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The P2X7 purinergic receptor in dogs

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School of Biological Sciences
Illawarra Health and Medical Research Institute

The P2X7 Purinergic Receptor in Dogs

Mari Spildrejorde

This thesis is presented as part of the requirement for the award of the Degree of Master of Science (by Research) of the University of Wollongong

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Disclaimer

This thesis is submitted in accordance with the regulations of the University of Wollongong in partial fulfilment of the degree of Master of Science (by Research). The thesis does not contain material that has been previously published or written by another person, unless otherwise referenced or acknowledged. The experimental work described in this thesis is my own, except where indicated, and has not been submitted for a degree to any other University.

Mari Spildrejorde
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Publications and Presentations Arising from this Thesis


Abbreviations

2-meSATP  2-methylthio-ATP
5’-NT   ecto-5’-nucleotidase
α,β-meATP  α,β-methylene ATP
ADP   adenosine 5’-diphosphate
AMP   adenosine 5’-monophosphate
AK   ecto-adenylate kinase
AP   alkaline phosphatase
APC   allophycocyanin
ASC   apoptosis-associated speck-like protein containing a C-terminal CARD
ATP   adenosine 5’-triphosphate
ATPγS   adenosine 5’-O-(3-thiotriphosphate)
BBG   brilliant blue G
Bp   base pair(s)
BSA   bovine serum albumin
BzATP   2,3-O-(4-benzoylbenzoyl)-ATP
cAMP   cyclic adenosine 5’-monophosphate
CARD   caspase recruitment domain
CV   coefficient of variation
EC_{50}   half-maximal effective concentration
EDTA   ethylenediaminetetraacetic acid
ELISA   enzyme-linked immunosorbent assay
E-NPP   ecto-nucleotide pyrophosphatase/phosphodiesterase
E-NTPDase   ecto-nucleoside triphosphate diphosphohydrolase
FCS   foetal calf serum
HEK 293 cells   human embryonic kidney 293 cells
HRP   horseradish peroxidase
IC\textsubscript{50}  
half-maximal inhibitory concentration

IFN-\textgreek{y}  
interferon-\textgreek{y}

IL  
interleukin

IPAF  
ICE-protease activating factors

kDa  
kiloDalton

LPS  
lipopolysaccharide

LRR  
leucine-rich repeats

LTA  
lipoteichoic acid

mAb  
monoclonal antibody

MDCK  
Madin-Darby canine kidney

MFI  
mean fluorescence intensity

min  
minute(s)

MW  
molecular weight

NALP  
NACHT-LRR-PYD-containing protein

NCBI  
National Center for Biotechnology Information

NDPK  
nucleotide diphosphate kinase

NLR  
NOD-like receptors

NMDG  
N-methyl-D-glutamine

pAb  
polyclonal antibody

PBMC  
peripheral blood mononuclear cells

PBS  
phosphate buffered saline

PCR  
polymerase chain reaction

PMSF  
Phenylmethylsulfonyl fluoride

PPADS  
Pyridoxal phosphate-6-azophenyl-2-4-disulphonic acid

PRR  
pattern recognition receptors

PYD  
pyrin domain

ROS  
reactive oxygen species

RT-PCR  
reverse Transcripntase polymerase chain reaction

SD  
standard deviation
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>sec</td>
<td>second(s)</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate-ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline tween-20</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine 5’-diphosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5’-triphosphate</td>
</tr>
</tbody>
</table>
Abstract

The P2X7 receptor is an adenosine 5'-triphosphate (ATP)-gated ion channel expressed on the cell-surface of many cell types, including cells of haematopoietic origin and epithelial cells. Upon prolonged activation by extracellular ATP, P2X7 has the ability to form pores permeable to large organic cations such as ethidium$^+$ and YO-PRO-1$^{2+}$. P2X7 activation leads to the stimulation of the NALP3 inflammasome and the subsequent release of the pro-inflammatory cytokines interleukin (IL)-1$\beta$ and IL-18.

The first aim of this study was to confirm the presence or absence of P2X7 and NALP3-related components in Madin-Darby canine kidney (MDCK) epithelial cells and lipopolysaccharide (LPS)-primed canine monocytes. Reverse transcriptase (RT)-PCR detected the presence of P2X7, NALP3, caspase-1, IL-1$\beta$ and IL-18 mRNA in MDCK cells and monocytes. The identity of each transcript, except IL-1$\beta$ due to its small size, was confirmed by sequencing. This data suggests that MDCK cells will provide a useful model cell line to study the role of P2X7 and NALP3-related components in kidney epithelial cells and renal disorders. Moreover, these results indirectly support our previous observations that P2X7 activation induces IL-1$\beta$ from LPS-primed canine monocytes.

The second aim of this study was to determine if ATP or the Toll-like receptor (TLR) ligands, LPS or lipoteichoic acid (LTA), induce IL-1$\beta$ release from MDCK cells and in whole canine blood. ELISA measurements failed to detect IL-1$\beta$ release from MDCK cells despite examining various combinations of ATP, LPS and/or nigericin (which induces IL-1$\beta$ release independently of P2X7). Further
studies are required however to determine whether MDCK cells can release IL-1β. In contrast to MDCK cells, LPS and ATP but not LTA induced IL-1β release in whole blood. Moreover, ATP-induced IL-1β release in whole blood required priming with LPS. A role for P2X7 in LPS- and ATP-induced IL-1β release in blood however could not be established, as the P2X7 antagonist AZ10606120 failed to impair IL-1β release. Further studies are required to determine if either or both of these processes require P2X7 activation.

The third aim of this study was to sequence and pharmacologically characterise a recombinant canine P2X7 receptor cloned from an English Springer Spaniel. Sequencing of the cloned receptor revealed two non-synonymous (missense) single nucleotide polymorphisms (SNPs): Leu440Phe and Pro452Ser. Immunoblotting confirmed the expression of P2X7 protein in canine P2X7-transfected HEK 293 cells. Moreover, flow cytometric measurements demonstrated that ATP induced ethidium+ (314 Da) uptake into P2X7-transfected but not mock-transfected HEK 293 cells in a time-dependent manner. The P2X7 agonists ATP, 3’-O-(4-benzoyl)benzoyl ATP (BzATP) and adenosine 5’-O-(3-thiotriphosphate) (ATPγS) induced ethidium+ uptake into P2X7-transfected HEK 293 cells in a concentration-dependent manner with EC50 values of 253 µM, 13 µM and 438 µM respectively. In contrast, adenosine 5’-diphosphate (ADP), uridine 5’-triphosphate (UTP) and α,β-methylene ATP (α,β-meATP) failed to induce ethidium+ uptake into these cells. The P2X7 antagonists A438079, AZ10606120, AZ11645373, BBG and KN-62 completely impaired ATP-induced ethidium+ uptake into P2X7-transfected HEK 293 cells with IC50 values of 190 nM, 11 nM, 7 nM, 1110 nM and 19 nM respectively. Finally, ATP induced YO-PRO-12+ (375 Da) and propidium2+ (415 Da) uptake
into P2X7-transfected cells. Collectively, the recombinant canine P2X7 receptor demonstrated similar characteristics to that of native canine and recombinant human P2X7. Thus, P2X7 drugs developed to block P2X7 in humans may also be of therapeutic value in dogs.

The last aim of this thesis was to confirm if the relative monocyte P2X7 function varies between dogs and to determine if this variation is due to SNPs in the \textit{P2RX7} gene. Flow cytometric measurements of ATP-induced YO-PRO-1\textsuperscript{2+} uptake into peripheral blood canine monocytes confirmed that the relative P2X7 function varied between dogs and within breeds. Flow cytometric measurements also demonstrated that the relative P2X7 function of human monocytes varies between subjects and over time. Amplification and sequencing of the canine \textit{P2RX7} gene of 19 dogs and MDCK cells identified four non-synonymous SNPs: Phe103Leu (exon 3), Arg270Cys (exon 8), Arg365Gln (exon 11), and Pro452Ser (exon 13). The dogs for which we had both functional data and genomic DNA (n = 62) were screened for the above SNPs and the resulting genotypes were compared to relative P2X7 function. Three of the SNPs (Phe103Leu, Arg365Gln and Pro452Ser) did not correspond with a change in P2X7 function. In contrast, the Arg270Cys SNP was associated with a loss-of-function in P2X7, however this finding was restricted to one dog, as well as MDCK cells which have low P2X7 function. Future studies using mutant P2X7 receptors, obtained by site-directed mutagenesis, are required to explore if any of these SNPs alter P2X7 function.

This thesis forms a part of an ongoing study investigating the role of canine P2X7 in inflammation and immunity. The confirmation of P2X7 in canine
monocytes and MDCK cells, and the identification of SNPs in the canine \textit{P2RX7} gene will provide future opportunities to investigate the role of the P2X7 receptor and its gene in canine health and disease.
Chapter 1: Introduction

1.1 Purinergic signalling

Extracellular purines and pyrimidines such as adenosine, adenosine 5’-triphosphate (ATP), adenosine 5’-diphosphate (ADP) and uridine 5’-triphosphate (UTP) act as signalling molecules through cell surface-bound purinoreceptors. These receptors are widely distributed throughout the body and mediate a wide range of biological responses upon activation (Ralevic and Burnstock, 1998). In 1978, Burnstock distinguished between two major subtypes of purinoreceptors; P1 and P2 receptors, which are selective for adenosine, or ATP and ADP respectively (Table 1.1) (Burnstock, 1978). P2 receptors have been further divided into two families consisting of ligand-gated ion channels (P2X) and G protein-coupled receptors (P2Y) (Burnstock and Kennedy, 1985).

Table 1.1: Purinoreceptor families

<table>
<thead>
<tr>
<th></th>
<th>P1 receptors</th>
<th>P2X receptors</th>
<th>P2Y receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Natural ligands</strong></td>
<td>Adenosine</td>
<td>ATP, ADP</td>
<td>ATP, ADP, UTP, Adenine dinucleotides</td>
</tr>
<tr>
<td><strong>Type</strong></td>
<td>G protein-coupled</td>
<td>Ion channel, Cation-selective pore*</td>
<td>G protein-coupled</td>
</tr>
<tr>
<td><strong>Subtypes</strong></td>
<td>A1, A2A, A2B, A3</td>
<td>P2X1-7</td>
<td>P2Y1,2,4,6,11,12,13,14</td>
</tr>
</tbody>
</table>

* P2X2,4,5 and 7 receptors. Adapted from Ralevic and Burnstock (1998).

1.1.1 The release of intracellular ATP

ATP is a purine nucleoside triphosphate, which is synthesized during oxidative
phosphorylation. It is utilized as a source of chemical energy available for all living cells, as well as an extracellular signalling molecule acting on purinoreceptors (Bodin and Burnstock, 2001). ATP can be released by cytolysis from damaged and dying cells in response to mechanical distortion, osmotic swelling, hypoxia, inflammatory conditions or to stimulation by some agents (Burnstock, 2007). Alternatively, ATP can be released as a physiological mechanism from healthy cells. The transport mechanism by which ATP is released from these cells is still being debated (Burnstock, 2007, Abbracchio et al., 2009). Exocytic vesicular release of ATP have been demonstrated in both neuronal cells, where ATP functions as a classic neurotransmitter, and in non-neuronal cells such as osteoblasts, epithelial and endothelial cells (Bodin and Burnstock, 2001). Other proposed mechanisms of ATP release include facilitated diffusion by nucleotide ATP-binding cassette transporters, electrodiffusional movement through ion channels such as connexin or pannexin hemi-channels, plasmalemmal voltage-dependent anion channels and P2X7 receptors (Burnstock and Verkhratsky, 2012). ATP can also be released from microbial flora and pathogens (Koshlukova et al., 1999), providing an alternative source of extracellular ATP other than that released from the host.

1.1.2 Regulation of extracellular ATP

The concentrations of extracellular ATP are closely regulated by a number of enzymes termed ectonucleotidases, which have their catalytic site on the extracellular side of the cell membrane (Novak, 2003). After release, ATP is
rapidly degraded to ADP, adenosine 5’-monophosphate (AMP) or adenosine. These nucleotides are ligands for a range of different purinoreceptors and thus can mediate a variety of physiological responses. The enzymes responsible for this degradation include ecto-nucleoside triphosphate diphosphohydrolases, ecto-nucleotide pyrophosphatase/phosphodiesterases, ecto-5’-nucleotidases and alkaline phosphatases (Yegutkin, 2008). Ecto-nucleoside triphosphate diphosphohydrolases and ecto-nucleotide pyrophosphatase/phosphodiesterases hydrolyse ATP and ADP to AMP (Fig. 1.1), and ecto-5’-nucleotidases further hydrolyze AMP to adenosine (Fig. 1.1). Alkaline phosphatases equally hydrolyze tri-, di- and monophosphates (Abbracchio et al., 2009). ATP can also be re-synthesized from AMP via ADP via backward ecto-phosphotransfer reactions by nucleotide diphosphate kinases and ecto-adenylate kinases (Fig. 1.1) (Yegutkin et al., 2002).

