 or 10 µM A-438079 or their respective diluents, DMSO or H₂O. For pannexin-1-antagonist related experiments, cells were pre-incubated (before nucleotide addition) for 15 mins at 37°C with 50 µM CBX, 100 µM MFQ, 50 µM FFA and 50 µM PRO or their respective diluents, DMSO, NaOH or H₂O. After 20 min of incubation with agonist, samples were centrifuged at 11000 g for 30 s, and cell-free supernatants collected and stored at -80°C until required. Amounts of IL-1β in
cell-free supernatants were quantified using an IL-1β ELISA kit according to the manufacturer’s instructions, with plates washed 3 times between incubations with 0.2 μM filtered wash buffer (8.0 g NaCl, 1.16 g Na₂HPO₄, 0.2 g KH₂PO₄, 0.2 g KCl, 0.05% Tween-20, pH 7.4) and measured at 450 nm on a Spectramax Plus 384 Spectrophotometer (Molecular Devices, Sunnyvale, CA).

3.1.6 Detection of P2X7 by immunoblotting

Immunoblotting of J774 and RAW264.7 cells was performed as previously described (Section 2.1.7).

3.1.7 Data presentation and statistical analyses

Results are expressed as means ± standard deviation (SD). All graphs were compiled using GraphPad Prism 5 software for Windows (GraphPad Software, Inc; La Jolla, CA). Differences between each treatment were compared using either the unpaired Student’s t-test for single comparisons to control samples or ANOVA for multiple comparisons (with Bonferroni post hoc test) using GraphPad Prism 5 software with \( P < 0.05 \) considered significant. Concentration response curves of normalised data (with variable slope assumed) were fitted using a least squares (ordinary) fit method using Prism 5; the correlation coefficient \( (R^2) \) ranged from 0.95 to 1.0.
3.2 Results

3.2.1 J774 cells express P2X7 and pannexin-1 mRNA

J774 cells have been previously shown to express P2X7 and pannexin-1 (Pelegrin and Surprenant 2006). To confirm that J774 cells express P2X7 and pannexin-1, total RNA was extracted from J774 cells and the expression of P2X7 examined using RT-PCR. J774 cells were found to express P2X7 and pannexin-1 mRNA (Fig. 3.1). H2O in place of RNA was used as a negative control and failed to yield any products (Fig 3.1).

**Fig. 3.1: J774 cells express P2X7 and pannexin-1 mRNA.** Total RNA was extracted from J774 cells, and P2X7 and pannexin-1 expression was examined using RT-PCR. Reactions were analyzed with 2% agarose gel electrophoresis and DNA was visualized after staining with ethidium bromide. H2O in place of mRNA was used as a negative control. Results are representative of three independent experiments.
3.2.2 J774 cells contain P2X7 protein

Immunoblotting was performed of whole lysates of J774 cells to determine if J774 cells contain P2X7 protein. As in Chapter 3, RAW264.7 macrophages were used as a positive control. Immunoblotting with an anti-P2X7 polyclonal antibody (Ab) of J774 and RAW246.7 cell lysates demonstrated the presence of a major band at 75 kDa (Fig. 3.2), the predicted size of glycosylated P2X7.

![Image of immunoblot](image.png)

**Fig. 3.2: J774 cells contain P2X7 protein.** Whole cell lysates of J774 and RAW246.7 (RAW) cells (containing an equal amount of protein) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with anti-P2X7 polyclonal Ab (extracellular epitope) and then with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG Ab, and visualised with chemiluminescence substrate. HRP-conjugated StreptActin was used for visualization of the molecular weight markers. Results are representative of four independent experiments.
3.2.3 ATP induces ethidium\(^+\) uptake into J774 cells in a concentration-dependent manner

Incubation with ATP has been previously shown to induce ethidium\(^+\) uptake into J774 cells (Pelegrin and Surprenant 2006). To confirm that ATP-induces ethidium\(^+\) uptake into J774 cells, and to determine the EC\(_{50}\) for ATP for further pharmacological studies, the effect of increasing concentration of ATP on ethidium\(^+\) uptake into J774 cells was assessed. ATP induced ethidium\(^+\) uptake into J774 cells in a concentration-dependent manner with a maximum response at 2 mM ATP and with an EC\(_{50}\) of 0.23 ± 0.01 mM (Fig. 3.3).

![Graph showing concentration-response curve for ethidium\(^+\) uptake](image.png)

**Fig. 3.3:** ATP induces ethidium\(^+\) uptake into J774 cells in a concentration-dependent manner. J774 cells in NaCl medium containing 25 µM ethidium\(^+\) were incubated in the absence (basal) or presence of varying concentrations of ATP as indicated at 37°C for 5 min. Incubations were stopped by addition of cold MgCl\(_2\) medium and centrifugation, and the MFI measured by flow cytometry. Ethidium\(^+\) uptake, calculated as the difference between ATP and basal treatments, was used to determine the maximum response to 2 mM ATP. Results are expressed as the mean ± SD (n = 3 replicates from one experiment); SD are too small to be seen.
3.2.4 P2X7 antagonists inhibit ATP-induced ethidium\(^+\) uptake into J774 cells in a concentration-dependent manner

KN-62 and A-438079 are well studied antagonists of P2X7 (McGaraughty et al. 2007; Gargett and Wiley 1997). Therefore, to confirm that the ATP-induced ethidium\(^+\) uptake into J774 cells was mediated by P2X7, J774 cells were pre-incubated with varying levels of P2X7 antagonists and the ATP-induced ethidium\(^+\) uptake measured. ATP was used at 250 µM, a concentration equivalent to the EC\(_{50}\) determined previously (Fig. 3.3). KN-62 and A-438079 impaired ATP-induced ethidium\(^+\) uptake in a concentration-dependent manner with a maximum inhibition at 10 µM for both KN-62 and A-438079, and an IC\(_{50}\) of 0.2 ± 0.03 µM and 1.3 ± 0.06 µM for KN-62 and A-438079 respectively (Fig. 3.4).

3.2.5 Pannexin-1 antagonists do not inhibit ATP-induced ethidium\(^+\) uptake into J774 cells

CBX, MFQ, PRO and FFA are antagonists of pannexin-1 (Iglesias et al. 2008; Silverman et al. 2008; Bruzzone et al. 2005). To determine if pannexin-1 is involved in P2X7-induced ethidium\(^+\) influx into J774 cells, cells in NaCl medium were pre-incubated with various concentrations of CBX, MFQ, PRO and FFA or their respective diluents H\(_2\)O, DMSO or NaOH (as indicated) before measurement of ethidium\(^+\) uptake in the presence or absence of 250 µM ATP. Each pannexin-1 antagonist failed to inhibit ATP-induced ethidium\(^+\) uptake into J774 cells in NaCl medium (Fig. 3.5).
Fig. 3.4: P2X7 antagonists inhibit ATP-induced ethidium\(^+\) uptake into J774 cells in a dose-dependent manner. J774 cell in NaCl medium were pre-incubated in the absence (basal) or presence of varying concentrations of KN62 or A-438079 as indicated at 37°C for 15 min. Ethidium\(^+\) was added (25 μM) and cells incubated with 250 μM ATP for 5 min. Incubations were stopped by addition of cold MgCl\(_2\) medium and centrifugation, and the MFI measured by flow cytometry. Ethidium\(^+\) uptake, calculated as the difference between ATP and corresponding basal treatments, was used to determine the maximum inhibition. Results are expressed as the mean ± SD (n = 3 replicates from one experiment).
Fig. 3.5: Pannexin-1 antagonists do not inhibit ATP-induced ethidium⁺ uptake into J774 cells. J774 cells in NaCl medium were pre-incubated with (A) 50, 20 and 10 μM CBX, (B) 100, 10 and 1 nM MFQ, (C) 50, 20 and 10 μM PRO or (D) 50, 20 and 10 μM FFA or their respective diluents (as indicated) at 37°C for 15 min. Cells were then incubated in the absence or presence of 250 μM ATP for 5 min, and incubations stopped by addition of cold MgCl₂ and centrifugation. Results are expressed as the mean ± SD (n = 3 replicates from one experiment). *** P < 0.001 compared to corresponding samples in the absence of ATP.
3.2.6 ATP-induces IL-1β release from LPS-primed J774 cells

The above results indicate that ATP-induced ethidium\textsuperscript{+} uptake in J774 cells is mediated by P2X7 (Fig. 3.3 and 3.4) but not by pannexin-1 (Fig. 3.5) despite the expression of pannexin-1 in these cells (Fig. 3.1). Pelegrin and Surprenant (2006) have previously shown that pannexin-1 is involved in ATP-induced IL-1β release from J774 macrophages. Therefore, as an alternate to ethidium\textsuperscript{+} uptake to study the role of pannexin-1 in P2X7-mediated processes, a series of experiments were performed to test the pannexin-1 antagonists on ATP-induced IL-1β release. First, to confirm that ATP induces IL-1β release from J774 cells, cells were primed with LPS (1 \textmu g/mL) for 4 h, then stimulated with ATP (5 mM) for 20 min as described (Pelegrin and Surprenant 2006) and the amount of IL-1β in cell-free supernatants measured by ELISA. As expected, ATP induced a significant release of IL-1β from LPS-primed J774 cells compared to control (basal) incubated cells (Fig. 3.6).

3.2.7 A-438079 but not KN-62 inhibits ATP-induced IL-1β release from LPS-primed J774 cells

To confirm that the ATP-induced IL-1β release was mediated by P2X7, LPS-primed J774 cells were pre-incubated with KN-62 and A-438079. As above (Fig. 3.6), ATP induced IL-1β release compared to control (basal) treatment (Fig. 3.7). Pre-incubation of cells with A-438079 significantly inhibited ATP-induced IL-1β release by 37\% (Fig. 3.7 B). In contrast, KN-62 failed to inhibit ATP-induced IL-1β release (Fig. 3.7 A).
Fig. 3.6: ATP induces IL-1β release from LPS-primed J774 cells. J774 cells in complete culture medium were primed with LPS (1 µg/mL) for 4 h at 37°C/5% CO₂. Cells were then incubated in physiological medium at 37°C/5% CO₂ for 20 min in the absence (basal) or presence of 5 mM ATP. The concentration of IL-1β in cell-free supernatant was analysed by ELISA. Results are expressed as the mean ± SD (n = 3 replicates from one experiment). ** P < 0.01 compared to basal.
Fig. 3.7: A-438079 but not KN-62 inhibits ATP-induced IL-1β release from LPS-primed J774 cells. J774 cells in complete culture medium were primed with LPS (1µg/mL) for 4 h at 37°C/5% CO₂. Cells were then pre-incubated with KN-62 or A-438079 in physiological medium at 37°C/5% CO₂ for 15 min followed by 20 min incubation in the absence or presence of 5 mM ATP. The concentration of IL-1β in cell-free supernatant was analysed by ELISA. Results are expressed as the mean ± SD (n = 3 replicates from one experiment). *** P < 0.001 compared to corresponding control; ††† P < 0.001 compared to corresponding ATP without antagonist.