**Figure 1.1 Mechanisms of ATP degradation and re-synthesis on the cell surface.** Extracellular adenosine 5’-triphosphate (ATP) can be degraded to adenosine 5’-diphosphate (ADP), adenosine 5’-monophosphate (AMP) and adenosine by ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDase), ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPP), ecto-5’-nucleotidases (5’-NT) and alkaline phosphatases (not shown). An opposite ATP-synthesising pathway is mediated by nucleotide diphosphate kinases (NDPK) and ecto-adenylate kinases (AK).
1.2 The P1 receptor family

P1 receptors are G-protein coupled cell surface receptors that are sensitive to adenosine (Ralevic and Burnstock, 1998). Four subtypes (A1, A2A, A2B and A3) have been described on the basis of their molecular structures, tissue distribution, biochemical and pharmacological properties. These receptors range in size from 266 to 412 amino acids with a mass of 30 to 45 kDa after glycosylation. P1 receptors, similar to other G-protein coupled receptors, have seven transmembrane domains, with the N-terminus on the extracellular side and the C-terminus on the cytosolic side (Ralevic and Burnstock, 1998). A1 and A3 subtypes primarily interact with Gi proteins to inhibit adenylyl cyclase and modulate ion channels. In addition, A1 receptor activation has been linked to various kinase pathways such as phosphoinositide 3 and mitogen-activated protein kinases (Fredholm et al., 2000). A2A and A2B subtypes are predominantly coupled with Gs proteins to cause an increase in intracellular cAMP (Brown et al., 2008).

P1 receptors are widely expressed on cells throughout the body, including neutrophils, mast cells, monocytes, macrophages, dendritic cells and lymphocytes (Brown et al., 2008). Their physiological role varies between tissues and includes reduction of neuronal activity (A1) (Ralevic and Burnstock, 1998), and downregulation of the production of pro-inflammatory cytokines and free radicals (A2A) during ischaemia (Hasko et al., 2008). P1 receptors are also involved in activation of mast cells (A2B), release of allergic mediators (A3), vasoconstriction, inhibition of renin secretion, inhibition of
neurotransmitter release in the kidney (A1), and vasodilation in smooth muscle and endothelial cells (A2B) (Ralevic and Burnstock, 1998).

1.3 The P2Y receptor family

P2Y receptors are G-protein coupled cell surface receptors that are sensitive to ATP, ADP, UTP, uridine 5’-diphosphate (UDP) and nucleotide sugars (Boeynaems et al., 2012). These receptors range in size from 308 to 377 amino acids with a mass of 41 to 53 kDa after glycosylation (Ralevic and Burnstock, 1998). All P2Y receptors have seven transmembrane domains with extracellular N-terminus and intracellular C-terminus (Abbracchio et al., 2006). The P2Y family consist of eight members (P2Y1,2,4,6,11,12,13 and 14) which are further subdivided into two groups based on structural features (protein sequence) and their binding to specific G-proteins. The P2Y1,2,4,6,11 subgroup is mainly coupled to G_q proteins to activate the phospholipase C inositol triphosphate signalling pathway to cause the release of Ca^{2+}, while the P2Y12,13,14 subgroup predominantly couple to G_i proteins to inhibit adenylyl cyclase and modulate ion channels (Abbracchio et al., 2009).

P2Y receptors are widely distributed throughout the body, with one or more subtype found virtually on all cells. This includes nerve, glial, muscle cells and immune cells such as eosinophils, mast cells, monocytes, macrophages, dendritic cells and lymphocytes. P2Y receptors can also be detected in endocrine, adipose, exocrine, gut, liver, kidney, bladder, lung, bone, epithelial and endothelial cells and platelets (Abbracchio et al., 2006). Known functions of P2Y receptors include various inflammatory responses such as chemotaxis
(P2Y2 and 12), phagocytosis (P2Y6), platelet aggregation (P2Y1 and 12), epithelial hydration (P2Y2 and 4) and bone formation and resorption (P2Y2, 6 and 13) (Boeynaems et al., 2012).

1.4 The P2X receptor family

P2X receptors are ATP-gated cation channels permeable to Na\(^+\), Ca\(^{2+}\) and K\(^+\) upon activation. Seven genes encoding for P2X subunits (P2X1-7) have been identified, with subunit proteins varying in size from 379 (P2X6) to 595 (P2X7) amino acids (Khakh et al., 2001). Functional receptors are formed as either homomeric (P2X1-5 and P2X7) or heteromeric (P2X1/2, P2X1/5, P2X2/3, P2X2/6 and P2X4/6) trimers (Abbracchio et al., 2009). All P2X receptor subunits have consensus sequences for N-linked glycosylation, some of which are required for trafficking to the cell surface (North, 2002).

The P2X receptors are widely but specifically distributed throughout the body. P2X receptors can be found on neurons, microglia, endothelial cells, colon, heart, liver, lung, kidney, endocrine glands, osteoblasts, skeletal muscle, fibroblasts and blood cells, including erythrocytes, platelets, and leukocytes (Burnstock et al., 2010). The physiological functions of P2X receptors include fast and slow neurotransmission at central synapses (P2X2, 4 and 6), initiation of sensory signalling pathways, such as taste, chemoreception and neuropathic pain (P2X2, 3, 4 and 7), control of blood flow by contraction or relaxation of smooth muscle cells (P2X1, 4 and 7), regulation and secretion of exocrine hormones (P2X4 and 7), intestinal motility (P2X2 and 3), regulation of proinflammatory mediators, cell proliferation and cell death (P2X7).
1.4.1 General structure of P2X receptors

Non-denaturing protein electrophoresis first showed that P2X receptors form stable oligomers of three subunits (Nicke et al., 1998). This trimeric structure was confirmed by crystallography of the P2X4 receptor in the zebrafish (Fig 1.2) (Kawate et al., 2009). Each P2X receptor subunit is comprised of two hydrophobic transmembrane domains, leaving most of the protein outside of the cell as a ~280 amino acid hydrophilic loop, and the N- and C-terminus on the cytoplasmic side (North, 2002, Ralevic and Burnstock, 1998). The ATP-binding site and sites for antagonists and modulators are found in the ectodomain (Khakh et al., 2001). Eight highly conserved residues (Lys69, Lys71, Phe183 and Thr184 from one subunit, and Asn288, Phe289, Arg290 and Lys308 from another subunit; numbering taken from rat P2X2) that are critical for ATP sensitivity have been identified by mutagenesis studies. These contribute to a positively charged pocket large enough to accommodate ATP (Browne, 2012). Recently, a crystal structure of the zebrafish P2X4 receptor in complex with ATP confirmed that ATP-binding sites are found at each of the three subunit interfaces (Hattori and Gouaux, 2012). The binding site for P2X antagonists appears to be antagonist- and receptor-specific. For example, in the case of P2X1 receptor sensitivity to suramin, the Lys138 residue is critical (Sim et al., 2008). Conversely, for the human P2X7 receptor, antagonism by GW791343, KN-62 and AZ11645373 is dependent on the Phe95 residue
Thus, the identification of residues involved in antagonist-binding is crucial for the development for potent and selective P2X receptor antagonists.

**Figure 1.2: The structure of P2X receptors.** (A,B) The crystal structure of the P2X4 receptor in zebrafish demonstrate that one P2X receptor consists of three subunits. The different subunits are shown in different colour, (A) viewed parallel to the membrane plane and (B) from the extracellular side. Adapted from Kawate et al. (2009).

### 1.5 The P2X7 receptor

Similarly to other P2X receptors, P2X7 exhibits ion channel activity upon activation by extracellular ATP, which allows the rapid flux of Na\(^+\), Ca\(^{2+}\) and K\(^+\) across the cell membrane. However, after sustained stimulation by ATP, P2X7 has the ability to form pores permeable to organic cations. This leads to a
range of inflammatory and other responses. P2X7 have been linked to a number of disorders, and thus is attracting attention as a therapeutic target.

1.5.1 Cloning of the P2X7 receptor

P2X7 was first named P2Z but was identified as a P2X receptor when cloned from rat brain macrophages by Surprenant et al. (1996). Subsequently, the P2X7 receptor was cloned from different tissues of various species including human monocytes (Rassendren et al., 1997), mouse microglia (Chessell et al., 1998), Xenopus Laevis stomach (Paukert et al., 2002), guinea pig brain (Fonfria et al., 2008) and finally from dog heart (Roman et al., 2009). The sequence similarities between the P2X7 receptors varies from 43-86%, with the canine P2X7 receptor having the highest sequence identity compared to the human P2X7 receptor (86%) (Fig. 1.3; Table 1.2). Similar to human, rat and mouse orthologues, the canine receptor contains 595 amino acids. Of note however, compared to human and rodent P2X7, canine P2X7 contains an additional residue in the extracellular loop (Thr284) and has a deletion of Val538 in the C-terminus (Roman et al., 2009). Compared to the reference sequence from National Center for Biotechnology Information (NCBI; accession no: NM_001113456.1) deduced from a Boxer (Lindblad-Toh et al., 2005), the canine P2X7 receptor cloned from a Beagle contained one non-synonymous single nucleotide polymorphism (SNP) in position 103 (Phe103Leu) (Roman et al., 2009) (Fig. 1.3).
Figure 1.3: ClustalW alignment of the amino acid sequence from the NCBI human P2X7 reference sequence (accession no: Q99572), canine P2X7 reference sequence (accession no: NM_001113456.1) and canine P2X7 cDNA cloned by Roman et al. (2009).
Table 1.2: Percent sequence identity between human, rat, mouse, guinea pig, dog and X. Laevis P2X7 receptors.

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Rat</th>
<th>Mouse</th>
<th>Guinea pig</th>
<th>Dog</th>
<th>X. Laevis</th>
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<tbody>
<tr>
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<td>81</td>
<td>77</td>
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<tr>
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<td>100</td>
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<td></td>
<td>43</td>
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<tr>
<td>X. Laevis</td>
<td></td>
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<td>100</td>
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</tbody>
</table>

1.5.2 The structure of P2X7 receptors

Crystallography studies have not been performed for P2X7, although the P2X4 structure together with alternative structure prediction indicate that the P2X7 subunit is structurally similar to other P2X receptors (section 1.4.1). P2X7 contains two transmembrane domains, and the N- and C-terminal domains on the intracellular side (Fig. 1.4) (Mager et al., 2006). The extracellular loop separating the two transmembrane domains contains 10 cysteine residues that form disulfide bonds and six-stranded β-pleated sheets (Freist et al., 1998). The main distinctive feature of P2X7 compared to other P2X receptors is the long intracellular C-terminal domain (244 amino acids), of which at least 177 amino acids are essential for the pore-formation (Surprenant et al., 1996b). Functional heterotrimeric P2X4/7 receptors have been observed (Guo et al., 2007), however homotrimeric P2X7 receptors appears to be the dominant assembly state of native P2X7 subunits (Nicke, 2008).
Figure 1.4: The schematic structure of a single P2X7 subunit. The P2X7 receptor subunit contains two transmembrane domains separated by an extracellular loop. The N-terminal and the long C-terminal domain are found intracellularly. Adapted from Wiley et al. (2011).

1.5.3 The distribution of P2X7 receptors

P2X7 is expressed in various cell types throughout the body. The highest levels of expression are found predominantly on cells of hematopoietic origin such as macrophages, dendritic cells, monocytes, natural killer cells, lymphocytes and erythrocytes. P2X7 is also present on mast cells, eosinophils, various epithelial and endothelial cells, fibroblasts and exocrine glands. In the nervous system, P2X7 is expressed on microglia, astrocytes, oligodendrocytes, ependymal cells,
Schwann cells, satellite cells and some neurons (Kaczmarek-Hájek et al., 2012, Wiley et al., 2011, Burnstock and Knight, 2004).

1.5.4 Activation of P2X7

Activation of P2X7 by extracellular ATP causes the opening of a non-selective cation channel within milliseconds, which allows the influx of Na\(^+\) and Ca\(^{2+}\), and the efflux of cellular K\(^+\) (Fig. 1.4) (Wiley et al., 2011). Compared to other P2X receptors, P2X7 require 10-100 times higher concentrations of extracellular ATP for activation (Jarvis and Khakh, 2009). Prolonged stimulation with ATP results in the slow development over tens of seconds of a reversible secondary non-selective pore permeable to large organic molecules with molecular weight up to 900 Da, including choline\(^+\) (104 Da), N-methyl-D-glutamine (NMDG; 195 Da) and fluorescent dyes such as ethidium\(^+\) (314 Da), YO-PRO\(^{2+}\) (375 Da), propidium\(^{2+}\) (415 Da) and lucifer yellow\(^{2-}\) (443 Da). This property has also been observed for P2X2, P2X4 and P2X5 receptors (Virginio et al., 1999b, Khakh et al., 1999, Bo et al., 2003), thus the ability to form pores is not unique to P2X7. Nevertheless the P2X7-induced uptake of dyes allows for the quantitative measurement of receptor function by fluorescent detection techniques such as flow cytometry (Jursik et al., 2007).

There has been much controversy regarding the mechanism of the P2X7 pore-formation (for review see Pelegrin, 2011). It has been suggested that the pore is a result of dilation of the intrinsic P2X7 channel itself (Jiang et al., 2005, Yan et al., 2010, Virginio et al., 1999a), however growing evidence suggest that P2X7-activation induces the activation of a second pore protein (directly or
indirectly via second messengers), such as the hemichannel pannexin-1 (Fig. 1.5) (Pelegrin and Surprenant, 2006, Iglesias et al., 2008, Locovei et al., 2007). Recently, however, a pannexin-1 knockout mice study demonstrated that pannexin-1 is not required for P2X7-mediated pore-formation (Qu et al., 2011). Although other evidence suggests that P2X7-activated pannexin-1 mediates YO-PRO\textsuperscript{2+} uptake into cells by releasing cellular ATP which then stimulates P2X7 thus providing a positive feedback loop (Qu et al., 2011, Dahl and Keane, 2012). Moreover, P2X7-activation has recently been shown to induce at least two selective permeation pathways that may involve an exchanger or transporter which mediates dye uptake instead of or in addition to a pore protein (Fig. 1.5) (Cankurtaran-Sayar et al., 2009, Marques-da-Silva et al., 2011, Schachter et al., 2008). In macrophages, but not in human embryonic kidney cells, separate pathways for cation and anion uptake were demonstrated (Cankurtaran-Sayar et al., 2009, Schachter et al., 2008). However, the true mechanism of the P2X7 pore formation remains unidentified.
Figure 1.5: Schematic representation of ATP-induced activation of P2X7. Activation of P2X7 by extracellular ATP causes the opening of a non-selective cation channel within milliseconds. Prolonged exposure to ATP results in the opening of a pore, by which the mechanism is controversial. The current model involves a dilation of the P2X7 channel itself, leaving it permeable to some larger molecules, such as N-methyl-D-glutamine (NMDG). In addition, at least one secondary pore protein is thought to be involved. Adapted from Pelegrin (2011).

1.5.5 Signalling events downstream of P2X7 activation

Activation of P2X7 mediates a range of downstream signalling pathways. A central role for the receptor in inflammatory responses has been established. P2X7-activation, together with a co-stimulus such as activation of Toll-like receptors (TLRs) by the bacterial products lipopolysaccharide (LPS) or lipoteichoic acid (LTA), leads to the upregulation and activation of the NALP3 inflammasome and the subsequent release the pro-inflammatory cytokines interleukin (IL)-1\(\beta\) and IL-18 (discussed further in section 1.7.3) (Piccini et al.,
The cellular efflux of K\(^+\) and influx of Ca\(^{2+}\) following P2X7-activation causes the activation of phospholipase A2 and phospholipase C, which is involved in processing and release of IL-1\(\beta\) (Andrei et al., 2004, Alzola et al., 1998). P2X7-activation also causes activation of phospholipase D which is required for killing of intracellular *Mycobacterium tuberculosis* (Kusner and Adams, 2000), the formation of reactive oxygen species (Sperlágh et al., 1998), activation of the transcription factor nuclear factor-κB via mitogen-activated protein kinase (Ferrari et al., 1997), and the activation of metalloproteases and subsequent shedding of cell surface molecules such as CD23, CD27 and CD62L (Gu et al., 1998, Moon et al., 2006). Membrane blebbing and subsequent apoptosis are also well established downstream effects following prolonged activation of P2X7 (Franceschi et al., 1996, Wilson et al., 2002).

### 1.6 The pharmacology of P2X7 receptors

The agonist and antagonist profile of each P2X receptor is unique. The pharmacology specific of P2X7 is discussed below.

#### 1.6.1 P2X7 receptor agonists

P2X7 is activated by a variety of agonists, although their potency and specificity varies. The most potent agonist of P2X7 is 2,3-O-(4-benzoylbenzoyl)-ATP (BzATP) which was previously considered selective for P2X7, however BzATP can also activate most other P2X receptors (Bianchi et al., 1999). ATP activates P2X7 at relatively high concentrations compared to other P2X
receptors, and the nucleotide is 10-fold less potent than BzATP at human P2X7 receptors (Donnelly-Roberts et al., 2009). Other nucleotides are weakly potent P2X7-agonists, including 2-methylthio-ATP (2-meSATP) and adenosine 5’-O-(3-thiotriphosphate) (ATPγS). Agonists for other P2X and P2Y receptors, including ADP, AMP, α,β-methylene-ATP (α,β-meATP), UTP and UDP are ineffective as P2X7 agonists (Donnelly-Roberts et al., 2009).

The agonist profile of P2X7 varies in a species-dependent manner. For instance, ATPγS and 2-meSATP are weak agonists at the human P2X7 receptor, but show no significant activity at rat or mouse P2X7 (Donnelly-Roberts et al., 2009). BzATP has similar potency at the human, rat and canine P2X7 receptor, however, this nucleotidie is 100-fold less potent at the mouse P2X7 receptor (Donnelly-Roberts et al., 2009, Stevenson et al., 2009, Roman et al., 2009). This increased sensitivity to BzATP in rat P2X7 compared to mouse P2X7 has been attributed to the Lys127 and Asn284 residues found in the rat P2X7 receptor (Young et al., 2007). In the human and canine P2X7 receptors polar or positively charged residues are also found in these positions; in contrast the mouse P2X7 receptor contains nonpolar (Ala127) and negatively charged (Asp284) residues at these sites. BzATP is considered a complete agonist of human, rat, mouse and canine P2X7 (Donnelly-Roberts et al., 2009, Stevenson et al., 2009), however the study by Roman et al. (2009) suggests that BzATP is only a partial agonist of canine P2X7. The reason for this difference with BzATP for the canine P2X7 receptor remains unknown, but may be due to differences in assays or dog breed. Or note however, BzATP is also considered a partial agonist of guinea pig P2X7 (Fonfria et al., 2008).
Human and rat P2X7 are 10-fold more sensitive to ATP compared to mouse P2X7 (Young et al., 2007, Donnelly-Roberts et al., 2009). The differences between rat and mouse receptor sensitivity have also been attributed to the Asn284 residue in rat P2X7 (Young et al., 2007). The study by Roman et al. (2009) reported that ATP showed similar agonist potency between human and canine P2X7, which contradicts the study by Stevenson et al. (2009), where human P2X7 was four-fold more sensitive to ATP compared to canine P2X7. Again, it should be noted, however, that the study by Roman et al. (2009) used recombinant P2X7 cloned from a Beagle, whereas the study by Stevenson et al. (2009) used native P2X7 from English Springer Spaniel erythrocytes. Thus this may account for the differences in ATP sensitivity between these two studies.

1.6.2 P2X7 receptor antagonists

P2X7 receptor function can be impaired by a range of antagonists of varying specificity. Pyridoxal phosphate-6-azophenyl-2-4-disulphonic acid (PPADS) can impair human, canine and rodent P2X7 receptors, as well as other homomeric and heteromeric P2X receptors. Of the non-specific P2X7 antagonists, KN-62 and calmidazolium, which also inhibit calmodulin-dependent kinases, are potent inhibitors of human, canine and mouse receptors, or human and rat receptors, respectively. (Gever et al., 2006, Donnelly-Roberts et al., 2009, Roman et al., 2009). Brilliant Blue G (BBG) impairs P2X7 at nonomolar concentrations for human, canine and rodent receptors (Roman et al., 2009, Donnelly-Roberts et al., 2009). Of note, BBG also impairs P2X4 and some P2Y receptors at low micromolar concentrations, and is a charged protein dye and
thus is likely to bind to other molecules to alter their function (Syed and Kennedy, 2012).

Recently, a number of potent and specific P2X7 antagonists have been made commercially available, including A438079 (Nelson et al., 2006), A740003 (Honore et al., 2006), AZ10606120 (Michel et al., 2008) and AZ11645373 (Stokes et al., 2006) (Table 1.3). Among these antagonists, A438079 and A740003 are the least species-selective P2X7 inhibitors with similar efficacy at the human, rat and mouse receptor (Donnelly-Roberts et al., 2009). AZ10606120 is a potent non-competitive inhibitor of human, rat and canine receptors (Michel et al., 2008, Roman et al., 2009). AZ11645373 is active at low to mid nanomolar concentrations against human and canine P2X7 receptors, whereas higher concentrations are needed to impair mouse and guinea pig receptors. Rat P2X7 receptors are essentially insensitive to this compound (Michel et al., 2009, Stokes et al., 2006). Lastly, GW791343 impairs human and canine P2X7 receptors with similar potencies, although it has no effect at mouse receptors and potentiates rat P2X7 receptors (Michel et al., 2008). A number of other P2X7-specific antagonists have also been developed, but these are patented and not readily available (Syed and Kennedy, 2012). In addition, anti-P2X7 monoclonal antibodies (clones L4 and 1F11) can also block human P2X7 receptor (Buell et al., 1998a) and mouse P2X7 receptor (Kurashima et al., 2012), respectively. Thus, this opens up the possibility of developing blocking anti-P2X7 antibodies with therapeutic potential.

P2X7 receptors are also sensitive to their extracellular milieu. The study by Virginio, et al. (1997) showed that divalent cations, such as Ca$^{2+}$, Mg$^{2+}$, Zn$^{2+}$
and Cu\(^{2+}\), impair rat P2X7-evoked currents with half-maximal inhibitory concentrations (IC\(_{50}\)) of 2.9 mM, 0.5 mM, 11 µM and 0.5 µM respectively. The relative potencies for blocking BzATP-induced YO-PRO\(^{2+}\) uptake were as follows: Cu\(^{2+}\) > Cd\(^{2+}\) = Zn\(^{2+}\) > Ni\(^{2+}\) >> Mg\(^{2+}\) = CO\(^{2+}\) > Mn\(^{2+}\) > Ca\(^{2+}\) = Ba\(^{2+}\) >> Sr\(^{2+}\) (Virginio et al., 1997). Thus, the relative amount of cations in media can affect P2X7 including signalling events downstream of P2X7 activation.

Table 1.3: Antagonist potencies against recombinant P2X7 from various species.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>IC(_{50}) (nM)</th>
<th>Human</th>
<th>Rat</th>
<th>Mouse</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPADS(^a)</td>
<td>1200</td>
<td>1200</td>
<td>4900</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>KN-62 (^a,b)</td>
<td>210</td>
<td>&gt; 100 000</td>
<td>190</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>BBG (^a,b)</td>
<td>1900</td>
<td>580</td>
<td>190</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>A438079(^a)</td>
<td>930</td>
<td>100</td>
<td>650</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>A740003(^a)</td>
<td>93</td>
<td>100</td>
<td>720</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>AZ10606120(^b)</td>
<td>2.6</td>
<td>28</td>
<td>ND</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>AZ11645373(^c)</td>
<td>35</td>
<td>3300</td>
<td>1500</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>GW791343(^b)</td>
<td>93</td>
<td>Potentiate</td>
<td>ND</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

Data obtained from \(^a\) Donnelly-Roberts et al. (2009); \(^b\) Roman et al. (2009); \(^c\) Michel et al. (2009). ND; Not Determined.

1.7 The physiological and pathophysiological role of P2X7 receptors

Studies of P2X7-deficient mice (Chessell et al., 2005, Solle et al., 2001b) together with the known signalling events downstream of P2X7-activation (section 1.5.5) have contributed to establishing a physiological role for the receptor in inflammation and pain. P2X7 receptors have shown to be involved in immune and inflammatory responses, including the processing and release of pro-inflammatory cytokines such as IL-1\(\beta\) and IL-18, and the killing of intracellular pathogens such as M. tuberculosis (tuberculosis) and Toxoplasma...
*gondii* (toxoplasmosis) (Wiley et al., 2011). A role for P2X7 receptors has also been established in bone formation (Ke et al., 2003). P2X7 has been linked to a range of inflammatory disorders such as rheumatoid arthritis, gout and chronic inflammatory and neuropathic pain (Skaper et al., 2010). In addition, P2X7 is known to modulate synaptic activity and neuron-glia signalling in the brain, and have been linked to neurodegenerative disorders such as multiple sclerosis (Narcisse et al., 2005), Alzheimer’s disease (Parvathenani et al., 2003) and Parkinson’s disease (Baranyi et al., 2011). High levels of P2X7 have been found in diverse tumors, and it has been suggested that the receptor has a role in tumor metastasis and invasiveness of cancer cells (Jelassi et al., 2011). The P2X7 receptor has also been linked to renal disorders, such as unilateral ureteral obstruction (Goncalves et al., 2006), glomerulonephritis (Turner et al., 2007, Taylor et al., 2009) and polycystic kidney disease (Xu et al., 2009). Lastly, P2X7 has been associated with neuropsychiatric illnesses, such as bipolar disease and major depression (Barden et al., 2006, Hejjas et al., 2009).

### 1.7.1 IL-1β

IL-1β is a member of the IL-1 cytokine family and is a key mediator of host response to infection and inflammatory processes. IL-1β is produced from a variety of cells, including activated monocytes, macrophages, dendritic cells, B lymphocytes, natural killer cells and microglia (Ferrari et al., 2006, Dinarello, 2009). IL-1β is synthesized as a precursor molecule (pro-IL-1β; 31 kDa) in response to bacterial products such as LPS, and host-derived inflammatory factors (Dinarello, 2009). These molecules mediate signalling pathways via
pattern recognition receptors (PRRs), including TLRs. However, a second stimulus such as ATP is required to activate the cysteine protease caspase-1 to mediate the proteolytic conversion of pro-IL-1β to mature, biologically active IL-1β (17 kDa) (Dinarello, 2009). Although processing of pro-IL-1β into active IL-1β is predominantly mediated by caspase-1, there are some caspase-1 independent pathways that process the precursor molecule extracellularly such as cleavage by elastase, the neutrophil protease proteinase-3, matrix metalloprotease 9, mast cell chymase and granzyme A (Coeshott et al., 1999, Taylor et al., 2009, Xu et al., 2009, Wilson et al., 2002).