3.2.8 Pannexin-1 antagonists CBX, FFA, PRO but not MFQ inhibit ATP-induced IL-1β release from LPS-primed J774 cells

To determine if pannexin-1 antagonists could impair ATP-induced IL-1β release, LPS-primed J774 cells were pre-incubated for 15 min with 50 µM CBX, 100 nM MFQ, 50 µM PRO and 50 µM FFA and their respective diluents H₂O, DMSO or NaOH, and ATP-induced IL-1β release measured as above. CBX, PRO and FFA significantly inhibited ATP-induced IL-1β cytokine release when compared to control samples by 39%, 32% and 22% respectively (Fig. 3.8). In
contrast, MFQ partially inhibited ATP-induced IL-1β release but this failed to reach statistical significant (Fig. 3.8).

**Fig. 3.8: Pannexin-1 antagonists CBX, FFA, and PRO but not MFQ inhibit ATP-induced IL-1β release from LPS-primed J774 cells.** J774 cells in complete culture medium were primed with LPS (1 µg/mL) for 4 h at 37°C/5% CO₂. Cells were then pre-incubated with (A) 50 µM CBX, (B) 100 nM MFQ, (C) 50 µM PRO, or (D) 50 µM FFA or their respective controls (as indicated) in physiological medium at 37°C/5% CO₂ for 15 min followed by 20 min incubation in the absence or presence of 5 mM ATP. The concentration of IL-1β in cell-free supernatant was analysed by ELISA. Results are expressed as the mean ± SD (n = 3 replicates from one experiment). **P < 0.01; ***P < 0.001 compared to corresponding control without ATP; †P < 0.05 as compared to corresponding ATP without antagonist.
3.2.9 P2X7 activation induces ethidium\(^+\) uptake into J774 cells in physiological medium

The above results indicate that pannexin-1 antagonists impair ATP-induced IL-1β release (Fig. 3.8), but not ethidium\(^+\) uptake (Fig. 3.5). One possible explanation for the discordance between these results is the differences in medium used. Therefore, the effect of pannexin-1 antagonists on ATP-induced ethidium\(^+\) uptake was re-examined in J774 cells as above (Fig. 3.5) except cells were resuspended in physiological medium. However, before examining this, the EC\(_{50}\) for ATP in physiological medium was determined, as well as the inhibitory effect of KN-62 and A-438079 in the medium (Fig. 3.9).

ATP induced ethidium\(^+\) uptake into J774 cells in physiological medium in a concentration-dependent manner with a maximum response at 1 to 2 mM ATP and with an EC\(_{50}\) of 3.4 ± 0.2 mM (Fig. 3.9A). Next, J774 cells in physiological medium were incubated with varying concentrations of P2X7 antagonists and the ATP-induced ethidium\(^+\) uptake measured. KN-62 and A-438079 impaired ATP-induced ethidium\(^+\) uptake in a concentration-dependent manner with a maximum inhibition at 3 µM KN-62 and >10 µM A-438079, and IC\(_{50}\) of 0.16 ± 0.5 µM for KN-62. However, a reliable IC\(_{50}\) value could not be determined for A-438079 since this compound failed to reach a plateau at the highest concentrations (Fig. 3.9B and C).
Fig. 3.9: P2X7 activation induces ethidium⁺ uptake into J774 cells in physiological medium.

(A) J774 cells were resuspended in physiological medium containing 25 µM ethidium⁺. (B, C) J774 cells in physiological medium were pre-incubated in the absence (basal) or presence of varying concentrations of KN62 or A-438079 as indicated at 37°C for 15 min, and 25 µM ethidium⁺ was then added. (A-C) Cells were incubated with 3.4 mM ATP (equal to EC₅₀ value obtained in A section) for 5 min. Incubations were stopped by addition of cold MgCl₂ medium and centrifugation, and the MFI measured by flow cytometry. (A) Ethidium⁺ uptake, calculated as the difference between ATP and basal treatments, was used to determine the maximum response to 2 mM ATP. (B, C) Ethidium⁺ uptake, calculated as the difference between ATP and corresponding basal treatments, was used to determine the maximum inhibition. Results are expressed as the mean ± SD (n = 3 replicates from one experiment).
3.2.10 The effect of pannexin-1 antagonists on P2X7-induced ethidium\textsuperscript{+} uptake into J774 cells in physiological medium

The above results (Fig. 3.9) confirm that P2X7 activation induces ethidium\textsuperscript{+} uptake into J774 cells in physiological medium. Therefore, the effect of the various pannexin-1 antagonists on P2X7-induced ethidium\textsuperscript{+} uptake into J774 cells in physiological medium was assessed. With the exception of CBX at 50 μM, all the pannexin-1 antagonists failed to inhibit ATP-induced ethidium\textsuperscript{+} uptake into J774 cells in physiological medium (Fig. 3.10).
Fig. 3.10: CBX but not other pannexin-1 antagonists impaired ATP-induced ethidium uptake into J774 cells in physiological medium. J774 cells in physiological medium were pre-incubated with (A) 50, 20 and 10 μM CBX, (B) 100, 10 and 1 nM MFQ, (C) 50, 20 and 10 μM PRO and (D) 50, 20 and 10 μM FFA or their respective diluents (as indicated) at 37°C for 15 min. Cells were then incubated in the absence or presence of 340 μM ATP for 20 min, and the incubation stopped by addition of cold MgCl₂ and centrifugation. Results are expressed as the mean ± SD (n = 3 replicates from one experiment). *** P < 0.001 compared to corresponding controls without ATP; † P < 0.05 compared to corresponding ATP without antagonist.
3.3 Discussion

Our group intends to study the role of P2X7 in NALP3 inflammasome activation, and the subsequent maturation and release of IL-1β and IL-18 from canine monocytes. Included in this broad aim, is to explore the role of canine pannexin-1 in this process. Pannexin-1 has been implicated in P2X7-induced pore formation and IL-1β release in murine macrophages, and to a lesser extent in human monocytes and macrophages (Pelegrin and Surprenant 2009; Locovei et al. 2007). However such reports are limited to only few groups. Therefore, this study attempted to confirm the role of pannexin-1 in P2X7-induced pore formation (ethidium+ uptake) and IL-1β release in murine J774 macrophages, as originally shown by Pelegrin and Surprenant (2006). It has been previously demonstrated that CBX, MFQ and FFA are able to block pannexin-1(Iglesias et al. 2008). Therefore, these antagonists, at the same concentrations to that used by Iglesias et al (2008) were used for the current study. The present study confirmed that J774 cells express P2X7 and pannexin-1 (Fig. 3.1), and that P2X7 induced ethidium+ uptake J774 and IL-1β release from these cells (Fig 3.3 and 3.6 respectively). However, a role for pannexin-1 in P2X7–induced ethidium+ uptake was limited to findings with the pannexin-1 antagonist, CBX, and could not be supported by the other pannexin-1 antagonists, PRO and FFA (Fig. 3.10). In contrast, a potential role for pannexin-1 in P2X7-induced IL-1β release was partly substantiated by the use of these three pannexin-1 antagonists (Fig. 3.8). MFQ completely failed to impair ATP-induced ethidium+ uptake (Fig. 3.10) and IL-1β release (Fig. 3.8) from J774 cells. However, during the course of this study, Iglesias et al. (2009) reported that MFQ obtained from either BioBlocks (San Diego, CA) or NCI-NHI (Bethesda, MD) but not Sigma Chemical Co, as used in the current study, inhibits pannexin-1 voltage-gated currents. This difference was
attributed to the different ratios of diastereomers of MFQ in each of the companies’ preparations (Iglesias et al. 2010). Thus, the feature of MFQ to block ATP-induced processes in the current study may be attributed to the source of MFQ. In contrast, CBX, PRO and FFA were all obtained from Sigma Chemical Co, as previously used by others (Ma et al. 2009; Pelegrin and Surprenant 2006) to study pannexin-1 downstream of P2X7 activation. It remains unclear as to why the inhibition of P2X7-induced ethidium+ uptake was limited to CBX, and when only used at 50 μM in physiological medium. Others (Ma et al. 2009; Pelegrin and Surprenant 2006) have previously shown that 20 μM CBX is sufficient to impair P2X7-induced ethidium+ uptake into J774 cells in this same medium. Moreover, others have shown that 20 μM CBX is sufficient to impair P2X7 functions in other cell systems (Pelegrin and Surprenant 2006). The differences between this study and other studies does not appear to be related to the source of cells, as all were originally obtained from the American Type Culture Collection. Moreover, the difference does not appear to be due to a lack of pannexin-1, as the J774 cells expressed pannexin-1 mRNA (Fig. 3.1), and CBX, PRO and FFA impaired P2X7-induced IL-1β release (Fig. 3.8). The inhibition of P2X7-induced ethidium+ uptake into J774 cells by 50 μM CBX in physiological but not NaCl medium however does suggest that the extracellular milieu (ion content) of the medium used may alter the potency of the various pannexin-1 inhibitors used. Consistent with this, the potency of various P2X7 antagonists is altered in the presence of different extracellular ions (Hibell et al. 2001). Moreover, increases in intracellular Ca2+ facilitates pannexin-1 activation (Locovei et al. 2006). Thus, the P2X7-induced channel in the presence of extracellular Ca2+ may lead to an increase in intracellular Ca2+ and the subsequent opening of pannexin-1. One notable difference between the study of Pelegrin and Surprenant (2006) and this study is that the J774 cells used for ethidium+ uptake were studied as an adherent-layer or in suspension respectively. Consistent
with this, is that P2X7-induced IL-1β from adherent J774 cells was impaired by pannexin-1 antagonists, contrasting the general lack of inhibition of P2X7-induced ethidium+ uptake by these compounds in suspended J774 cells.