IL-1β exerts a wide range of pro-inflammatory biological activities, including the induction of acute-phase protein synthesis and subsequent activation of the complement system, phagocytosis, fever induction, vasodilation, local tissue destruction and activation of lymphocytes (Gosslau et al., 2011). The cytokine also plays a role in angiogenesis and tumor metastasis (Dinarello, 2009). Autoinflammatory diseases were originally characterised by recurrent episodes of fever with inflammatory responses in multiple organs. These diseases are now a part of a broader group that show elevated levels of IL-1β and include familial Mediterranean fever, arthritis, systemic lupus erythematosus, pericarditis, urate crystal arthritis (gout) and type 2 diabetes (Dinarello, 2009, Ferrari et al., 2006). This demonstrates the importance of IL-1β as a therapeutic target.

1.7.2 IL-18

IL-18 was originally identified as an interferon (IFN)-γ-inducing factor, and is
an important regulator of both innate and acquired immune responses (Okamura et al., 1995). Similar to IL-1β, IL-18 is produced as a precursor molecule (pro-IL-18; 23 kDa), which is cleaved by caspase-1 to the biologically active cytokine (18 kDa) (Ferrari et al., 2006). In contrast to pro-IL-1β, pro-IL-18 is synthesized and stored in the cytosol independent of an external signal such as LPS (Puren et al., 1999).

IL-18 is secreted from a variety of cells, including monocytes, macrophages, dendritic cells, fibroblasts and osteoblasts (McInnes et al., 2000). Secreted IL-18 subsequently induces the synthesis of other cytokines, including IL-6, IL-8, tumor necrosis factor (TNF)-α, IFN-γ and IL-1β. Moreover, the production of several chemokines and adhesion molecules is induced by IL-18. Other functions of IL-18 include the direct modulation of the activity of B- and T-lymphocytes, natural killer cells, dendritic cells and macrophages (McInnes et al., 2000). Furthermore, similar to IL-1β, overproduction of IL-18 have been shown to play a role in autoinflammatory and autoimmune diseases such as rheumatoid arthritis, Crohn’s disease, macrophage activation syndrome, type 1 diabetes, psoriasis and graft-versus-host disease (Dinarello, 2009).

1.7.3 NALP3 inflammasome activation

Inflammasomes are large macromolecular complexes containing NOD-like receptors (NLRs) such as NACHT-LRR-PYD-containing protein (NALPs) (Fig. 1.6) or ICE-protease activating factors (IPAFs), which act as intracellular sensors for danger signals, including microbes and cell disruption. The NALP3 inflammasome is particularly well studied due to its prominent function in
sterile inflammatory responses, antimicrobial responses, adjuvanticity and hereditary autoinflammatory syndromes (Bauernfeind et al., 2011).

**Figure 1.6: Schematic structure of the NALP3 inflammasome.** NALP3 (NACHT-LRR-PYD-containing protein) consist a leucine-rich repeat (LRR) domain, a nucleotide binding domain (NACHT) and a pyrin domain (PYD). Upon activation, NALP3 recruits apoptosis-associated speck-like protein containing a C-terminal CARD (ASC) via PYD-PYD interactions and caspase-1 via caspase recruitment domain (CARD)-CARD interactions to form a macromolecular complex – the NALP3 inflammasome. Adapted from Bauernfeind et al. (2011).

Upon activation, the NALP3 inflammasome is formed by assembly of NALP3, the adaptor protein apoptosis-associated speck-like protein containing a C-terminal CARD (ASC) via pyrin domain (PYD)-PYD interactions. The leucine-rich repeats (LRR) are thought to sense the signals that lead to oligomerization of the NACHT domain. Pro-caspase-1 is then recruited to the inflammasome by via homotypic caspase recruitment domain (CARD)-CARD interactions and subsequently activated. Activation of the NALP3 inflammasome and caspase-1
results in the processing and release of the pro-inflammatory cytokines IL-1β and IL-18 (Bauernfeind et al., 2011, Martinon et al., 2009).

NALP3 inflammasome activation can be triggered by a wide range of stimuli. Microbes that can activate the inflammasome include influenza A virus, adenovirus, Staphylococcus aureus, Escherichia coli and Neisseria gonorrhoeae (Bauernfeind et al., 2011). Although it is not completely understood how microbes activate the NALP3 inflammasome, influenza A virus encodes an ion channel protein (M2), which is thought to be involved in NALP3 inflammasome-activation (Ichinohe et al., 2010). Moreover, activation by bacteria has been attributed to specific bacterial pore-forming toxins, which allows the efflux of cellular K+ (Fig. 1.7). These include Listerolysin O, nigericin (Mariathasan et al., 2006), streptolysin (Harder et al., 2009) and maitotoxin (Gurcel et al., 2006).

Phagosomal materials such as crystals, particles and endogenous protein aggregates can also activate NALP3. Gout-associated uric acid crystals, including monosodium urate and calcium pyrophosphate dehydrate crystals, have been shown to cause the activation of the NALP3 inflammasome and the release of IL-1β (Martinon et al., 2006). Furthermore, it has been shown that environmental pollutants consisting of inorganic crystalline material such as crystalline silica and asbestos, which cause the pulmonary fibrotic disorders silicosis and asbestosis respectively, work in a NALP3-dependent manner (Hornung et al., 2008, Dostert et al., 2008). In addition, aluminium salts, which are commonly used as adjuvants in vaccines, and endogenous protein aggregates such as amyloid-β linked to Alzheimer’s disease induce the
production of pro-inflammatory cytokines via the NALP3 inflammasome (Hornung et al., 2008, Halle et al., 2008).

**Figure 1.7: Molecular mechanisms involved in the activation of the NALP3 inflammasome.** An initial priming step, such as the activation of pattern recognition receptors, leads to the enhanced transcription of NALP3 (NLRP3) and pro-interleukin-1β (pro-IL-1β). A second stimulus, such as ATP-induced P2X7-activation or pathogen-derived pore-forming toxins, leads to the efflux of K+ and subsequent assembly and activation of the NALP3 inflammasome comprising of NALP3, the protein apoptosis-associated speck-like protein containing a C-terminal CARD (ASC) and pro-caspase-1. Caspase-1 is then activated and subsequently cleaves pro-IL-1β into mature IL-1β. Lysosomal damage and reactive oxygen species (ROS) can also activate the NALP3 inflammasome (Bauernfeind et al., 2011).
Lastly, the inflammasome can be activated by endogenous signals, such as extracellular ATP via the activation of P2X7 (Fig. 1.7). The resulting drop in cytosolic K\(^+\) concentrations stimulates the NALP3 inflammasome by a poorly understood mechanism. However evidence indicates that the process may involve the production of reactive oxygen species (ROS) and lysosomal disintegration (Bauernfeind et al., 2011).

The level of NALP3 expression is also important for activation of the inflammasome. Monocytes and macrophages require an initial priming step to cause the enhanced transcription of NALP3. For example, microbial components such as LPS, single-stranded RNA, peptidoglycans and CpG DNA stimulate cell signalling through various PRRs including TLRs (Fig. 1.7). This leads to the synthesis of NALP3 via the transcription factor NF-κB (Bauernfeind et al., 2011, Martinon et al., 2009).

Following activation of the NALP3 inflammasome and the subsequent generation of mature IL-1β in the cytosol (Fig. 1.7), the biologically active cytokine is released from the cell via various mechanisms. These include the: exocytosis of secretory lysosomes (Andrei et al., 2004) or endosome-derived recycling multivesicular bodies (Qu et al., 2007); shedding of plasma membrane microvesicles (Milius et al., 2007); or direct efflux via membrane transporters (Panupinthu et al., 2008).

### 1.8 P2X7 receptor splice variants

The gene encoding the P2X7 receptor (P2RX7) consists of 13 exons and is located on the human chromosome 12 (12q24.31) (Buell et al., 1998b). The
full-length protein defined as P2X7A is well characterised. In addition, nine different naturally occurring splice variants have been identified in humans (P2X7B-J) (Cheewatrakoolpong et al., 2005, Adinolfi et al., 2010, Feng et al., 2006). Four of these, namely P2X7B, P2X7C, P2X7E and P2X7G, contain a premature stop-codon in exon 10 resulting in a truncated C-terminal domain (Cheewatrakoolpong et al., 2005). P2X7G and P2X7H contain an alternative start codon and additional exon (N3) in the intron between exon 2 and 3, which results in a non-functional protein lacking the first transmembrane domain. P2X7B is of particular interest due to its wide tissue expression and involvement in cell-growth and cancer. Recently, P2X7B was found to be co-expressed with P2X7A, forming modulated receptors that lose its proapoptotic activity typical of P2X7 (Adinolfi et al., 2010). P2X7J lacks exon 8 resulting in a truncated protein with 10 alternate amino acids at the C-terminus. This variant has also been identified in cancer cells, and has been shown to inhibit P2X7A-mediated apoptosis (Feng et al., 2006). The P2X7I variant results from a rare point mutation between exon 1 and 2, causing a null allele (Skarratt et al., 2005), and has been linked to increased susceptibility to tuberculosis (Fernando et al., 2005). Other splice variants lack one or more of the 13 exons of P2X7A (Cheewatrakoolpong et al., 2005), but their role remains undetermined.

Three P2X7 splice variants have been identified in mice (Nicke et al., 2009, Masin et al., 2012). A fully functional variant, P2X7K, express an alternative exon 1, and subsequently a different N-terminus and first transmembrane domain. This isoform is more sensitive to agonists and has increased pore-forming activity. Interestingly, P2X7K escapes silencing in a commonly used
P2X7 knockout mouse strain (Glaxo) (Nicke et al., 2009). More recently, two novel splice variants with alternative exon 13 (P2X713b and P2X713c) were identified (Masin et al., 2012). These truncated C-terminal variants were expressed in a different P2X7 knockout mouse strain (Pfizer) and showed decreased receptor function compared to the full-length protein. When co-expressed with P2X7A, P2X713b exhibited a dominant negative effect on surface expression, indicating that it may play a role in suppressing normal P2X7 function (Masin et al., 2012).

Several splice variants have also been found in cloned canine P2X7 receptors (Roman et al., 2009). One isoform contains an alternative splice site within exon 13, resulting in a truncated C-terminal domain lacking amino acid residues 432-522. Another isoform lacks exon 7, causing a frame-shift and the introduction of a premature stop-codon. If translated, this causes a truncated P2X7 receptor consisting of residues 1-205 and an additional 13 unique residues at the C-terminus (Roman et al., 2009).

1.9 P2X7 single nucleotide polymorphisms

The human P2RX7 gene is highly polymorphic, containing over 1500 single nucleotide polymorphisms (SNPs) (http://www.ncbi.nlm.nih.gov/snp). The majority of these SNPs are intronic or synonymous, however over 100 non-synonymous amino-acid altering SNPs have been identified, of which 17 have been characterised for phenotype (Fig. 1.8; Table 1.4) (Sluyter and Stokes, 2011). The most extensively studied variant is the Glu496Ala (rs3751143) loss-of-function SNP, which has shown to impair P2X7 pore formation (Gu et al.,
2001) without affecting channel activity (Boldt et al., 2003). A variety of functional defects have been observed in the presence of this mutation including impaired P2X7-mediated IL-1β and IL-18 release from monocytes (Sluyter et al., 2004a, Sluyter et al., 2004b), mycobacterial and toxoplasma killing within macrophages (Fernando et al., 2007, Lees et al., 2010) and apoptosis of macrophages and lymphocytes (Fernando et al., 2005, Wiley et al., 2002). Glu496Ala has been associated with increased susceptibility to extrapulmonary tuberculosis (Fernando et al., 2007). Recently, Glu496Ala was linked to decreased risk of ischemic heart disease in smokers (Gidlöf et al., 2012). Other loss-of-function SNPs include the null allele (rs35933842), Arg270His (rs7958311), Arg307Gln (rs28360457), Thr357Ser (rs2230912) and Ile568Asn (rs1653624), and are also known to impair P2X7-mediated apoptosis and killing of intracellular pathogens (Sluyter and Stokes, 2011).

Gain-of-function SNPs include His155Tyr (rs208294) and Ala348Thr (rs1718119) and result in increased P2X7-mediated IL-1β release from monocytes (Stokes et al., 2010). A recent study showed an association between the valine residue at position 76 (rs175225809) and predisposition to multiple sclerosis (Oyanguren-Desez et al., 2011). Moreover, Gln460Arg (rs2230912) has been associated with the mood disorder bipolar disease (Barden et al., 2006), and although this SNP itself does not alter P2X7 function, association with this SNP to the gain-of-function variants Arg270 and 155Tyr suggests a possible link of these SNPs with this disease (Sluyter et al., 2010).

Although several of the non-synonymous SNPs have been characterised phenotypically, the exact mechanism by which human P2X7 function is altered
is largely unknown. The Arg307Gln (rs28360457) SNP in the extracellular loop is thought to decrease the sensitivity to ATP by removing the positive residue from the ATP-binding site (Fig. 1.8) (Gu et al., 2004). In contrast, the Ile568Asn (rs1653624) SNP located at the C-terminus prevents P2X7 trafficking to the cell surface (Wiley et al., 2003), while the Ala348Thr SNP within the second transmembrane domain is thought to result in altered gating properties (Stokes et al., 2010).

Figure 1.8: Schematic representation of selected single nucleotide polymorphisms (SNPs) in the primary sequence of the human P2X7 receptor. Loss-of-function and gain-of-function SNPs are identified by colour (red and green respectively). Adapted from Wiley et al. (2011).
### Table 1.4: Single nucleotide polymorphisms of the human P2RX7 gene characterised for phenotype.


<table>
<thead>
<tr>
<th>dbSNP ID</th>
<th>Amino Acid Change</th>
<th>Effect on function</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs35933842</td>
<td>Null Allele</td>
<td>LoF</td>
</tr>
<tr>
<td>rs17525809</td>
<td>Val76Ala</td>
<td>Partial LoF</td>
</tr>
<tr>
<td>rs28360445</td>
<td>Arg117Trp</td>
<td>LoF</td>
</tr>
<tr>
<td>rs28360447</td>
<td>Gly150Arg</td>
<td>LoF</td>
</tr>
<tr>
<td>rs208294</td>
<td>His155Tyr</td>
<td>GoF</td>
</tr>
<tr>
<td>rs28360451</td>
<td>Glu186Lys</td>
<td>LoF</td>
</tr>
<tr>
<td>rs11263122</td>
<td>Asn187Lys</td>
<td>Possible LoF</td>
</tr>
<tr>
<td>rs28360452</td>
<td>Leu191Pro</td>
<td>Partial LoF</td>
</tr>
<tr>
<td>rs7958311</td>
<td>Arg270His</td>
<td>Partial LoF</td>
</tr>
<tr>
<td>rs7958316</td>
<td>Arg276His</td>
<td>LoF</td>
</tr>
<tr>
<td>rs28360457</td>
<td>Arg307Gln</td>
<td>LoF; associated with accelerated lumbar spine bone loss and risk of hip arthroplasty failure</td>
</tr>
<tr>
<td>rs1718119</td>
<td>Ala348Thr</td>
<td>GoF; associated with anxiety disorder and protection against toxoplasmosis</td>
</tr>
<tr>
<td>rs2230911</td>
<td>Thr357Ser</td>
<td>Partial LoF; decreased in vitro killing of <em>M. bovis</em> by infected macrophages</td>
</tr>
<tr>
<td>rs2230912</td>
<td>Gln460Arg</td>
<td>Neutral effect; association with mood disorders</td>
</tr>
<tr>
<td>rs3751143</td>
<td>Glu496Ala</td>
<td>LoF; decreased in vitro killing of <em>Mycobacterium ssp</em> and <em>T. gondii</em>. Association with tuberculosis susceptibility and risk of bone fracture</td>
</tr>
<tr>
<td>rs2230913</td>
<td>His521Gln</td>
<td>Neutral effect</td>
</tr>
<tr>
<td>rs1653624</td>
<td>Ile568Asn</td>
<td>LoF; Associated with risk of bone fracture</td>
</tr>
</tbody>
</table>

Abbreviations: LoF; Loss-of-function, GoF; Gain-of-function.

SNPs in the P2RX7 gene have also been found in other species. Various laboratory mouse strains were shown to contain a Pro451Leu (rs48804829) mutation located in the C-terminal domain (Adriouch et al., 2002). Receptors containing this SNP are less sensitive to ATP and demonstrate severely impaired P2X7 pore-formation, similar to the Glu496Ala SNP in the human
*P2RX7* gene (Adriouch *et al.*, 2002, Young *et al.*, 2006). Three additional non-synonymous SNPs have been identified in mouse cDNA cloned by Aldrich *et al.* (2002) from C57BL/6 and Balb/c mice compared to the sequence reported originally from NTW8 microglial cells (Chessell *et al.*, 1998). However, only one of these, the Met283Thr SNP, appears to alter P2X7 function. The study by Young *et al.* (2006) showed that the presence of a methionine residue at position 283 significantly reduced channel currents and eliminated ethidium uptake. In addition, four non-synonymous SNPs have been reported online: Gly113Asp (rs37541158); Pro396Arg (rs37237164); Arg486His (rs36331015); and His514Arg (rs36859091) (www.ensembl.org).

With the exception of Phe103Leu (rs23314713) in the canine *P2RX7* gene (section 1.5.1) SNPs in the *P2RX7* gene of other species have not been published. One other non-synonymous SNP in the canine *P2RX7* gene however has been reported online, Pro452Ser (rs23315462) (www.ensembl.org). Whether these variants alter canine P2X7 function remains unknown. Of note, a recently identified SNP in a different P2 receptor, the P2Y12 receptor, has been associated with postoperative haemorrhage in Greater Swiss Mountain dogs (Boudreaux and Martin, 2011). This study, together with the association between SNPs in the *P2RX7* gene and human disorders, may support a role for variations in the *P2RX7* gene in canine disorders.

### 1.10 Dog as a model for human disease

Approximately 400 inherited disorders have been characterised in dogs, most of which are relevant to humans. Dogs may provide ideal models of human
diseases due to factors such as five- to eight-fold faster aging compared to humans, shared environment with their owners, high level of health care provided and that they are usually kept until old age. The selective breeding of dogs has caused a reduced genetic variation, making genomic mapping of diseases notably simpler (Rowell et al., 2011). A range of studies related to human disorders have made use of dog models. Several studies used dog as a model for Alzheimer’s disease (Butterfield et al., 2012, Barone et al., 2012, Head et al., 2012), one of which used aged canine brain to study early amyloid-β plaque formation (Cummings et al., 1993). Dog models have also been used for studying childhood blindness (Acland et al., 2001), type 2 diabetes (Jeong et al., 2012), collagen type III glomerulopathy (Rortveit et al., 2012), chronic hepatitis and fibrosis (Favier et al., 2012), cancer (Rowell et al., 2011), Parkinson’s disease (Choi et al., 2011), muscular dystrophy (Wadosky et al., 2011), Hurler syndrome (Shull et al., 1994) and osteoarthritis (McDevitt et al., 1977). These studies highlight the potential of using the dog as a model for P2X7-related disorders.

1.10.1 The native canine P2X7 receptor

Parker and Snow (1972) demonstrated 40 years ago that extracellular ATP increased the permeability for cations in dog erythrocytes. Some three decades later, the canine genome project revealed the gene encoding P2RX7 in domestic dogs (Lindblad-Toh et al., 2005). Shortly after, P2X7 mRNA was identified in the canine brain, confirming the presence of P2X7 within the domestic dog (Lee et al., 2005). A more recent study using blood from English Springer Spaniels
showed that P2X7 was responsible for the ATP-induced cation flux in erythrocytes (Sluyter et al., 2007). Similar to human P2X7, BzATP was a complete agonist of canine P2X7 compared to ATP. Canine P2X7 was also shown to be sensitive to 2-meSATP and ATPγS, although the agonist potencies were lower than for BzATP and ATP (Sluyter et al., 2007). ATP-induced ethidium\(^+\) uptake into English Springer Spaniel erythrocytes and lymphocytes was 40- and five-fold greater compared to equivalent human cell types (Stevenson et al., 2009). In contrast, ATP-induced ethidium\(^+\) uptake into canine monocytes was three-fold lower compared to human monocytes (Sluyter et al., 2007). Comparison of dog and human erythrocytes suggested that this difference in P2X7 function was directly due to differences in P2X7 expression (Sluyter et al., 2007).

Recently, our group cloned canine P2X7 from an English Springer Spaniel in order to further characterise the receptor pharmacologically (Jalilian, 2011). Moreover, our laboratory confirmed the presence of functional P2X7 in canine monocytes, and B- and T-lymphocytes by an ATP-induced YO-PRO-1\(^{12+}\) uptake assay (Jalilian, 2011). The ATP-induced YO-PRO-1\(^{12+}\) uptake into canine monocytes was impaired by the P2X7 antagonists A438079 and KN-62, indicating that the dye uptake is attributed to P2X7 (Jalilian, 2011). Similar to the previously studied English Springer Spaniel breed (Stevenson et al., 2009) the relative P2X7 function in T-lymphocytes was higher compared to B-lymphocytes and monocytes from five different dog breeds (Jalilian, 2011). Moreover, ATP-induced YO-PRO-1\(^{12+}\) uptake into canine monocytes was determined in 43 dogs of various pure and cross breeds. The relative P2X7 function of monocytes varied between and within dog breeds (Peranec, 2011,
Jalilian, 2011). The cause of this variation was not determined but it is possible that SNPs in the canine *P2RX7* gene may be responsible for variation in canine P2X7 function. In addition, LPS-primed canine monocytes were shown to express P2X7, TLR4, NALP3, caspase-1, IL-1β and IL-18 mRNA (Jalilian, 2011), and that P2X7 activation could induce IL-1β release from these cells (Peranec, 2011). Functional P2X7 was also identified in the Madin-Darby canine kidney (MDCK) cell line (Jalilian, 2011). However, the expression was relatively low, with functional responses only observed in sucrose medium which is known to potentiate P2X7-mediated responses (Michel *et al.*, 1999, Gadeock *et al.*, 2012). MDCK cells were also shown to express TLR4, NALP3 and caspase-1; IL-1β and IL-18 however were not detected (Jalilian, 2011).

1.11 Aims

This thesis forms a part of an ongoing study investigating the role of canine P2X7 in inflammation and immunity. Specifically, this thesis aims to:

1. Confirm the presence or absence of P2X7 and NALP3-related components in LPS-primed canine monocytes and MDCK cells (Chapter 2);
2. Determine if ATP or TLR ligands induce IL-1β release from MDCK cells and in whole canine blood (Chapter 2);
3. Sequence and pharmacologically characterise a recombinant canine P2X7 receptor cloned from an English Springer Spaniel (Chapter 3);
4. Confirm if the relative monocyte P2X7 function varies between dogs and to determine if this variation is due to SNPs in the *P2RX7* gene.
Chapter 2: P2X7 and IL-1β release in canine monocytes and kidney cells

2.1 Materials and Methods

2.1.1 Materials

RPMI-1640 medium, Dulbecco’s modified Eagle’s/F12 (DMEM/F12) medium, L-glutamine, GlutaMAX, YO-PRO-1²⁺ and 0.25% trypsin were from Invitrogen Corporation (Auckland, New Zealand). Foetal calf serum (FCS) was from Bovogen (East Keilor, Australia). Penicillin-streptomycin, ethidium bromide, lipopolysaccharide (LPS) from Escherichia coli serotype 055:B5, adenosine 5’-triphosphate (ATP), nigericin, 100% ethanol and MgCl₂ were from Sigma Chemical Company (St Louis, MO). Ficoll-Paque PLUS and ExoSAP-IT PCR Clean-up Kit were from GE Healthcare (Uppsala, Sweden). Primers were from Geneworks (Hindmarsh, Australia). BigDye® Terminator V3 was from Applied Biosystems (Carlsbad, CA). Agarose was from Bioline (Alexandria, Australia). AZ10606120 was obtained from Tocris Bioscience (Ellisville, MO). Lipoteichoic acid (LTA) from Staphylococcus aureus was from Invivogen (San Diego, CA). Bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), Tris base and other general grade chemicals were from Amresco (Solon, OH).

2.1.2 Cell lines

Madin-Darby canine kidney (MDCK) epithelial cells were purchased from the European Collection of Cell Cultures (Porton Down, UK). Cells were cultured in DMEM/F12 medium supplemented with 10% FCS, 2 mM GlutaMAX, 100
units/mL penicillin and 10 µg/mL streptomycin (complete MDCK medium). Cells were incubated at 37°C/5% CO₂ and were passaged two times per week by limited trypsinisation, as required.

2.1.3 Collection of dog blood

Peripheral blood from 9 mixed or pure breed dogs was collected into vacutainer tubes containing lithium heparin (Greiner Bio-One, Frickenhausen, Germany), from either Albion Park Veterinary Hospital (Albion Park, Australia) or Oak Flats Veterinary Clinic (Oak Flats, Australia). Written consent was obtained from informed owners with approval by the University of Wollongong animal ethics committee.

2.1.4 LPS-primed canine peripheral blood monocytes

Blood was centrifuged (450 x g for 15 min; with brake off) and the buffy coat was collected. Buffy coats were diluted with RPMI-1640 medium, underlaid with Ficoll-Paque PLUS and centrifuged (560 x g for 20 min; with brake off). The peripheral blood mononuclear cells (PBMCs) were collected and washed once with RPMI-1640 medium (450 x g for 10 min followed by 300 x g for 5 min) and twice with RPMI-1640 medium (300 x g for 5 min). PBMCs were then suspended in 20 mL RPMI-1640 medium containing 10% FCS and 5 mM L-glutamine (complete monocyte medium) and cultured in a T75 flask (Greiner Bio-One, Germany) for 2 hours at 37°C/5% CO₂. Non-adherent mononuclear cells were removed by swirling the flask six times and removing the medium.
This process was repeated using 10 mL RPMI-1640 medium to remove the remaining non-adherent cells. The cells were then incubated in 20 mL complete monocyte medium containing 0.1 µg/mL LPS for 4 hours at 37°C/5% CO₂.