The current study demonstrates that of the tested pannexin-1 antagonists, CBX is the most potent in impairing P2X7-induced processes followed by PRO and FFA and finally MFQ, which as discussed above was completely ineffective. Although, Pelegrin and Surprenant (2006) did not originally test PRO and FFA, they did demonstrate that CBX was the only gap junction antagonist tested which could impair ATP-induced cation fluxes. Moreover, Ma et al. (2009) revealed that, in both oocytes and mammalian cells, activation of pannexin-1 can be rapidly and reversibly inhibited by CBX at concentrations 5- to 20-fold less than required to inhibit connexin-mediated currents. Further they have demonstrated that CBX is the most potent pannexin-1 antagonist in both human and mice cells compared to other compounds. Similarly, CBX is a more potent inhibitor of pannexin-1 currents compared to FFA (Bruzzone et al. 2005). In contrast, Iglesias et al. (2008) demonstrated that P2X7-induced currents were most potently impaired by MFQ, then CBX and finally FFA. Finally, PRO, which was originally shown to block pannexin-1 in frog oocytes (Silverman et al. 2008), has not been shown to impair mammalian pannexin-1 nor has it been compared to CBX, FFA or MFQ in relation to P2X7-induced events. Nevertheless, the inhibition of P2X7-induced IL-1β release from J774 cells suggests that this compound, which is used to treat gout (Rider and Jordan 2009; Moolenburgh et al. 2006), may in part mediate its mechanism of action by impairing IL-1β release in this disease. In conclusion, the results indicate that, in decreasing order of potency, CBX, PRO, and FFA can impair P2X7-induced processes.
This study confirmed that J774 cells express functional P2X7 as originally reported by others (Pelegrin and Surprenant 2006; Coutinho-Silva et al. 2005; Chiozzi et al. 1997). First, RT-PCR and immunoblotting demonstrated the presence of P2X7 mRNA and protein respectively in these cells (Fig. 3.1 and 3.2). Second, ATP-induced ethidium$^+$ uptake into J774 cells with a $EC_{50}$ of $\sim0.23$ and $\sim3.4$ mM in NaCl medium nominally free of or containing extracellular Ca$^{2+}$ (Fig. 3.3 and 3.9A), typical of ATP-induced cation fluxes mediated by recombinant mouse P2X7 (Donnelly-Roberts et al. 2009; Chessell et al. 1998). This difference in the $EC_{50}$ values for ATP between the two media is also typical of P2X7, and is most likely as a result of Ca$^{2+}$ binding ATP and reducing the availability of ATP$^+$ (North 2002). Third, the P2X7 antagonists, KN-62 (Gargett and Wiley 1997) and A-438079 (McGaraughty et al. 2007), impaired ATP-induced ethidium$^+$ uptake into J774 cells with $IC_{50}$ values of $\sim0.2$ and $\sim2$ μM (Fig. 3.4A and B and 3.9B and C). Of note, these $IC_{50}$ values are similar to the inhibition of ATP-induced Yo-Pro-1$^{2+}$ uptake in 1321N1 cells transfected with recombinant murine P2X7 (Donnelly-Roberts et al. 2009). Finally, ATP induced IL-1$\beta$ release from LPS-primed J774 cells (Fig. 3.6), and this process was impaired by A-438079 (Fig. 3.7B). The inability of KN-62 to impair ATP-induced IL-1$\beta$ release from J774 cells (Fig. 3.7A) however was unexpected, especially given its greater potency in impairing ATP-induced ethidium$^+$ uptake compared to A-438079. One possible explanation is that KN-62 may be ineffective at blocking murine P2X7 when ATP nears 5 mM ATP as used in the IL-1$\beta$ release assay.

In conclusion, this study confirms that J774 cells express P2X7 and pannexin-1. Moreover, the pannexin-1 antagonists, CBX, can impair P2X7-induced ethidium$^+$ uptake and IL-1$\beta$ release from these cells, but its mechanism of action is modulated by the extracellular milieu. Finally,
the pannexin-1 antagonists, PRO and FFA can also inhibit P2X7-induced IL-1β release from J774 cells but are unable to impair P2X7-mediated ethidium\(^+\) uptake.
Chapter 4: Cloning of the Canine P2X7 Receptor

4.1 Materials and Methods

4.1.1 Materials

General materials are described in Chapter 2. Additional materials are listed below. Wizard® plus SV Minipreps DNA Purification Systems and Wizard® SV Gel and PCR Clean-up Systems were from Promega (Madison, WI). QIAGEN-100 tip maxi columns were obtained from QIAGEN (Valencia, CA). SacII, NheI and NotI restriction enzymes, 1X Tango buffer, buffer O and B and T4 ligase were from Fermentas (Glen Burnie, MD). OPTI-MEM® I reduced serum medium and Lipofectamine™ 2000 reagent were obtained from Invitrogen (Carlsbad, CA).

4.1.2 Plasmids and cDNA

Plasmid phP2X7-AcGFP-N1 was kindly provided by Dr. Leanne Stokes (University of Sydney, Sydney, Australia). Canine P2X7 cDNA was extracted from canine (English Springer Spaniel) peripheral blood mononuclear cells (PBMCs) and kindly provided by Dr. Peter Williamson (University of Sydney). PCR primers to amplify the canine P2RX7 gene were designed by Dr. Jason McArthur (University of Wollongong, Wollongong, Australia).

4.1.3 Cell lines

Human 1321N1 astrocytoma cells, originally obtained from the American Type Culture Collection (Manassas, VA), were kindly provided by Dr. Leanne Stokes. Human embryonic kidney 293 (HEK293) cells were kindly provided by Dr. Justin Yerbury (University of Wollongong). 1321N1 and HEK293 cells were maintained in DMEM-high glucose and RPMI-
1640 medium respectively supplement with 10% FCS and 5 mM L-glutamine (complete culture medium). Cells were incubated at 37°C/5% CO₂ and were passaged twice a week, as required. 1321N1 and HEK293 cells were harvested using trypsin-EDTA.

4.1.4 Bacterial strains and culture conditions

*Escherichia coli* JM109 was grown in Luria-Bertani (LB) (Appendix) broth or on LB agar (Appendix) as described by Sambrook et al (1998). Media was supplemented with 50 μg/mL of kanamycin when required. Bacteria were grown at 37°C, on agar plates incubated in a Thermoline (Sydney, Australia) Laboratory Incubator and in liquid cultures shaken at 200 rpm in a Bioline (Sydney, Australia) 472 Shaking Incubator to facilitate aeration.

4.1.5 Amplification of canine P2X7 fragment by PCR

Canine P2X7 cDNA was amplified using two different sets of primers, one forward primer (SacII) and two reverse primers (NheI and NotI). The primer sequences are shown in Table 4.1. The PCR was prepared in a 20 μL volume containing 10 x buffer (2 μL), 2.5 mM dNTP (5 μL), 6 mM MgCl₂ (2.6 μL), 10 μM of each primers (2 μL), DNA template (1 μL), *Taq* polymerase (0.2 μL) and dH₂O (7.6 μL). The cycling parameters for PCR were 94° for 2 min, 30 cycles of 94° for 30 s, 59° for 30 s, 72° for 3:30 min.
Table 4.1: Primers used for amplification of canine P2X7 fragment. Restriction enzyme sites used to facilitate cloning are underlined (P2X7 forward contains NheI, P2X7 reverse contains NotI or SacII).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>NotI (Reverse)</td>
<td>CTAGCGGCCGCTCAGTAAGGACTCTCTGAAGCCACTGTA</td>
</tr>
<tr>
<td>SacII (Reverse)</td>
<td>GAGCGCGGGTAAGGACTCTCTGAAGCCACTGTA</td>
</tr>
<tr>
<td>NheI (Forward)</td>
<td>TGCACGCTAGCATGTCTGCTGCTGCAGCTGTAAC</td>
</tr>
</tbody>
</table>

4.1.6 Plasmid extraction

Plasmids were extracted using two different techniques. Plasmids containing positive recombinant gene clones for sub-cloning and DNA sequencing were extracted using the Wizard® plus SV Minipreps DNA Purification System according to manufacturer’s instructions.

Large scale extraction of plasmids for transfection experiments was conducted using QIAGEN-100 tip maxi columns as specified by the manufacturer. Briefly, a single colony of E. coli JM109 containing the plasmid of interest was used to inoculate 3 mL of LB as a starter culture and incubated at 37°C for 6 h with vigorous shaking. The starter culture was then used to inoculate 97 mL of LB and grown at 37°C for 12-16 h with vigorous shaking until an optical density (OD$_{600}$) of 1 was reached. Cells were harvested by centrifugation at 6000 x g for 15 min at 4°C. The resulting cell pellet was resuspended in buffer P1 (lysis buffer, Appendix). Then buffers P2 (Appendix) and P3 (Appendix) were added into the solution and cells were centrifuged at 20000 x g for 30 min at 4°C to remove cellular debris. Supernatant was removed and centrifuged for the second time at 20000 x g for 15 min at 4°C. The cell lysate supernatant was run through a QIAGEN-100 tip column. The column was washed with 20 mL buffer QC (Appendix) and
plasmid DNA was eluted from the column using 5 mL buffer QF (Appendix) and stored at -20°C.

4.1.7 Restriction enzyme digestion of DNA

Restriction enzyme digestion was performed using NheI/SacII and NheI/NotI restriction enzymes. For removal of the full length human P2X7 gene, phP2X7-AcGFP-N1 was double digested with NheI/SacII or NheI/NotI in a 46 μL reaction volume containing buffer B or buffer B and O, respectively. Digestion of the phP2X7-AcGFP-N1 vector with NheI/SacII released the human P2X7 open reading frame alone, while digestion with NheI/NotI released the human P2X7 open reading frame encoding the hP2X7-GFP fusion protein from the vector. All digestions were incubated at 37°C for 4 h and restriction enzymes were heat inactivated by incubation at 80°C for 20 min.

4.1.8 DNA ligation

Ligations were performed using T4 ligase. Each ligation contained ~100 ng of digested plasmid DNA, ~500 ng of insert DNA and 20 U of T4 ligase in a final reaction volume of 30 μL containing 1X T4 ligase buffer. Ligations were incubated at 15°C over night, followed by T4 ligase inactivation by incubation at 65°C for 15 min. Ligation reactions were purified via Wizard® SV Gel and PCR Clean-up systems according to the manufacturer’s instructions.

4.1.9 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using 0.86% agarose gel in 1X TAE buffer (Appendix). Electrophoresis was conducted at 90 V for 1 h in a MiniSub Cell GT electrophoresis tank (BioRad, Hercules, CA). DNA was visualised using Novaline Gel Documentation System
(Novex, Melbourne, Australia) under ultraviolet light (UVP, Upland, CA) after staining in ethidium bromide solution (1μg/mL) for 20 min and de-staining in H_{2}O for 10 min. Approximate DNA fragment size and concentration was determined by comparison to the migration of HyperLadder™ I DNA ladder. When required, DNA fragments were extracted from agarose gels via the Wizard® SV Gel and PCR Clean-up kit according to the manufacturer’s instructions.

4.1.10 Preparation of electro-competent *E. coli* JM109

To prepare electro-competent *E. coli* JM109 for transformation with recombinant plasmid DNA, a single colony was inoculated into 3 mL of LB broth and grown overnight at 37°C with vigorous shaking. This culture was then used to inoculate 400 mL of LB broth and grown at 37°C with vigorous shaking to an optical density (OD_{600}) of 0.6. The culture was then chilled on ice for 20 min before centrifugation at 4,000 x g for 15 min at 4°C to harvest the cells. The cells were resuspended in 400 mL of cold milliQ water and pelleted under the same conditions. The pellet was washed by re-suspension in 200 mL of cold milliQ water and centrifuged again. Cells were resuspended in 4 mL of chilled 10% glycerol and centrifuged under the same conditions, before final resuspension in 600 μL of chilled 10% glycerol. Cells were distributed into 40 μL aliquots for storage at -80°C.

4.1.11 Transformation of electro-competent *E. coli* JM109

Transformation of *E. coli* JM109 was performed by adding 2 μL (~50 ng) of plasmid DNA to a 40 μL aliquot of electro-competent cells. The transformation mixture was transferred to a cold 1 mL, 2 mm gap, eletro-transformation cuvette (Eppendorf, Hamburg, Germany) and transformed using MicroPulsar™ (BioRad) using settings defined by the manufacturer for bacterial electroporation (2.5 μF capacitance, 2.5 kV single pulse, 20 Ω in series, 200 Ω in parallel).
Following transformation, 500 μL of LB broth was added to the transformation mixture which was then transferred to a 1.5 mL microcentrifuge tube for incubation at 37°C for 1 h. To select for successfully transformed bacteria, dilution series of the transformation culture were plated onto LB agar containing appropriate antibiotics and incubated over-night at 37°C.

4.1.12 Cloning strategy for construction of canine P2X7-AcGFP-N1

To clone canine P2X7, the human P2X7 sequence was removed from phP2X7-AcGFP-N1 using NheI/SacII or Nhel/NotI restriction enzyme combinations (Fig. 4.1). Digestion with Nhel/SacII will remove the human P2X7 gene and will leave GFP gene in the vector enabling the expression of a recombinant–GFP fusion protein. Digestion with Nhel/NotI will remove both the hP2X7-GFP sequence from the vector. Digested plasmids fragments were separated by 0.86% agarose gel electrophoresis and the DNA fragments corresponding to the plasmid vector were gel purified using Wizard® SV Gel and PCR Clean-up Systems according to the manufacturer’s instructions. The canine P2X7 cDNA was PCR amplified, digested with Nhel/SacII or Nhel/NotI, and was ligated with the appropriately digested and purified vector DNA. Ligation reactions were transformed into electro-competent *E. coli* JM109 and cultured overnight at 37°C on agar containing kanamycin. Positive clones containing canine P2X7 were identified by extraction, digestion and electrophoresis analysis of purified plasmid DNA followed by DNA sequence analysis.
Fig. 4.1: Schematic diagram summarising the cloning strategy used to create canine P2X7-AcGFP-N1 and canine P2X7-Ac-N1 constructs. phP2X7-AcGFP-N1 was double digested using NheI/SacII, or NheI/NotI restriction enzymes. Digestion, with NheI/NotI will remove GFP gene from the vector, while digestion with NheI/SacII will leave GFP gene in the vector. Vector fragments were separated by 0.86% agarose gel electrophoresis and purified from the gel. Then, PCR amplified and appropriately digested canine P2X7 was ligated with the appropriately digested vector.
4.1.13 DNA sequence analysis

To confirm the construction of the canine pcP2X7-Ac-N1 and pcP2X7-AcGFP-N1 DNA sequencing was conducted using Big Dye Terminator v3.1 Cycle Sequencing Kit. Each sequencing reaction contained 0.5 μL of Big Dye Terminator Ready Reaction Mix (RR-100) (Perkin-Elmer, USA), in 10X sequencing buffer (1 μL) (Appendix), template DNA (5 μL), 3.2 pmol sequencing primer (2 μL) (Table 4.2) and 1.5 μL of dH2O to a total volume of 10 μL. Sequencing reactions were conducted in a thermocycler using 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min with a final extension period of 10 min at 60°C.

Table 4.2: Primers used for sequencing of cloned canine P2X7

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X7 first reverse</td>
<td>ATCTTCTTGATTCATTCTCTCC</td>
</tr>
<tr>
<td>P2X7 first forward</td>
<td>CCGCCTGGTGTCGCCATCG</td>
</tr>
<tr>
<td>P2X7 second reverse</td>
<td>CAGCTGCATCTCCTCTCTG</td>
</tr>
<tr>
<td>P2X7 second forward</td>
<td>CCACCTCAGAGCCGTCCTCTG</td>
</tr>
</tbody>
</table>

DNA sequencing reactions were purified via ethanol precipitation. 3 μL of 2 M sodium acetate (pH 4.5) and 35 μL of cold 95% (v/v) ethanol was added to each reaction. The solution was incubated on ice for 10 min then centrifuged at 14000 x g for 20 min to pellet precipitated DNA. The supernatant was removed and the DNA pellet was washed by adding 250 μL of cold 70% (v/v) ethanol. The tube was centrifuged at 14000 x g for another 20 min and the supernatant removed. The pellet was allowed to air dry away from light and stored at -20°C. DNA sequencing was performed by Margaret Phillips (University of Wollongong) using a 3130xl Genetic Analyser (Applied Biosystems, Carlsbad, CA), as per the manufacturer’s instructions.
Sequence data were analysed using ChromasPro v1.33 (Technelysium, Brisbane, Australia) and BioEdit v7.0.9.0 (Hall, Carlsbad, CA).

4.1.14 Cell transfection

1321N1 or HEK293 cells were plated in 6-well plates (Greiner Bio-One, Frickenhausen, Germany) at 5 x 10^5/mL/well and incubated overnight at 37°C/5% CO₂. Different concentrations of constructed pcP2X7-AcGFP-N1 DNA (1, 2, 4, and 8 µg) was prepared in OPTI-MEM® I reduced serum medium, mixed with an equal volume of Lipofectamine™ 2000 reagent in OPTI-MEM® I reduced serum medium and incubated at room temperature for 20 min. Meanwhile, cells were washed once with 1 mL OPTI-MEM® I reduced serum medium. Then, each mixture of pcP2X7-AcGFP-N1 DNA and Lipofectamine was added into each well containing OPTI-MEM® I reduced serum medium containing 5% FCS, and the plate was incubated at 37°C/5% CO₂ for 48 h. Both untreated and mock-transfected cells (treated with Lipofectamine alone) were used as controls. Then, cells from each well were harvested by scraping and washed once with NaCl medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 5 mM glucose, 0.1% BSA, pH 7.4). Cells were resuspended in NaCl and data was collected using a LSR II flow cytometer and FacsDiva software (BD Bioscience, San Jose, CA) with a total of 3 x 10^4 events collected using an emission window of 515 ± 20 nm. The percentage of GFP⁺ cells was determined using FlowJo Software (TreeStar, Ashland, OR).
4.2 Results

4.2.1 Amplification of canine P2X7 fragment

Canine P2X7 was successfully amplified from English Springer Spaniel cDNA using PCR. *NheI/SacII*, or *NheI/NotI* primers were used for production of fragments with two different ends. Reaction mixtures were purified and visualised via agarose gel electrophoresis. Fragments were observed at ~1800 base pairs (bp) corresponding closely to the size of canine P2X7 cDNA which is ~1800 bp (accession number XM_534669) (Fig. 4.1).

![Amplification of canine P2X7 fragment](image)

**Fig. 4.1: Amplification of canine P2X7 fragment.** DNA present in PCR reactions was resolved using 0.86% agarose gel and visualized after staining with ethidium bromide. Lane 1, HyperLadder™ I DNA ladder; lane 2 amplified canine P2X7 digested with *NheI/SacII*; lane 3, amplified canine P2X7 digested with *NheI/NotI*. Size of the DNA fragments and molecules weight markers are indicated in base pairs (bp).
4.2.2. **Purified vector fragments from digested phP2X7-AcGFP-N1**

To remove hP2X7 from phP2X7-AcGFP-N1, the plasmid was separately double digested with *NheI/SacII* and *NheI/NotI* restriction enzymes. The DNA digestions were separated via 0.86% agarose gel electrophoresis, and the DNA fragments corresponding to vector DNA at ~4665 base pairs for the *NheI/SacII* digests (Lane 3) and at ~3923 base pairs for the *NheI/NotI* digests (Lane 4) were purified from the gel (Fig. 4.2). Undigested phP2X7-AcGFP-N1 is also shown in lane 2 (Fig. 4.2).

![Agarose Gel Electrophoresis Image](image.png)

**Fig. 4.2: Purified vector fragments from digested phP2X7-AcGFP-N1.** Purified *NheI/SacII* and *NheI/NotI* digested vector fragments were visualised using 0.86% agarose gel electrophoresis. Lane 1, HyperLadder™ I DNA ladder; lane 2, undigested phP2X7-AcGFP1; lane 3, *NheI/SacII* fragments of phP2X7-AcGFP-N1; lane 4, *NheI/NotI* fragments of phP2X7-AcGFP-N1. Sizes of the DNA fragments are 4665 base pairs for the *NheI/SacII* digests (Lane 3) and at ~3923 base pairs for the *NheI/NotI* digests (Lane 4).
4.2.3 Cloning of the canine P2X7 gene

To clone the cP2X7 gene, *NheI/SacII* and *NheI/NotI* digested PCR amplified DNA fragment were ligated into pAc-GFP-N1 digested with *NheI/SacII* and *NheI/NotI* to produce the recombinant plasmids pcP2X7-AcGFP-N1 and pcP2X7-Ac-N1 respectively. To confirm the correct construction of these plasmids, pcP2X7-AcGFP-N1 and pcP2X7-Ac-N1 plasmids were both extracted and digested with the same restriction enzymes originally used for digestion of the hP2X7-AcGFP-N1 plasmid (*NheI/SacII* and *NheI/NotI*, respectively). DNA fragments were then separated by 0.86% agarose gel electrophoresis and visualised by staining with ethidium bromide (Fig. 4.3). pcP2X7-AcGFP-N1 fragment (at ~4665 bp; lane 3) and pcP2X7-Ac-N1 (at ~3923 bp; lane 5) fragment, as well as cP2X7 sequence (at ~1800 bp; lanes 3 and 5) were observed at the right size. Also, an undigested plasmid was run as a control (lanes 2 and 4).