### 2.1.5 Detection of P2X7 and NALP3-related components

Total RNA was isolated from LPS-primed monocytes or MDCK cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Equal amounts of RNA for each cell type were amplified by reverse transcription (RT)-PCR using the MyTaq One-Step RT-PCR Kit (Bioline, Sydney Australia) using primer pairs specific for various canine mRNA transcripts (Table 2.1). Each RT-PCR reaction contained 2X MyTaq One-Step mix, reverse transcriptase, 2 U RiboSafe RNase Inhibitor, 0.2 µM forward and reverse primer, 1-2 µg RNA template and DEPC-treated H₂O to a final volume of 12 µL. The RT-PCR was performed using a Mastercycler Pro S thermocycler (Eppendorf, Germany) using the cycle conditions described in Table 2.1. The amplicons were then separated via electrophoresis using a 1.5% agarose gel at 100 V for 1 hour in Tris acetate-EDTA (TAE) buffer (20 mM acetic acid, 1 mM EDTA, 40 mM Tris, pH 8) and stained with ethidium bromide.

### 2.1.6 Sequencing of P2X7 and NALP3-related components

Amplicons from RT-PCR (section 2.1.5) were purified using 1 µL of diluted (1:4) ExoSAP-IT enzyme per 2.5 µL of cDNA, and the samples were incubated using
a Mastercycler Pro S thermocycler at 37°C for 15 min, and then 80°C for 15 min. Each sequencing reaction contained 3.4 µL sequencing buffer, 1 µL BigDye® Terminator v3.1, 1 µL cDNA, 0.6 µM forward or reverse primer (Table 2.1) and sterile deionised H₂O to a final volume of 20 µL. PCR amplification was then performed using a Mastercycler Pro S thermocycler with 30 repeats of denaturation at 96°C for 30 sec, annealing at 55°C for 15 sec and extension at 60°C for 4 min, followed by a single cycle of final extension at 30°C for 30 sec.

**Table 2.1: Primers and cycle conditions for P2X7 and NALP3-related components**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer 5’-3’</th>
<th>Reverse primer 5’-3’</th>
<th>Cycle conditions</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>GTCTGGCTGGCTTAAGATC</td>
<td>CTGCAATCTAGGATCTGGAG</td>
<td>95°C for 20 s, 52.4°C for 20 s, 72°C for 60 s</td>
<td>910</td>
</tr>
<tr>
<td>P2X7</td>
<td>TGGCTCCCATCAGCTCCC</td>
<td>GTCCTGGAGGCAAAGGCCC</td>
<td>95°C for 10 s, 62°C for 10 s, 72°C for 30 s</td>
<td>240</td>
</tr>
<tr>
<td>NALP3</td>
<td>CACTGTACGCTCTGTGGCAGGGT</td>
<td>GTCCTCCAGGCGTTGTGCC</td>
<td>95°C for 10 s, 63.7°C for 10 s, 72°C for 30 s</td>
<td>273</td>
</tr>
<tr>
<td>Caspase-1</td>
<td>ACAGACGCTGGGGCTCTCCTC</td>
<td>CCCAGGCCCTCCACGAGACT</td>
<td>95°C for 10 s, 63.7°C for 10 s, 72°C for 30 s</td>
<td>339</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGCAAAAACAGATGGGAAATAA</td>
<td>GTAAGTTGCAGTCCACCGATT</td>
<td>95°C for 10 s, 53°C for 10 s, 72°C for 30 s</td>
<td>64</td>
</tr>
<tr>
<td>IL-18</td>
<td>CCTGGAAATCGATTACTTTGCC</td>
<td>GTTTTGTTTCTTACAGGAGAGAG</td>
<td>95°C for 10 s, 57°C for 10 s, 72°C for 30 s</td>
<td>251</td>
</tr>
</tbody>
</table>

a Primers for P2X7 (Accession no: NM_001113456.1), NALP3 (Accession no: XM_843284.2) and caspase-1 (Accession no: NM_001003125.1) were designed from the corresponding reference sequences (http://www.ncbi.nlm.nih.gov) using Primer 3 (http://frodo.wi.mit.edu/ primer3/). Primers for TLR4 (Asahina et al., 2003), IL-1β (Maccoux et al., 2007) and IL-18 (Chamizo et al., 2001) were obtained from previous publications.

b PCR mixtures were incubated at 45°C for 20 min (except TLR4 which was incubated for 30 min), then at 95°C for 60 sec (s), followed by 42 cycles as indicated, and then a final extension at 72°C for 5 min.
To purify PCR products, amplicons were mixed with 2 µL 125 mM EDTA (pH 8), 2 µL sodium acetate (pH 5.1) and 50 µL 100% ethanol. The mix was then incubated at room temperature for 15 min followed by centrifugation (13200 x g for 15 min). The precipitated products were then washed with ice-cold 70% ethanol (13200 x g for 15 min) and dried using a heat block at 60ºC. All products were kindly sequenced using a 3130xl capillary sequencer (Applied Biosystems, Carlsbad, CA) by Margaret Phillips (University of Wollongong, Australia). Resulting sequences were compared to the corresponding NCBI (http://www.ncbi.nlm.nih.gov) reference sequences using Geneious Pro Software (Biomatters, Auckland, New Zealand).

2.1.7 IL-1β release from MDCK cells

MDCK cells were seeded in 24-well plates (Greiner Bio-One) at 2 x 10^5 cells/well and incubated overnight at 37ºC/5% CO₂. Cells were then incubated with fresh complete MDCK medium in the absence or presence of LPS (as indicated) (1 mL/well) for 24 hours at 37ºC/5% CO₂. Alternatively, MDCK cells were seeded in 24-well plates at 1 x 10^5 cells/well and incubated overnight at 37ºC/5% CO₂. Cells were then incubated with fresh complete MDCK medium in the absence or presence of 10 µg/ml LPS for 4 or 24 hours. These cells were then pre-incubated in RPMI-1640 medium (containing 0.1% BSA) or in sucrose medium (280 mM sucrose, 5 mM KCL, 10 mM N-methyl-D-glucamine, 5 mM glucose, 10 mM HEPES, 0.1% BSA, 2 mM Ca^{2+}, 1 mM Mg^{2+}, pH 7.4) (0.3 ml/well) at 37ºC/5% CO₂ for 15 min, followed by 60 min incubation in the absence or presence of 3 or 5 mM ATP or 20 µM nigericin. Following
incubations, samples were centrifuged (11,000 x g for 30 sec), and cell-free supernatants collected and stored at -80°C until required. The amount of IL-1β in cell-free supernatants was quantified using a Canine IL-1β VetSet™ ELISA Development Kit (Kingfisher Biotech, St. Paul, MN) according to the manufacturer's instructions and measured at 450 nm on a Spectramax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA).

2.1.8 ATP-induced IL-1β release from LPS- or LTA-primed canine whole blood

Whole blood (100 µL) was plated in 96-well plates (Greiner Bio-One). Equal volumes of RPMI-1640 medium (supplemented with 2 mM GlutaMAX, 100 units/mL penicillin and 10 µg/mL streptomycin; incomplete culture medium) were added to each well and sterile H2O was added to surrounding wells. The diluted blood was then incubated in the absence or presence of 100 ng/mL LPS or LTA for 2 hours at 37°C/5% CO2. In some experiments 1 µM AZ10606120 was added after 1 hour and 45 min incubation with LPS or LTA. The blood was then co-incubated in the absence or presence of 6 mM ATP for a further 30 min or 2 hours at 37°C/5% CO2. Following incubations, plates were centrifuged (700 x g for 10 min), and cell-free supernatants collected and stored at -80°C until required. The amount of IL-1β in cell-free supernatants was quantified as described in section 2.1.7.
2.1.9 IL-1β release from LPS- or LTA-primed canine whole blood

Whole blood was plated in 96-well plates and diluted with equal volumes of incomplete culture medium as described in section 2.1.8. The diluted blood was then incubated in the absence or presence of LPS or LTA (as indicated) for 24 hours at 37°C/5% CO₂. In some experiments diluted blood was also incubated in the absence or presence of 1 µM AZ10606120 for 24 hours at 37°C/5% CO₂. Following incubations, plates were centrifuged (700 x g for 10 min) and cell-free supernatants collected and stored at -80°C until required. The amount of IL-1β in cell-free supernatants was quantified as described in section 2.1.7.

2.1.10 Presentation of data and statistical analysis

Results are expressed as mean ± standard deviation (SD). All graphs were prepared using GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, CA). Differences between each treatment were compared using ANOVA for multiple comparisons (with Tukey’s post hoc test) using GraphPad Prism 5 software with \( P < 0.05 \) considered significant.
2.2 Results

2.2.1 Canine monocytes and MDCK cells express TLR4, P2X7, NALP3, caspase-1, IL-1β and IL-18

Our group has previously shown that MDCK cells contain TLR4, P2X7, NALP3 and caspase-1 mRNA. In contrast, mRNA of the pro-inflammatory cytokines IL-1β and IL-18 were not detected (Jalilian, 2011). These experiments however did not include a positive control, such as LPS-primed canine monocytes, therefore the absence of IL-1β and IL-18 in MDCK cells remains in doubt. Moreover, although the RT-PCR products corresponded to the predicted size of each transcript in canine monocytes and MDCK cells (Jalilian, 2011), sequencing of these amplicons is required to confirm the identity of each transcript. RT-PCR was used to confirm if canine monocytes and MDCK cells express P2X7, NALP3 and caspase-1, and if canine monocytes express IL-1β and IL-18 and determine if MDCK cells express IL-1β and IL-18. Since MDCK cells express TLR4 mRNA and cell-surface TLR4 (Jalilian, 2011), this molecule was also included as a positive control. Sufficient cDNA was not available to test the expression of TLR4 in monocytes. However these cells have previously been shown to express this molecule (Jalilian, 2011). As expected MDCK cells expressed TLR4 mRNA (Fig. 2.1). Both MDCK cells and LPS-primed canine monocytes expressed P2X7, NALP3, caspase-1, IL-1β and IL-18 mRNA (Fig. 2.1), with transcripts corresponding to the predicted size for each molecule (Table 2.1).

The identity of each above transcript, except IL-1β due to its small size (64 bp), was then confirmed by sequencing. The nucleobase sequences deduced from the TLR4 (accession no: NM_001002950.2; Fig. 2.2), P2X7 (accession no:
NM_001113456.1; Fig. 2.3), NALP3 (accession no: XM_843284.2; Fig. 2.4) and IL-18 (accession no: NM_001003169.1; Fig. 2.6) amplicons were 100% identical to the corresponding reference sequence. The nucleobase sequence deduced from the caspase-1 amplicon was 99% identical to the reference sequence (accession no: NP_001003125.1; Fig. 2.5).

**Figure 2.1: MDCK cells express TLR4, P2X7, NALP3, caspase-1, IL-1β and IL-18 mRNA.** RNA was isolated from MDCK cells and LPS-primed canine monocytes (positive control) and TLR4, P2X7, NALP3, caspase-1, IL-1β and IL-18 were amplified using RT-PCR. Products were separated by agarose gel electrophoresis and stained with ethidium bromide. Results are representative of at least 2 experiments.

**Figure 2.2: Partial sequence of canine TLR4.** Amplicons of TLR4 from MDCK cells from Fig. 2.1 were sequenced. The deduced nucleobase sequence was compared to the NCBI reference sequence (accession no: NM_001002950.2).
Figure 2.3: Partial sequence of canine P2X7. Amplicons of P2X7 from LPS-primed canine monocytes and MDCK cells from Fig. 2.1 were sequenced. The deduced nucleobase sequence was compared to the NCBI reference sequence (accession no: NM_001113456.1).

Figure 2.4: Partial sequence of canine NALP3. Amplicons of NALP3 from LPS-primed canine monocytes and MDCK cells from Fig. 2.1 were sequenced. The deduced nucleobase sequence was compared to the NCBI reference sequence (accession no: XM_843284.2).
2.2.2 IL-1β release could not be detected from MDCK cells

To determine if MDCK cells release IL-1β, cells were first incubated in the
absence or presence of 0.1, 1 or 10 µg/mL LPS for 24 hours, and IL-1β in cell-free supernatants was measured using a canine IL-1β ELISA. Cells incubated in the absence of LPS for 24 hours failed to release IL-1β (0 ± 0 pg/mL; n = 3). Similarly LPS at all concentrations failed to induce detectable IL-1β release from these cells (0 ± 0 pg/mL; n = 3). Next, unprimed MDCK cells or cells primed with 10 µg/ml LPS for 4 or 24 hours (in RPMI-1640 or sucrose medium) were incubated with 3 mM ATP, 5 mM ATP or 20 µM nigericin, which induces IL-1β release from human monocytes independently of P2X7 (Sluyter et al., 2004b), for 60 min, and IL-1β in cell-free supernatants was measured by ELISA. None of these conditions induced detectable IL-1β release from MDCK cells (Table 2.2).

Table 2.2: ATP and nigericin do not induce IL-1β release from LPS-primed MDCK cells. Unprimed MDCK cells (Nil) or MDCK cells in complete MDCK medium containing 10 µg/mL LPS were incubated at 37°C for 4 or 24 hours. Cells were then incubated in RPMI-1640 medium (containing 0.1% BSA) or sucrose medium in the absence (Control) or presence of 3 or 5 mM ATP, or 20 µM nigericin at 37°C for 60 min. The amount of IL-1β in cell-free supernatants was quantified using a canine IL-1β ELISA (n = 3).