4.2.4 ClustalW alignment of the deduced amino acids sequences of three canine P2X7 sequences

The cloned canine P2X7 from pcP2X7-AcGFP-N1 was sequenced and the deduced amino acid sequence was then compared to other canine P2X7 sequences in the NCBI database using ClustalW2 tool (European Bioinformatics Institute, www.ebi.ac.uk). Two single nucleotide polymorphisms which produce amino acid changes were identified at positions Pro452Ser and Cys507Gly when compared to the other canine P2X7 sequences in the NCBI/GeneBank database (accession number EU334661 and XM_534669) (Fig. 4.4).
Fig. 4.3: Construction of pcP2X7-Ac-N1 and pcP2X7-AcGFP-N1. Both pcP2X7-Ac-N1 and pcP2X7-AcGFP-N1 were digested with *Nhe*I/*Not*I and *Nhe*I/*Sac*II, respectively. DNA fragments were separated using agarose gel electrophoresis. Lane 1, HyperLadder™ I DNA ladder; lane 2, pcP2X7-AcGFP-N1; lane 3, pcP2X7-AcGFP-N1 digested with *Nhe*I/*Sac*II and canine P2X7; lane 4, pcP2X7-Ac-N1; lane 5, pcP2X7-Ac-N1 digested with *Nhe*I/*Not*I.
Dog_EU: MSACCSCNDI FQYETNKKIR IQSMNYGTIK WIFHVIIFSY ISFALISDKR 50
Dog_XM: MSACCSCNDI FQYETNKKIR IQSMNYGTIK WIFHVIIFSY ISFALISDKR

Dog_EU: YQQKEPLISS VHTKVKGTAE VKMEILENGI KKMVSTVFDT ADYTFPFLQGN 100
Dog_XM: YQQKEPLISS VHTKVKGTAE VKMEILENGI KKMVSTVFDT ADYTFPFLQGN

Dog_EU: SFFVMTNFLK TEGQQQGFCP EFPTTRTLCS NDWGCKKGWM DPQSKGIQTG 150
Dog_XM: SFFVMTNFLK TEGQQQGFCP EFPTTRTLCS NDWGCKKGWM DPQSKGIQTG

Dog_EU: RCIEYKGKQK TCEVSAWCPH EAVEEAPRPA LLNGAENFTV LIKNNIDFPG 200
Dog_XM: RCIEYKGKQK TCEVSAWCPH EAVEEAPRPA LLNGAENFTV LIKNNIDFPG

Dog_EU: FIDFLINTYS SKCCRSHIYP CFKCCEYCAV NEYYKKKCE TIVEPKPTLK 400
Dog_XM: FIDFLINTYS SKCCRSHIYP CFKCCEYCAV NEYYKKKCE TIVEPKPTLK

Dog_EU: SPIPQSCSEEM QLLSEEVPTR SSNSPDWCQC GHCLPSQLPE SQRCLEELCC 500
Dog_XM: SPIPQSCSEEM QLLSEEVPTR SSNSPDWCQC GHCLPSQLPE SQRCLEELCC

Dog_EU: RKKAGACITTT SEPFRKLILS RQVQLQFLLYY QEPPLLVDEN SNSRLRCAY 550
Dog_XM: RKKAGACITTT SEPFRKLILS RQVQLQFLLYY QEPPLLVDEN SNSRLRCAY

Dog_EU: RCTYTTWRFGS QDLADFAILP SCCRWRIRRE FPKSEGQYSG FRSPY 595
Dog_XM: RCTYTTWRFGS QDLADFAILP SCCRWRIRRE FPKSEGQYSG FRSPY

89
Fig. 4.4: ClustalW alignment of the deduced amino acids sequences of three canine P2X7 sequences. Two single nucleotide polymorphisms which produce amino acid changes at positions Pro452Ser (CCG to AGT) and Cys507Gly (TGC to GGC), were identified in the sequence of the P2X7 cloned in the current study (cP2X7). Dog_EU and Dog_XM are the sequences from previous studies (accession number EU334661 and XM_534669). Amino acids which differ between sequences are shaded in yellow.

4.2.5 Canine P2X7-AcGFP-N1 transfection into 1321N1 cells

The above agarose gel electrophoresis and sequencing results indicate that canine P2X7 was successfully cloned into the pAcGFP-N1 vector (Fig. 4.3 and 4.4 respectively). To determine if the cP2X7-GFP fusion protein would be expressed in mammalian cells, 1321N1 cells were transfected with different concentrations of pcP2X7-AcGFP-N1 (1, 2, 4 and 8 µg). After 48 h the percentage of GFP+ cells was determined by flow cytometry. Untreated and mock-transfected cells expressed low amounts of auto fluorescent cells (Fig. 4.5). In contrast, a small but greater amount of GFP+ cells were observed in cells transfected with pcP2X7-AcGFP-N1, with transfection efficiencies similar for 2, 4 and 8 µg (Fig. 4.5).

4.2.6 Canine P2X7-AcGFP-N1 transfection into HEK293 cells

The above results indicate that 1321N1 cells, can express low amounts of P2X7 following transfection (Fig. 4.5). However, transfection efficiency of human P2X7-GFP is greater in HEK293 cells compared to 1321N1 cells (Stokes et al. 2010). Therefore, pcP2X7-AcGFP-N1
was transfected into HEK293 cells (as above for 1321N1) and analysed by flow cytometry. In contrast to 1321N1 cells, pcP2X7-AcGFP-N1 failed to transfect HEK293 cells at any of the concentrations tested, with the percentage of autoflourescence/GFP\textsuperscript{+} cells similar to that of untreated or mock-transfected cells (Fig. 4.6).

![GFP expression plots](image)

**Fig. 4.5: Canine P2X7-AcGFP-N1 transfection into 1321N1 cells.** Canine P2X7-AcGFP-N1 plasmid was transfected into 1321N1 cells. Cells were cultured in 6-well plate for 24 h and transfected with different concentrations of P2X7-AcGFP-N1 (1, 2, 4 and 8 µg) and Lipofectamine for 48 h. Both untreated cells and cells treated with Lipofectamine alone (mock-transfected) were used as negative controls. Cells were harvested and analysed by flow cytometry. Values in boxes represent percentage of autofluorescent (Nil, mock) and autofluorescence\textsuperscript{+}/GFP\textsuperscript{+} cells (1-8 µg).
Fig. 4.6: Canine P2X7-AcGFP-N1 transfection into HEK293 cells. Canine P2X7-AcGFP-N1 plasmid was transfected into HEK293 cells. Cells were cultured in 6-well plate for 24 h and transfected with different concentrations of P2X7-AcGFP-N1 (1, 2, 4 and 8 µg) and Lipofectamine for 48 h. Both untreated cells and cells treated with Lipofectamine alone (mock-transfected) were used as negative controls. Cells were harvested and analysed by flow cytometry. Values in boxes represent percentage of autofluorescent (Nil, mock) and autofluorescent+/GFP+ cells (1-8 µg).
4.3 Discussion

The current study cloned the P2X7 receptor from an English Springer Spaniel. This breed was selected as our group has already obtained pharmacological data for native P2X7 in leukocytes and erythrocytes from this breed (Stevenson et al. 2009; Sluyter et al. 2007). Comparing the sequence of the canine P2X7 cloned in this study with a previously cloned canine P2X7 from a Beagle (Roman et al. 2009) and a P2X7 sequence (boxer) described in the NCBI/GeneBank database (accession number XM_534669) identified two single nucleotide polymorphisms at positions Pro452Ser and Cys507Gly in the P2RX7 gene of the English Spring Spaniel (Fig. 4.4). The functional significance of these amino acid changes is unknown but could be studied further by pharmacological studies of the recombinant receptor in transfected cells. Of note, all canine sequences contain the additional residue (Asp282) in the extracellular loop, and the deleted residue (Val538) from the C-terminus compared to other mammalian P2X7 receptors.

The recombinant P2X7 cloned in the current study was expressed at low amounts in transfected 1321N1 cells, but not to transfected HEK293 cells (Fig. 4.5 and 4.6). The low amount of P2X7 expression in 1321N1 cells was not totally unexpected as these cells are often more difficult to transfect than HEK293 cells (Merucci et al. 2011). However, it remains unknown why the recombinant P2X7 failed to express in HEK293 cells (Fig. 4.6) and due to time constraints this could not be explored further. It also remains to be explored if the low amount of transfected 1321N1 cells observed in the current study is reproducible and whether this low amount of expression results in the presence of functional P2X7 in these cells. If functional P2X7 is present following transfection of 1321N1 cells, pharmacological characterisation of the cloned receptor can take place. In contrast, if functional P2X7 receptors are absent, further consideration needs to
be given to improve transfection rates (as discussed in Chapter 6). Of note, the two amino acid changes Pro452Ser and Cys507Gly identified in the cloned P2X7 sequence of the current study occur at the highly conserved residues (Pro452 and Cys507) in human, rat and mouse P2X7. Moreover, it is noteworthy, that the Pro452Ser SNP in canine P2X7 occurs at a corresponding position of a SNP in mouse P2X7 (Pro451Leu) (Adriouch et al. 2002). These changes also need to be considered in future attempts to transfect cells with this construct.
Chapter 5: P2X7 Function Varies Between Dogs

5.1 Materials and Methods

5.1.1 Materials

Materials are described in Chapter 2. Additional materials are listed below. YO-PRO-1\(^{2+}\) was from Invitrogen (Eugene, OR). Allophycocyanin (APC)- and PerCP/Cy5.5-conjugated anti-human/canine CD14 (clone M5E2) monoclonal antibody (mAb) were from BioLegend (San Diego, CA). This mAb also recognises canine CD14 (Jacobsen et al. 1993). Murine anti-canine CD3 (clone CA17.2A12) and B-cell (clone CA2.1D6) mAb were from Serotec (Raleigh, NC). APC conjugated-donkey anti-mouse IgG was from eBioscience (San Diego, CA). 7-Aminoactinomycin (7AAD) was from Enzo life sciences (Plymouth Meeting, PA). Standard SPHERO™ Rainbow Fluorescent particles were from Spherotech (Libertyville, IL). Wizard® Genomic DNA Purification Kit was from Promega (Madison, WI).