<table>
<thead>
<tr>
<th>LPS Priming</th>
<th>Assay Medium</th>
<th>IL-1β (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Nil</td>
<td>RPMI-1640</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>4 h</td>
<td>RPMI-1640</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>24 h</td>
<td>RPMI-1640</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>24 h</td>
<td>Sucrose</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>
2.2.3 ATP-induced IL-1β release in canine whole blood requires priming with LPS

Our laboratory has previously shown that ATP induces IL-1β release from LPS-primed monocytes (Peranec, 2011) and has previously used a whole blood assay to measure human IL-1β release (Sluyter et al., 2004b). Therefore this whole blood assay was used to determine if priming with LPS or LTA induces canine IL-1β release. Peripheral blood from three dog breeds was incubated with 100 ng/mL LPS or LTA for 2 hours followed by incubation in the absence or presence of ATP for 30 min or 2 hours. IL-1β release in cell-free supernatants was measured by ELISA. Incubation of blood for 2.5 or 4 hours in the absence of TLR ligand and ATP resulted in IL-1β release from only one of three dogs (Fig. 2.7). Similar results were observed for samples incubated in the presence of LTA or ATP alone (at both 2.5 and 4 hours). In contrast, incubation with LPS alone induced IL-1β release from two of three dogs (at 2.5 hours) and from all three dogs (at 4 hours), however this increase failed to reach statistical significance. Incubation with ATP for 30 min or 2 hours caused a five-fold and significant increase in IL-1β release from LPS-primed blood compared to LPS-primed blood incubated in the absence of ATP (Fig. 2.7). Of note, the amount of ATP-induced IL-1β release varied between dogs, each of which were a different breed (Fig. 2.7). In contrast, ATP did not induce any further IL-1β release from LTA-primed blood compared to LTA-primed blood incubated in the absence of ATP (Fig. 2.7).
Figure 2.7: Priming with LPS but not LTA mediates ATP-induced IL-1β release from canine whole blood. (A,B) Whole blood in incomplete culture medium was incubated in the absence (Nil) or presence of 100 ng/mL LPS or LTA for 2 hours at 37°C/5% CO₂ and then in the absence or presence of 6 mM ATP for (A) 30 min or (B) 2 hours at 37°C/5% CO₂. (A,B) Incubations were stopped by centrifugation and the amount of IL-1β in cell-free supernatants was quantified by ELISA. * P < 0.05 compared to nil and ATP alone.

2.2.4 LPS induces IL-1β release in canine whole blood

To determine if stimulation with LPS or LTA can induce IL-1β release from canine whole blood in the absence of exogenous ATP, canine whole blood was incubated in the absence (control) or presence of 0.01, 0.1, 1 or 10 µg/mL LPS or LTA from 24 hours, and IL-1β release in cell-free supernatant was measured by ELISA. Incubation of blood for 24 hours in the absence of TLR ligands resulted in IL-1β release from one of three dogs (Fig. 2.8). LTA had no effect on IL-1β release from canine whole blood compared to blood incubated in the absence of TLR ligands (control) (Fig. 2.8). In contrast, LPS induced IL-1β release from canine whole blood at all concentrations compared to control (Fig. 50.
A five-fold average increase in IL-1β release was found at 0.01 and 0.1 µg/mL LPS compared to control. At incubations with 1 or 10 µg/mL LPS, the IL-1β concentrations were six- and eight-fold higher, respectively, compared to control. As for ATP-induced IL-1β release (Fig. 2.7), LPS-induced IL-1β release varied between dog breeds (Fig. 2.8).

Figure 2.8: Stimulation with LPS but not LTA induces IL-1β release from canine whole blood. Whole blood in incomplete culture medium was incubated in the absence (Nil) or presence of varying concentrations of LPS or LTA (as indicated) for 24 hours at 37°C/5% CO₂. Incubations were stopped by centrifugation and the amount of IL-1β in cell-free supernatants was quantified by ELISA. ** P < 0.01 compared to nil.

2.2.5 AZ10606120 failed to impair ATP-induced IL-1β release in canine whole blood

To determine if the ATP-induced IL-1β release from LPS-primed canine whole blood was due to P2X7 activation, LPS-primed canine whole blood was
incubated in the absence or presence of 1 µM AZ10606120, followed by incubation in the absence or presence of 6 mM ATP for 30 min. As above (Fig. 2.7), ATP induced significant IL-1β release from LPS-primed blood compared to LPS-primed blood incubated in the absence of ATP (Fig. 2.9). Of note, incubation with AZ10606120 did not alter the amount of IL-1β release in the presence of ATP compared to blood incubated in the presence of ATP alone (Fig. 2.9). In the presence of AZ10606120 ATP-induced IL-1β release however failed to reach significance compared to IL-1β release in the presence of AZ10606120 alone. This was most likely due to the variation in IL-1β release from Labrador 3. IL-1β release in the absence or presence of AZ10606120 (in the absence or presence of ATP) was similar (Fig. 2.9).

**Figure 2.9: AZ10606120 does not impair ATP-induced IL-1β release in LPS-primed canine whole blood.** Whole blood in incomplete culture medium was incubated with 100 ng/mL LPS for 2 hours at 37°C/5% CO₂, with 1 µM AZ10606120 (AZ10) added in the final 15 min as indicated. Samples were then incubated for a further 30 min at 37°C/5% CO₂ in the absence or presence of 6 mM ATP. Incubations were stopped by centrifugation and the amount of IL-1β in cell-free supernatants was quantified by ELISA. * P < 0.05 compared to nil.
2.2.6 AZ10606120 failed to impair LPS-induced IL-1β release in canine whole blood

To determine if the LPS-induced IL-1β release from canine whole blood was due to P2X7 activation, canine whole blood was co-incubated in the absence or presence of 10 µg/mL LPS and in the absence or presence of 1 µM AZ10606120 for 24 hours. As above (Fig. 2.8), LPS induced IL-1β release in blood compared to blood incubated in the absence of LPS however this failed to reach significance (Fig. 2.10). Moreover, co-incubation with AZ10606120 did not alter LPS-induced IL-1β release (Fig. 2.10). IL-1β release in the absence or presence of AZ10606120 (in the absence of LPS) was similar (Fig. 2.9).

Figure 2.10: AZ10606120 does not impair LPS-induced IL-1β release from canine whole blood. Whole blood in incomplete culture medium was incubated with in the absence (Nil) or presence of 10 µg/mL LPS and in the absence or presence of 1 µM AZ10606120 (AZ10) for 24 hours at 37° C/5% CO₂. Incubations were stopped by centrifugation and the amount of IL-1β in cell-free supernatants was quantified by ELISA.
2.3 Discussion

The current study confirms the expression of P2X7 and NALP3-related components in canine monocytes as previously observed by Jalilian (2011). Expression of P2X7, NALP3, caspase-1, IL-1\(\beta\) and IL-18 mRNA was identified by RT-PCR with amplicons corresponding to the predicted size and the identity of each confirmed by sequencing (Fig 2.1 and 2.3-6). Our laboratory recently showed that ATP induces IL-1\(\beta\) release from LPS-primed canine monocytes via activation of the P2X7 receptor (Peranec, 2011). P2X7-induced IL-1\(\beta\) release from LPS-primed canine monocytes however varied between dogs (Peranec, 2011). Therefore, due to this variation, IL-1\(\beta\) release was examined in a whole blood assay as previously used by our group (Sluyter et al., 2004b). This assay is simpler to perform and contain less experimental manipulations. Thus, this method is potentially less prone to experimental variation. The current study demonstrated that ATP also induces IL-1\(\beta\) release in LPS-primed canine whole blood (Fig. 2.7 and 2.9). As for LPS-primed monocytes (Peranec, 2011), ATP-induced IL-1\(\beta\) release in LPS-primed blood varied between dogs. In contrast to LPS-primed monocytes however, a role for P2X7 in ATP-induced IL-1\(\beta\) release in LPS-primed blood could not be established, as 1 µM AZ10606120 failed to impair IL-1\(\beta\) release (Fig. 2.9). This result was unexpected as AZ10606120, as well as other P2X7 antagonists, have been shown to impair 2 mM ATP-induced IL-1\(\beta\) release in LPS-primed canine whole blood (Roman et al., 2009). However, this prior study demonstrated that the sensitivity for AZ10606120 decreases in IL-1\(\beta\) release assays with whole blood compared to ethidium\(^+\) uptake assays with cells in HEPES-buffered saline solutions (Roman et al., 2009). Thus, it is likely that higher concentrations of AZ10606120 are required to impair IL-1\(\beta\)
release from LPS-primed canine whole blood, however due to time constraints this was not examined. Alternatively, the batch of AZ10606120 used in the current study may have expired. Unfortunately no other aliquots of this compound were available for further testing. New AZ10606120 and additional P2X7 antagonists should be examined in the future to determine if P2X7 is responsible for ATP-induced IL-1β release in LPS-primed canine whole blood.

Stimulation with LPS has been shown to induce IL-1β release in human whole blood (Van Der Poll and Lowry, 1997). The current study demonstrated that LPS also induces IL-1β release in canine whole blood in the absence of exogenous ATP (Fig. 2.8 and 2.10). As for ATP-induced IL-1β release in LPS-primed blood (Fig. 2.7 and 2.9), LPS-induced IL-1β release in whole blood varied between dogs. Moreover, a role for P2X7 in LPS-induced IL-1β release in whole blood could not be established, as 1 µM AZ10606120 failed to impair IL-1β release (Fig. 2.10). As for ATP-induced IL-1β release in LPS-primed blood, higher concentrations of a new batch of AZ10606120 and additional P2X7 antagonists should be examined in the future to determine if P2X7 is responsible for LPS-induced IL-1β release in canine whole blood. However, the possibility remains that LPS induces IL-1β release in whole blood via a mechanism independent of P2X7.

Stimulation with LTA has also been shown to induce IL-1β release in human whole blood (Hermann et al., 2002). In the current study LTA failed to induce IL-1β release from canine whole blood following stimulation with ATP over 30 min or 2 hours (Fig. 2.7). Moreover, incubation with LTA over 24 hours failed to induce IL-1β release from canine whole blood (Fig. 2.8). However, these
findings need to be verified by using a positive control, such as examining LTA-induced IL-1β release in human whole blood to ensure the LTA stock is working.

This study also aimed to confirm if MDCK cells express P2X7 and NALP3-related components to possibly provide a model cell line to study the role of P2X7 in kidney epithelial cells and renal disorders. Expression of TLR4, P2X7, NALP3 and caspase-1 mRNA in MDCK cells was confirmed by RT-PCR with amplicons corresponding to the predicted size and the identity of each confirmed by sequencing (Fig. 2.1-5). The expression of IL-1β and IL-18 mRNA in MDCK cells was also demonstrated by RT-PCR and sequencing (Fig. 2.1 and 2.6), contradicting a recent study in our laboratory (Jalilian, 2011). These differences are readily explained by the absence of a positive control in the original experiments by Jalilian (2011), suggesting that the RT-PCR of the cytokines in this previous study failed. Prior to the current study and that of Jalilian (2011), the expression of P2X7, NALP3, caspase-1 and IL-18 had not been directly observed in MDCK cells, although pharmacological blockade of caspase-1 was shown to prevent apoptosis in MDCK cells (Feldenberg et al., 1999) indirectly supporting the presence of this enzyme in these cells. In contrast, TLR4 and IL-1β mRNA had been demonstrated previously in MDCK cells (Gröne et al., 2002, Sun et al., 2007).

Despite the presence of the above molecules in MDCK cells and various attempts, IL-1β release could not be detected from these cells using a commercially available IL-1β ELISA kit (minimum assay sensitivity of 0.053 ng/mL). MDCK cells failed to induce detectable IL-1β release following 24 hour
incubation in the absence or presence of 0.1-10 μg/ml LPS (section 2.2.2). Moreover, neither 60 min incubation with 3 mM ATP, 5 mM ATP nor 20 μM nigericin could induce detectable IL-1β release from MDCK cells primed with 10 μg/ml LPS for 4 or 24 h, or from unprimed MDCK cells (Table 2.2). The inability of ATP to induce IL-1β from MDCK cells could not be solely attributed to the low amount of P2X7 on MDCK cells, as nigericin, which induces human and murine IL-1β release independently of P2X7 activation (Sluyter et al., 2004b, Solle et al., 2001a), also failed to induce IL-1β from MDCK cells. Thus, it is likely that IL-1β and/or other NALP3 inflammasome-associated molecules are present at low amounts in MDCK cells, and that the LPS-priming conditions employed in this study are insufficient to activate or up-regulate these molecules to cause detectable IL-1β release. Moreover, it is possible that other components responsible for the release of IL-1β are absent in MDCK cells.