5.1.2 Detection of YO-PRO-1\(^{2+}\) uptake by fixed-time flow cytometry

Peripheral blood mononuclear cells (PBMCs), isolated as previously described (Section 2.4), were washed once in NaCl medium (145 mM NaCl, 5 mM KCl, 5 mM D-glucose, 0.1 % BSA, 10 mM HEPES, pH 7.5) at 300 g for 5 min. Cells were resuspended in NaCl medium containing 1 μM YO-PRO-1\(^{2+}\), and incubated in the absence or presence of 1 mM adenosine 5’-triphosphate (ATP) at 37\(^\circ\)C for up to 5 min. For P2X7 antagonist-related experiments, cells were pre-incubated (before ATP addition) for 15 mins at 37\(^\circ\)C with 1 μM KN-62 or 10 μM A-438079 or their respective diluents, dimethyl sulphoxide (DMSO) or H\(_2\)O. Incubations with ATP were
terminated by addition an equal volume of ice-cold MgCl\textsubscript{2} medium (20 mM MgCl\textsubscript{2}, 145 mM NaCl, 5 mM KCl and 10 mM HEPES, pH 7.5) and centrifugation at 300 g for 5 min. Cells were washed with NaCl medium and incubated with APC- or PerCP/Cy5.5-conjugated anti-CD14 mAb for 15 min at room temperature. Where indicated, cells were also co-incubated with 7AAD. In some experiments, cells were incubated with anti-CD3 or B-cell mAb for 15 min at room temperature, washed once with NaCl medium and incubated with APC-conjugated anti-murine IgG for 15 min at room temperature. After incubation, cells were washed once with NaCl medium, and the data was collected using a LSR II flow cytometer and FacsDiva software (both BD Bioscience, San Diego, CA). A total of 2 x 10\textsuperscript{5} cells were collected using emission window at 515 ± 20 nm (for YO-PRO-1\textsuperscript{2+}). The emission window at 515 ± 20 nm was calibrated by standard fluorescent particles before each experiment to minimise day-to-day variations between samples. Data was analysed using FlowJo software (Tree Star Inc., Ashland, CA).

5.1.3 DNA extraction from canine monocytes

Genomic DNA was extracted from canine whole blood using the Wizard\textsuperscript{®} Genomic DNA Purification Kit according to the manufacturer’s instructions. To check the quality and relative concentration of DNA, samples were ran on a 0.86% agarose gel (90 V for 50 min) and visualized by ethidium bromide staining.

5.1.4 Data presentation and statistical analyses

Data is presented as mean ± standard deviation (SD) or as mean of duplicate values (except in Table 5.1 where duplicate data is presented as mean ± SD with coefficient of variance (CV)). CV was calculated as described by Synek (2008). All graphs were compiled using Prism 5 software
for Windows (GraphPad Software, Inc; La Jolla, CA). Differences between each treatment were compared using either the unpaired or paired Student’s t-test for single comparisons to control samples, or ANOVA for multiple comparisons using GraphPad Prism 5 software with \( P < 0.05 \) considered significant.

5.2 Results

5.2.1 ATP-induced YO-PRO-1\(^{2+}\) uptake into canine monocytes from one-day aged blood is higher than in monocytes from fresh (day 0) blood

Korpi-Steiner et al. (2008) have previously used a fixed-time flow cytometric assay to measure the function of P2X7 in human monocytes within whole blood. Therefore, this assay was attempted initially to detect P2X7 function in canine monocytes in whole blood. However, due to the large ratio of red blood cells to monocytes in canine blood it was not possible to measure ATP-induced YO-PRO-1\(^{2+}\) uptake in CD14\(^+\) monocytes (results not shown). Thus, to increase the proportion of monocytes in samples, PBMCs were isolated from whole blood by density centrifugation and then the ATP-induced YO-PRO-1\(^{2+}\) uptake into CD14\(^+\) monocytes was assessed by flow cytometry. Moreover, an advantage of the method of Korpi-Steiner et al. (2008) is that monocyte P2X7 function does not significantly change in whole human blood stored up to 4 days. Thus, to determine if this also held for canine monocytes, ATP-induced YO-PRO-1\(^{2+}\) uptake into monocytes from fresh blood (day 0) and one day-old blood (day 1; stored overnight at 4\(^\circ\)C) was compared using blood from four dogs. ATP-induced YO-PRO-1\(^{2+}\) uptake into monocytes isolated from day 0 blood (194 ± 75 MFI; Fig. 5.1). ATP also induced YO-PRO-1\(^{2+}\)
uptake into monocytes from day 1 blood (257 ± 64 MFI; Fig. 5.1). ATP-induced YO-PRO-1^{2+} uptake was higher in day 1 compared to day 0 monocytes, however this failed to reach statistical significance ($P = 0.051$). Nevertheless, given these differences between days, it was decided to only assess ATP-induced YO-PRO-1^{2+} uptake into canine monocytes from fresh (day 0) blood samples.

Fig. 5.1: ATP-induced YO-PRO-1^{2+} uptake into canine monocytes from one-day aged blood is higher than in monocytes from fresh (day 0) blood. PBMCs were isolated from fresh blood (Day 0) or blood stored overnight at 4°C (day 1). PBMCs were suspended in NaCl medium containing 1 µM YO-PRO-1^{2+}, and incubated in the absence or presence of 1 mM ATP for 5 min. Incubations were stopped by MgCl$_2$ addition and centrifugation. Washed cells were labelled with APC-conjugated anti-CD14 mAb, and the MFI of YO-PRO-1^{2+} uptake examined by flow cytometry. The voltage setting at 515/20 was adjusted using SPHERO™ Rainbow Fluorescent particles to obtain the same readings before days 0 and 1. Results are presented as means of duplicate samples for each day ($n = 4$). $P = 0.05$ (paired student’s t-test).
5.2.2 ATP-induced YO-PRO-1^{2+} uptake into CD14^{+}/7AAD{^-} and total CD14^{+} monocytes is similar

As performed by Korpi-Steiner et al (2008), the above experiments utilised the vital dye, 7AAD, to exclude dead monocytes which would also potentially take up YO-PRO-1^{2+}. Therefore, to determine if inclusion of 7AAD{^-} was necessary the amount of ATP-induced YO-PRO-1^{2+} into CD14^{+}/7AAD{^-} monocytes and total CD14^{+} monocytes was compared. As above, ATP induced YO-PRO-1^{2+} uptake into CD14^{+}/7AAD{^-} monocytes (168 ± 38; Fig. 5.2). ATP-induced YO-PRO-1^{2+} uptake into total CD14^{+} cells was similar (177 ± 35; Fig. 5.2) and not significantly different ($P = 0.051$). Therefore, it was decided for subsequent studies to exclude 7AAD from the protocol, and measure ATP-induced YO-PRO-1^{2+} into total CD14^{+} monocytes.

5.2.3 P2X7 antagonists inhibit ATP-induced YO-PRO-1^{2+} uptake into canine monocytes

To confirm that the ATP-induced YO-PRO-1^{2+} uptake into canine monocytes is mediated by P2X7, cells in NaCl medium were pre-incubated with 1 μM KN-62 or DMSO (diluent control), or in the absence or presence of 10 μM A-438079 or an equal volume of H_{2}O for 15 mins at 37°C before measurement of YO-PRO-1^{2+} uptake in the absence or presence of 1 mM ATP. As above, ATP induced significantly higher amounts of YO-PRO-1^{2+} uptake into monocytes cells compared to basal (Fig. 5.1). Pre-incubation of monocytes with KN-62 or A-438079 impaired ATP-induced YO-PRO-1^{2+} uptake by 75% and 90%, respectively (Fig. 5.3). Although the difference with KN-62 failed to reach statistical significance. Neither antagonist significantly altered YO-PRO-1^{2+} uptake in the absence of ATP (Fig. 5.3).
Fig. 5.2: ATP-induced YO-PRO-1\(^{2+}\) uptake into CD14\(^+\)/7AAD\(^-\) and total CD14\(^+\) monocytes is similar. PBMCs were isolated from fresh blood and suspended in NaCl medium containing 1 µM YO-PRO-1\(^{2+}\), and incubated in the absence or presence of 1 mM ATP for 5 min. Incubations were stopped by MgCl\(_2\) addition and centrifugation. Washed cells were labelled with APC-conjugated anti-CD14 mAb and 7AAD. The MFI of YO-PRO-1\(^{2+}\) uptake into CD14\(^+\)/7AAD\(^-\) or total CD14\(^+\) monocytes examined by flow cytometry. The voltage setting at 515/20 was adjusted using SPHERO™ Rainbow Flourescent particles to obtain the same readings. Results are presented as means of duplicate samples for each day \((n = 7)\). \(P = 0.05\) (paired student’s t-test).

5.2.4 Comparison of ATP-induced YO-PRO-1\(^{2+}\) uptake into monocytes between dogs and breeds

The above experiments provided a standardised ATP-induced YO-PRO-1\(^{2+}\) uptake assay for detection of P2X7 function in canine monocytes. Therefore, this assay was used to measure the P2X7 function in monocytes from different dog breeds, to determine the relative variation in P2X7 function between these animals. ATP induced YO-PRO-1\(^{2+}\) uptake into monocytes from
15 different dogs (Table 5.1). There was a 2.6-fold difference between the dogs with the lowest and highest ATP-induced YO-PRO-1^{2+} uptake (MFI of 134 vs. 347). Moreover, data obtained for monocytes from the dogs of the same breed (Staffordshire Bull Terrier) demonstrated a 2.3-fold difference between the lowest and highest ATP-induced YO-PRO-1^{2+} uptake (MFI of 153 vs. 347). In contrast, ATP-induced YO-PRO-1^{2+} uptake in monocytes from two Golden Retriever was similar (MFI of 164 vs. 188).

Fig. 5.3: P2X7 antagonists inhibit ATP-induced YO-PRO-1^{2+} uptake into canine monocytes. Canine PBMCs in NaCl medium were pre-incubated with (A) 1 μM KN-62 or an equal volume of DMSO or (B) in the absence or presence 10 μM A-438079 or an equal volume of H_{2}O for 15 mins at 37°C. Cells were then incubated in the absence or presence of 1 mM ATP for 5 mins, and the incubations stopped by addition of MgCl_{2} medium and centrifugation. Washed cells were labelled with APC-conjugated anti-CD14 mAb and the MFI of YO-PRO-1^{2+} uptake into CD14^{+} monocytes was examined by flow cytometry. Results are expressed as mean ± SD (n = 3 individual dogs). ***P < 0.001 compared to corresponding basal; †††P < 0.001 compared to ATP without antagonist.
Table 5.1: Comparison of ATP-induced YO-PRO-1$^{2+}$ uptake into monocytes between dogs and breeds. P2X7 function in total CD14$^+$ monocytes from different dogs, was determined by duplicate measurements of ATP-induced YO-PRO-1$^{2+}$ uptake as described in Fig. 5.2. Results are expressed as the mean, SD and CV ($n = 15$). Standard fluorescent beads were used to adjust the emission at 515 ± 20 nm. The MFI of the beads provided a mean of 480 (SD, 12, $n = 15$).

<table>
<thead>
<tr>
<th>Breed</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australian Bull Dog</td>
<td>182</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Border Collie</td>
<td>202</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Bull Terrier</td>
<td>181</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Corgi cross</td>
<td>175</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>German Shorthaired Pointer</td>
<td>273</td>
<td>55</td>
<td>20</td>
</tr>
<tr>
<td>Golden Retriever</td>
<td>188</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Golden Retriever</td>
<td>164</td>
<td>51</td>
<td>10</td>
</tr>
<tr>
<td>Jack Russell Terrier cross</td>
<td>134</td>
<td>9</td>
<td>7</td>
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<tr>
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<td>257</td>
<td>66</td>
<td>26</td>
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<tr>
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<td>2</td>
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<tr>
<td>Maltese cross</td>
<td>163</td>
<td>79</td>
<td>49</td>
</tr>
<tr>
<td>Rhodesian Ridgeback cross/ Cattle Dog</td>
<td>176</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Staffordshire Bull Terrier</td>
<td>234</td>
<td>13</td>
<td>6</td>
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<tr>
<td>Staffordshire Bull Terrier</td>
<td>347</td>
<td>28</td>
<td>8</td>
</tr>
<tr>
<td>Staffordshire Bull Terrier</td>
<td>153</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

| All Dogs                                   | 197  | 23  | 10 |


5.2.5 P2X7 function in canine T cells is higher than in canine B cells and monocytes obtained for various breeds

It has been previously demonstrated that the relative P2X7 function is greater in T cells than in monocytes or B cells from English Springer spaniels, and that the relative P2X7 function between these latter two cell types from this breed is similar (Stevenson et al. 2009). Therefore, to determine if this relative pattern of P2X7 function in leukocytes is true of other breeds, ATP-induced YO-PRO-1<sup>2+</sup> uptake into monocytes, B cells and T cells was compared among five different dog breeds. ATP induced YO-PRO-1<sup>2+</sup> uptake into T cells with a MFI of 555 ± 73 (Fig. 5.4). This was significantly greater than ATP-induced YO-PRO-1<sup>2+</sup> uptake into monocytes (MFI of 170 ± 26) and B-cells (MFI of 251 ± 106). The ATP-induced YO-PRO-1<sup>2+</sup> uptake into monocytes or B-cells was not significantly different (P = 0.052).

5.2.6 Examination of genomic DNA from different dog breeds

To allow for the future comparison of P2RX7 gene sequences between different dog breeds and the possible detection of single nucleotide polymorphisms (SNPs), which may alter function, genomic DNA was collected from a total of 49 dogs (Table 5.1), and the DNA quality and relative concentration checked by agarose gel electrophoresis and ethidium bromide staining. DNA was found to be of high molecular weight and the relative concentrations similar between samples. Examples of examined DNA are illustrated in Fig. 5.5.
Fig. 5.4: P2X7 function in canine T cells is higher than in canine B cells and monocytes obtained for various breeds. PBMCs were isolated from fresh blood and suspended in NaCl medium containing 1 µM YO-PRO-1^{2+}, and incubated in the absence or presence of 1 mM ATP for 5 min. Incubations were stopped by MgCl₂ addition and centrifugation. Cells were labelled with APC-conjugated anti CD14 mAb or anti-CD3 or B-cell mAb and APC-conjugated antimurine IgG for 15 min at room temperature. The MFI of YO-PRO-1^{2+} uptake into each subpopulation was examined by flow cytometry. Results are expressed as the mean of duplicate values (n = 5). *** P < 0.001 compared to corresponding monocytes and B-cell MFI.
Fig. 5.5: Examination of genomic DNA from different dog breeds. Genomic DNA was isolated from whole blood of different dogs, and analysed by 0.86% agarose gel electrophoresis and ethidium bromide staining. Results are representative of 49 samples.

5.3 Discussion

The current study established a fixed-time flow cytometry assay to measure P2X7 function in canine monocytes and to compare the relative P2X7 function between different dog breeds. A fixed-time flow cytometric assay has previously been used to measure P2X7 function in human monocytes in whole blood (Korpi-Steiner et al. 2008). However, due to the large ratio of red
blood cells to monocytes in canine blood it was not possible to measure ATP-induced YO-PRO-1\(^{2+}\) uptake into canine monocytes in whole blood (results not shown). Thus, this protocol was altered and eventually led to the reliable measurement of ATP-induced YO-PRO-1\(^{2+}\) uptake into CD14\(^+\) monocytes in isolated PBMCs (Fig. 5.2 and Fig. 5.1). Preincubation of PBMCs with the P2X7 antagonists, KN-62 and A-438079 inhibited ATP-induced YO-PRO-1\(^{2+}\) uptake into CD14\(^+\) monocytes (Fig. 5.3), confirming that this process was mediated by P2X7.

Use of standard fluorescent particles were used to calibrate the voltage setting at 515/20 nm for each run ensured comparable measurements of P2X7 function between samples. However, due to ethical restrictions and pet owner confidentiality, samples from the same dog collected at different time points was not possible. Therefore, intervariation in P2X7 function of the same dog collected at different times could not be assessed.

P2X7 function between monocytes isolated from fresh and one day-aged blood was different. In contrast, Korpi-Steiner (2008) found that the daily difference in human monocyte P2X7 function is minimal. Reasons for the differences between the two studies were not examined. However, we are confident that the variations in dog samples are not due to variations in the flow cytometer, as the instrument was calibrated before each run as explained above. Further, canine P2X7 function changes after one-day blood storage were not related to the percentage of nonviable cells as P2X7 function was similar in total CD14\(^+\) cells or CD14\(^+\)/7AAD\(^-\) (viable) cells (Fig. 5.2). Non-viable cells have been shown to affect human monocyte P2X7 function due to YO-PRO-1\(^{2+}\) uptake associated with loss of plasma membrane integrity (Korpi-Steiner et al. 2008). Thus, it can be concluded that the increasing P2X7 function of monocytes after one-day storage is not due to changes in the instrument between days nor the proportion of nonviable cells. Thus, these differences between the two studies may be due to the use of density gradient
centrifugation in the current study, as opposed to whole blood, which would remove a number of dead cells. Finally, besides the obvious species differences, the current study used heparin as the anti-coagulant and ATP as the P2X7 agonist; in contrast to the study by Korpi-Steiner et al. (2008), which used citrate and BzATP respectively. It is difficult to reconcile why the use of ATP or BzATP would produce a difference between the two studies, as both were used at supermaximal concentrations (Stevenson et al. 2009). It is more likely that monocyte P2X7 function is better preserved in citrate blood, or that P2X7 function is better maintained in humans than in dogs.

ATP-induced YO-PRO-1^{2+} uptake assay into canine monocytes was performed to measure and compare P2X7 function among dog breeds. P2X7 function varies between breeds and in some cases between dogs of the same breeds, with a 2.6-fold difference between dogs with the lowest and highest P2X7 function (Table 5.1). Moreover, no dog displayed a complete loss of function. This variation in P2X7 function in dogs is similar to that of mice, where approximate two-fold differences have been observed between different mouse strains (Adriouch et al. 2002) and no complete loss-of-function has been reported. This difference between mouse strains has been largely attributed to the Pro451Leu SNP in the C-terminus of murine P2X7 (Adriouch et al. 2002). Thus, it is possible that partial loss-of-function SNPs may also account for the variation in P2X7 function between dog breeds. In contrast to dogs and mice, P2X7 function in human is much more variable including gain-of-function SNPs (Stokes et al. 2010) and complete loss-of-function SNPs (Gu et al. 2004; Wiley et al. 2003; Gu et al. 2001). In the latter case, P2X7 function is only partially impaired in subjects heterozygous for loss-of-function SNPs. Thus, the observed variation in P2X7 function between dogs in the current study, may be the result of dogs heterozygous for gain- or loss-of-function SNPs. Therefore, establishment of a canine DNA bank
will allow for future opportunities to examine the $P2RX7$ gene sequence between individual dogs or breeds, and to determine if SNPs exist.

The current study also demonstrated that P2X7 function is greater in canine T-cells, followed by B-cells and monocytes in five different breeds (Fig. 5.5). This pattern is the same as previously observed for a sixth breed, the English Springer Spaniel (Stevenson et al. 2009). In contrast, P2X7 function is highest in human monocytes, followed by B and T cells (Gu et al. 2000). The reasons for these variations are unknown. Differences in P2X7 function between canine monocytes, and T and B cells are most likely responsible due to differences in P2X7 expression between these cells, as observed for human leukocytes (Gu et al. 2000). Differences in P2X7 function in each leukocyte population between canines and humans may also be the result of different cell sub-types within each population. For example, P2X7 function is higher in leukemic B-cells, which are predominantly CD5$^+$, compared to normal B-cells which are predominantly CD5$^-$ (Gu et al. 2000). Additionally, P2X7 function is greater on murine CD4$^+$ T regulatory cells compared to CD4$^+$ T helper cells (Schenk et al. 2011; Hubert et al. 2010; Aswad et al. 2005). Thus, it is tempting to suggest that the ratio of given lymphocytes sub-types in dogs differs to that of humans, and in particular that dogs contain a much higher population of CD4$^+$ T regulatory cells.

In conclusion, this study developed a fixed-time flow cytometry assay to measure relative P2X7 function in dogs. Moreover, P2X7 function varies among dog breeds. The establishment of a canine genomic DNA bank will be useful for the future detection of SNPs which may explain variations in P2X7 function.
Chapter 6: General Discussion

Our group is currently studying the role of canine P2X7 in inflammation and immunity. The main objectives of this group project are (i) to investigate processes downstream of P2X7 activation in canine monocytes such as interleukin (IL)-1β release; (ii) to clone the canine P2X7 receptor for future pharmacological and mutagenesis studies; (iii) to determine if polymorphic variants which alter P2X7 expression or function are present in dogs. Collectively, these objectives will provide further insight into the mammalian P2X7 receptor and its potential role as therapeutic target in dogs. The current study was part of this larger group study and in which the following outcomes were observed.

First, Madin-Darby canine kidney (MDCK) cells were found to express P2X7, TLR4, NALP3 and caspase-1, but not IL-1β and IL-18 (Chapter 2). Moreover, these cells were found to express low amounts of functional P2X7 (Chapter 2). Second, various pannexin-1 antagonists inhibited P2X7-induced IL-1β release from J774 cells but were generally unable to impair P2X7-induced uptake (Chapter 3). Third, canine P2X7 was cloned from an English Spring Spaniel and could be transfected into human 1321N1 astrocytoma cells, however whether this receptor could form functional P2X7 channels/pores was not examined (Chapter 4). Finally, a fixed-time flow cytometry assay to measure relative P2X7 function in dogs was developed and this assay showed variation in P2X7 function between dogs and dog breeds (Chapter 5). Moreover, a canine genomic DNA bank was established (Chapter 5), which will be used for the future direction of single nucleotide polymorphisms (SNPs) which may explain variations in P2X7 function.

The current study aimed to determine the presence or absence of P2X7 in MDCK cells (Chapter 2) for potential use for transfection studies with the cloned canine P2X7 (Chapter 4). The
detection of P2X7 in MDCK cells prevents their use in transfection studies, however these cells provide a useful future model to study P2X7 in canine epithelial cells. The detection of NALP3 and caspase-1, but not IL-1β and IL-18 provides future opportunities to study the role of P2X7 in NALP3 inflammasome activation independent of cytokine release. There is emerging evidence that inflammasomes and caspases are involved in effector mechanisms independently of IL-1β and IL-18 (Lamkanfi, 2011). The presence of NALP3 and caspase-1 in MDCK cells, however this needs to be confirmed at the protein level.

Lipopolysaccharide (LPS)-primed canine monocytes were used as a positive control for the MDCK cell study, and were found to express NALP3, caspase-1, IL-1β and IL-18 mRNA (Chapter 2). This supports our group’s future plan to determine if P2X7 activation can induce the release of IL-1β and IL-18 from canine monocytes. However, the presence of these molecules also needs to be confirmed at the protein level. As a starting point for future IL-1β release assays, the ability of P2X7 activation to induce IL-1β release from J774 macrophages was examined (Chapter 3). Moreover, a role for pannexin-1 in this process was investigated in these cells (Chapter 3), to potentially examine a role for pannexin-1 in P2X7-induced cytokine release from canine monocytes. Although a role for pannexin-1 in inflammasome activation has been recently questioned (Qu et al. 2011), the inhibition of P2X7-induced IL-1β release from J774 macrophages by probenicid (Chapter 3) may be of future interest to canine health. Probenecid, as an inhibitor of renal transport, has been used for decades to treat gout (Silverman et al. 2008), but recent data (Lamkanfi et al. 2009), as well as data in the current study (Chapter 3) indicate an additional role for this drug in impairing IL-1β release. Therefore, it will be of future importance
to determine if P2X7 activation can induce IL-1β release from canine monocytes, and whether this process is impaired by probenecid.

The current study used two P2X7 antagonists, KN-62 and A-438079, to confirm the presence of function P2X7 in canine and murine cells (Chapters 2 and 3). KN-62 has been previously reported to block recombinant and native canine and murine P2X7 (Donnelly-Roberts et al. 2009; Roman et al. 2009; Stevenson et al. 2009). To my knowledge the use of A-438079 has not been used to impair canine P2X7, however, it has been previously shown to impair human, mouse and rat P2X7 (Donnelly-Roberts et al. 2009). Moreover, results from this study indicate that A-438079 may be a more effective inhibitor of canine P2X7 than KN-62. However, it should be noted that it was used at a 10-fold higher concentration, thus a direct comparison cannot be fully made. Establishment of the cloned P2X7 receptor, will assist in the future pharmacological characterization of this receptor to various P2X7 agonists and antagonists indicating KN-62 and A-438079.

P2X7 has been cloned from many mammalian species (Roman et al. 2009; Fonfria et al. 2008; Chessell et al. 1998; Surprenant et al. 1996). Among these cloned mammalian P2X7 receptors, canine P2X7 has the closest homology to human P2X7 (Roman et al. 2009). P2X7 has been cloned from a Beagle (Roman et al. 2009), however the cloning of another P2X7 from a different breed seemed important for future pharmacological and functional studies in dogs. Moreover, our group has previously obtained a range of pharmacological data on P2X7 on leukocytes and erythrocytes from English Springer Spaniels (Stevenson et al. 2009; Sluyter et al. 2007). Therefore, canine P2X7 was cloned from an English Spring Spaniel for the first time (Chapter 4). The expression of this cloned receptor in transfected 1321N1 cells was low. However, these
cells may still be a suitable candidate for transfection with this cloned P2X7, but optimization of
the transfection technique is warranted.

MDCK cells have been previously transfected with cloned P2X7 to study the structure,
expression and function of this receptor (Zhou et al. 2008; Feng et al. 2006). However, because
these cells express functional endogenous P2X7 (Chapter 2) these former studies should be
interpreted with caution. Nevertheless, more functional and structural analysis should be
performed for better understanding of endogenous P2X7 canine in epithelial cells. KN-62 and A-
438079, which are the specific P2X7 antagonists, were used for measuring the pharmacological
properties of P2X7 in MDCK, mouse J774 macrophages and canine monocytes. KN-62 could
inhibit ATP-induced ethidium$^+$ uptake into MDCK cells significantly which is consistent with
pharmacological properties of cloned canine P2X7 performed by Roman et al. (2009). However,
KN-62 failed to significantly block P2X7 in canine monocytes although it inhibited ATP-
induced ethidium$^+$ uptake by 75%. In contrast, A-438079 could significantly inhibit ATP-
induced ethidium$^+$ uptake in MDCK, J774 and canine monocytes. Further investigation of P2X7
pharmacological properties can be performed on the cloned canine P2X7 and on monocytes for
better comparison between recombinant P2X7 and endogenous P2X7.

The relative P2X7 function varies among between dogs and breeds (Chapter 5). Future studies
are planned to sequence the P2RX7 gene from the samples within the established canine
genomic DNA bank (Chapter 5). The sequences will then be compared to the P2X7 function data
in an attempt to find SNPs which potentially alter P2X7 function. Those SNPs will then be
introduced into the cloned P2X7 through site-directed mutagenesis and the mutant receptor
transfected into mammalian cells for further investigation of P2X7 expression and function.
In conclusion, this study was a small part of a larger study investigating the role of the canine P2X7 receptor in health and disease. Having access to canine monocytes and DNA, as well as cloned P2X7 will provide future opportunities to study canine P2X7.
References


APPENDIX

Agarose Gel Electrophoresis

1 X TAE Buffer
Tris-base 40 mM
Glacial Acetic Acid 20 mM
EDTA (pH 8.0) 1 mM

TE Buffer
Tris-HCl 10 mM
EDTA (pH 8.0) 1 mM

0.86% TAE Agarose Gel
Agarose 0.86% (w/v)
40 mM Tris
1 mM EDTA
Glacial Acetic Acid 0.142% (v/v)

2% TAE Agarose Gel
Agarose 2% (w/v)
40 mM Tris
1 mM EDTA
Glacial Acetic Acid 0.142% (v/v)

Agarose Loading Buffer
Bromophenol blue 0.005% (w/v)
Glycerol 75% (v/v)
TE Buffer 25% (v/v)

Ethidium Bromide Solution
Ethidium Bromide 1μg/mL in dH₂O

SDS-PAGE Gel Electrophoresis

2 X Cracking Buffer
Tris-HCl 90 mM
Bromophenol blue 0.02% (w/v)
Glycerol 20% (v/v)
SDS 2% (w/v)
β-mercaptoethanol 1% (v/v)

10% Resolving Gel
Bis-acrylamide (37.5:1) 10% (w/v)
Tris-HCl 1.5 M (pH 8.8) 25% (v/v)  
SDS 0.1% (w/v)  
TEMED (add last) 0.05% (v/v)  
Ammonium Persulphate (add last) 0.0015% (w/v)  

4% Stacking Gel  
Bis-acrylamide (37.5:1) 4% (w/v)  
Tris-HCl 1.5 M (pH 6.8) 25% (v/v)  
SDS 0.1% (w/v)  
TEMED (add last) 0.05% (v/v)  
Ammonium Persulphate (add last) 0.0015% (w/v)  

1 X SDS-Gel Running Buffer  
Tris-base 50 mM  
Glycine 196 mM  
SDS 0.1% (w/v)  
pH 8.3  

Western Blot Analysis  

Western Transfer Buffer  
Methanol 20% (v/v)  
Tris base 23 mM  
Glycine 192 mM  
SDS 0.001% (v/v)  

PBS  
NaCl 137 mM  
Na₂HPO₄ 4.3 mM  
KCl 2.7 mM  
KH₂PO₄ 1.4 mM  
pH 7.4  

TBS  
Tris.Cl 10 mM  
NaCl 350 mM  
pH 7.4  

TBST  
Tris.Cl 20 mM  
NaCl 500 mM  
Tween-20 0.05% (v/v)
**Blocking Buffer**
Skim milk powder 5% (w/v)
Made up in TBST

**Growth and Nutrient Media**

**LB Broth**
Bactotryptone 10% (w/v)
Yeast Extract 5% (w/v)
NaCl 10% (w/v)

**LB Agar**
Bactotryptone 10% (w/v)
Yeast Extract 5% (w/v)
NaCl 10% (w/v)
Agar 15% (w/v)

**QIAGEN-100 Tip Column Buffer**

**Buffer P1 (resuspension buffer)**
Tris-HCl 50 mM (pH 8.0)
EDTA 10 mM
RNase A 100 µg/mL

**Buffer P2 (lysis buffer)**
NaOH 200 mM
SDS 1% (w/v)

**Buffer P3 (neutralization buffer)**
Potassium Acetate 3.0 M (pH 5.5)

**Buffer QBT (Equilibration buffer)**
NaCl 700 mM
MOPS 50 mM (pH 7.0)
Isopropanol 15% (v/v)
Triton® X-100 0.15% (v/v)

**Buffer QC (wash buffer)**
NaCl 1.0 M
MOPS 50 mM (pH 7.0)
Isopropanol 15% (v/v)
Buffer QF (elution buffer)
NaCl 1.25 M
Tris.Cl 50 mM (pH 8.5)
Iopropanol 15% (v/v)

Wizard® Plus SV Minipreps DNA Purification

Cell Resuspension Solution
Tris-HCl 50 mM (pH 7.5)
EDTA 10 mM
RNase A 100 μg/mL

Cell Lysis Solution
NaOH 0.2 M
SDS 1% (w/v)

Neutralisation Solution
Guanidine Hydrochloride 4.09 M
Potassium Acetate 0.759 M
Glacial Acetic Acid 2.12 M

Column Wash Solution
Aotassium Acetate 60 mM
Tris-HCl 8.3 mM (pH 7.5)
EDTA 0.04 mM (pH 8.0)
Ethanol 60% (v/v)

Wizard® SV Gel and PCR Clean-up Systems

Membrane Wash Solution
Potassium acetate 10 mM (pH 5.0)
Ethanol 80%
EDTA 16.7 μM (pH 8.0)

Membrane Binding Solution
Guanidine isothiocyanate 4.5 M
Potassium acetate 0.5 M (pH 5.0)

1 X TE Buffer
Tris-HCl 10 mM (pH 7.5)
EDTA 1mM (pH 8.0)
1 X TBE Buffer
Tris base 89 mM
Boric acid 89 mM
EDTA 2 mM (pH 8.0)

1 X TAE Buffer
Tris base 40 mM
Sodium acetate 5 mM
EDTA 1 mM (pH 8.0)