The presence of NALP3 in MDCK cells is noteworthy. Recent evidence using knockout mice indicates that kidney epithelial-expressed NALP3, independently of inflammasome activation and cytokine release, mediates the inflammation and associated tissue damage during renal ischemia/reperfusion injury (Shigeoka et al., 2010). Whether the autocrine release of ATP and subsequent activation of P2X7 is involved in this process in vivo remains unknown, however MDCK cells can release ATP following mechanical stimulation (Ostrom et al., 2000). Thus these cells may serve as a suitable model cell line to study this potential mechanism further.
In conclusion, the current study demonstrates that P2X7 and NALP3-related components are expressed in canine monocytes and MDCK cells. Further studies however are required to determine whether or not MDCK cells release IL-1β. In addition, ATP induces IL-1β release in canine whole blood and this process requires priming with LPS. Moreover, stimulation with LPS over 24 hours induces IL-1β release in the absence of exogenous ATP. Further studies are required, however, to determine if either or both of these processes require P2X7 activation, or if LTA can induce IL-1β release in canine whole blood.
Chapter 3: Genetic and pharmacological characterisation of a recombinant canine P2X7 receptor

3.1 Materials and Methods

3.1.1 Materials

OPTI-MEM reduced serum medium and Lipofectamine 2000 were from Invitrogen Corporation (Auckland, New Zealand). 3’-O-(4-benzoyl)benzoyl ATP (BzATP), adenosine 5’-diphosphate (ADP), uridine 5’-triphosphate (UTP), α,β-methylene ATP (α,β-meATP), Brilliant Blue G (BBG) and propidium iodide were purchased from Sigma Chemical Company (St Louis, MO). Primers were from Geneworks (Hindmarsh, Australia). EDTA-free Protease Inhibitor Cocktail Tablets were from Roche (Mannheim, Germany). Phenylmethylsulfonyl fluoride (PMSF), n-dodecyl-β-D-maltoside, Tween-20, D-glucose and other general grade chemicals were from Amresco (Solon, OH). Sample buffer and 4-20% Tris-Glycine gel were from NuSep (Lane Cove, Australia). Rabbit anti-rat P2X7 (intracellular epitope) polyclonal Antibody (pAb) and rabbit anti-mouse P2X7 (extracellular epitope) pAb were purchased from Alomone labs (Jerusalem, Israel). Horseradish peroxidise (HRP)-conjugated goat anti-rabbit IgG pAb was from Rockland Immunochemicals Inc. (Gilbertsville, PA). Precision Plus Protein Standards, nitrocellulose membrane and Precision StrepTactin-(HRP) conjugate were from Bio-Rad (Hercules, CA). SuperSignal West Pico Chemiluminescent Substrate was from Pierce (Rockford, IL). Amersham Hyperfilm ECL was from GE Healthcare (Uppsala, Sweden). Developer and fixer were from Kodak (Rochester, NY). AZ10606120, AZ11645373 and A-438079 were obtained from Tocris Bioscience (Ellisville, MO). Adenosine 5’-O-(3-thiotriphosphate) (ATPγS)
and KN-62 were from Alexis Biochemicals (Lausen, Switzerland). Diploma milk powder was from Fonterra Foodservices (Mount Waverly, Australia). cDNA of the canine P2X7 construct (pcP2X7-Ac-N1) (Jalilian, 2011) was provided by Iman Jalilian (University Of Wollongong, Wollongong, Australia). The remainder of materials are described in Chapter 2.

### 3.1.2 Cell lines

The human embryonic kidney (HEK) 293 cell line (originally obtained from the American Type Culture Collection, Manassas, VA) was kindly provided by Dr Leanne Stokes (University of Sydney, Penrith, Australia). The human RPMI 8226 cell line (originally obtained from the European Collection of Cell Cultures, Porton Down, UK) was kindly provided by Associate Professor Marie Ranson (University of Wollongong, Wollongong, Australia). Cells were cultured in DMEM/F12 medium supplemented with 10% foetal calf serum (FCS), 2 mM Glutamax, 100 units/mL penicillin and 10 µg/mL streptomycin (complete culture medium). Cells were incubated at 37°C/5% CO₂ and were passaged two times per week by limited trypsinisation (HEK 293 cells), as required.

### 3.1.3 Sequencing of the canine P2X7 construct

Sequencing reactions were performed as described in section 2.1.5, using the primers described in Table 3.1. Purification of PCR products was performed as described in section 2.1.5. Resulting sequences were compared to the NCBI
reference sequence (Accession no: NP_001106927) using Geneious Pro Software (Biomatters, Auckland, New Zealand).

Table 3.1: Primers used for sequencing of the canine P2X7 cDNA construct.

<table>
<thead>
<tr>
<th>Primer Pair&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Forward Primer 5’-3’</th>
<th>Reverse Primer 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCGCCTGGTGTCCCCATCG</td>
<td>ATCTTCTTGATTCCATTCTCC</td>
</tr>
<tr>
<td>2</td>
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<td>CAGCTGCATCTCCTCTG</td>
</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>4</td>
<td>AGACAATTGTGGAGCCAA</td>
<td>CTAGGTGCTGGGGAGCAAAG</td>
</tr>
</tbody>
</table>

<sup>a</sup>Primers were designed using the online primer design program Primer3 (http://frodo.wi.mit.edu/prime3/).

3.1.4 Transfection of canine P2X7 cDNA into HEK 293 cells

HEK 293 cells were transfected with canine P2X7 cDNA as previously described (Peranec, 2011). HEK 293 cells (0.25 - 0.5 x 10^6 cells/well) in complete culture medium were incubated overnight at 37°C/5% CO₂ in 6-well plates. The following day, Lipofectamine 2000 diluted 1:20 in OPTI-MEM medium was incubated at room temperature for 5 min. Canine P2X7 cDNA was diluted in OPTI-MEM medium (4 µg/120 µL) and incubated as above. Equal volumes of Lipofectamine 2000 and diluted cDNA (P2X7) or OPTI-MEM alone (mock) were incubated at room temperature for 20 minutes prior to incubation with cells. Complete culture medium on cells was replaced with fresh complete culture medium, and 240 µL Lipofectamine 2000/OPTI-MEM mixtures (with or without canine P2X7 cDNA) were added drop-wise to cells. Plates were incubated at 37°C/5% CO₂ for 24 hours. The transfection medium was then removed and replaced with fresh complete culture medium and incubated at 37°C/5% CO₂ for another 24 hours.
3.1.5 Detection of P2X7 protein by immunoblotting

Cell lysates from RPMI 8226 cells (positive control) and mock- or P2X7-transfected HEK293 cells were prepared as previously described (Constantinescu et al., 2010). RPMI 8226 cells were collected by centrifugation (300 x g for 5 min) and washed three times with PBS. HEK 293 cells were washed three times with PBS and harvested by mechanical scraping. Harvested cells (1 x 10^7 cells/mL) were then incubated in lysis buffer (50 mM Bis-Tris, 750 mM 6-aminohexanoic acid, 1% n-dodecyl-β-D-maltoside, 1 mM PMSF, 1 EDTA-free protease inhibitor cocktail tablet, pH 7.0) on ice for 60 min. Cells were sheared 10 times through a 1 mL syringe fitted with an 18 G needle and stored at -20ºC until required. Cell lysates were thawed on ice and centrifuged at 16,000 x g at 4ºC for 10 min. Cleared cell lysates were collected and protein concentrations measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA) according to the manufacturer’s instructions. Cell lysates were then incubated in NuSep reducing sample buffer containing 5% 2-mercaptoethanol at 100ºC for 5 minutes. Proteins were separated in SDS glycine running buffer (2.4 mM Tris base, 19.2 mM Glycine, 3.5 mM SDS) by electrophoresis using 4-20% Tris-Glycine gels (75V for 30 min, then 120V for 60 min) and transferred to nitrocellulose membrane in transfer buffer (2.5 mM Tris base, 19.2 mM glycine, 2% methanol and deionised H₂O) at 4ºC (80V for 90 min). The membrane was then washed in Tris-buffered saline containing Tween-20 (TBST) (500 mM NaCl, 20 mM Tris, 0.2% Tween-20, pH 7.5), and blocked in 20 mL TBST containing 5% milk powder at 4ºC overnight. The membrane was washed three times with TBST over 30 min and then incubated with rabbit anti-P2X7 pAb (diluted 1:500) in TBST containing 5% milk powder
at room temperature for 2 hours. The membrane was washed as above and incubated with HRP-conjugated goat anti-rabbit IgG antibody (diluted 1:1000) and StrepTactin-HRP conjugate (1:5000) in TBST containing 5% milk powder at room temperature for 1 hour. The membrane was washed as above, followed by incubation with chemiluminescent substrate according to the manufacturer’s instructions. The membrane was visualised using Amersham Hyperfilm ECL, following development with developer and fixer.

3.1.6 Detection of fluorescent cation uptake into transfected HEK 293 cells by fixed-time flow cytometry

Agonist-induced fluorescent cation uptake into mock- or P2X7-transfected HEK 293 cells was performed as previously described (Gadeock et al., 2012). HEK 293 cells were washed three times in NaCl medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 0.1% bovine serum albumin and 5 mM glucose pH 7.4) and harvested by mechanical scraping. Cells were pre-incubated at 37°C for 5 min, or for P2X7 antagonist studies in the absence or presence of antagonists (as indicated) at 37°C for 15 min. Cells were then incubated in 25 µM ethidium²⁺ (or 1 µM YO-PRO-1²⁺ or 25 µM propidium²⁺ as stated) and in the absence or presence of ATP for 5 min (unless otherwise stated). The incubations were stopped by the addition of 1 mL of ice-cold MgCl₂ solution (NaCl medium containing 20 mM MgCl₂) and centrifugation (300 x g for 5 min). Cells were washed once in NaCl medium (300 x g for 5 min) and data was acquired by flow cytometry, using a LSR II flow cytometer and FACSDiva software (BD Bioscience, San Diego, CA). A total of 1 x 10⁴ events were collected and the
mean fluorescence intensity (MFI) was measured using an emission window of 575/26 for ethidium\(^{+}\) (or 515/20 for YO-PRO-1\(^{2+}\) or 695/40 for propidium\(^{2+}\)). FlowJo 8.7.1 software (Tree Star Inc., Ashland, CA) was used to analyse collected data.

### 3.1.7 Presentation of data and statistical analysis

Results are expressed as mean ± standard deviation (SD). All graphs were prepared using GraphPad Prism 5 (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 Tukey’s post hoc test) using GraphPad Prism 5 software with \(P < 0.05\) considered significant.
3.2 Results

3.2.1 Canine P2X7 cDNA contains two single nucleotide polymorphisms

Canine P2X7 has previously been cloned from a Beagle (Roman et al., 2009) with near identical amino acid sequence (except a Phe103Leu SNP; rs23314713) compared to the NCBI reference sequence for P2X7 deduced from a Boxer (Accession no: NP_001106927). Our group recently cloned the canine P2X7 receptor from an English Springer Spaniel and inserted into a pcP2X7-Ac-N1 plasmid (Jalilian, 2011). This construct was sequenced and the amino acid sequence was compared to the National Centre for Biotechnology Information (NCBI) canine P2X7 reference sequence. Two non-synonymous (missense) single nucleotide polymorphisms (SNPs) were identified in the P2X7 construct (Fig. 3.1). The first SNP changed the leucine at amino acid position 440 (TTG \rightarrow TTT) to phenylalanine (Leu440Phe; Fig. 3.1). The second SNP changed the proline at amino acid position 452 (CCT \rightarrow TCT) to serine (Pro452Ser; Fig. 3.1). A synonymous SNP was found at amino acid position 415 (CTG \rightarrow TTG).
Figure 3.1: ClustalW alignment of the amino acid sequence from the NCBI canine P2X7 reference sequence and the canine P2X7 construct. The canine P2X7 cDNA construct was sequenced by PCR and the sequence was compared to the NCBI reference sequence (Accession no: NP_001106927). Two non-synonymous SNPs were identified at positions Leu440Phe and Pro452Ser as indicated.

3.2.2 Canine P2X7-transfected HEK 293 cells express P2X7 protein

HEK 293 cells were transfected without (mock) or with canine P2X7 cDNA as previously described within our laboratory (Peranec, 2011). To determine if the mock- or canine P2X7-transfected cells contained P2X7 protein,
immunoblotting of whole cell lysates was performed using anti-P2X7 Abs. Human RPMI 8226 multiple myeloma cells, which contain P2X7 protein (Farrell et al., 2010), were used as a positive control. Using anti-P2X7 polyclonal Abs (targeting either intracellular or extracellular epitope) a major band was detected at 68-72 kDa, which corresponds to the molecular weight of non-glycosylated P2X7, in RPMI 8226 cells and canine P2X7-transfected HEK 293 cells but not mock-transfected HEK 293 cells (Fig. 3.2). A second major band at 155-160 kDa, possibly a P2X7 dimer, was also present in RPMI 8226 cells and canine P2X7-transfected HEK 293 cells, but not in mock-transfected HEK 293 cells (Fig. 3.2). A faint 75 kDa band was detected in mock-transfected HEK 293 cells by the anti-P2X7 polyclonal Ab targeting the intracellular epitope but not the anti-P2X7 polyclonal Ab targeting the extracellular epitope (Fig. 3.2).

3.2.3 ATP induces ethidium$^+$ uptake into canine P2X7-transfected but not mock-transfected HEK 293 cells

To determine if P2X7-transfected HEK 293 cells express functional canine P2X7, mock- or canine P2X7-transfected HEK 293 cells were incubated with ethidium$^+$ in the absence or presence of 1 mM ATP for 5 min and the amount of ethidium$^+$ uptake measured by flow cytometry. As expected, ATP did not induce ethidium$^+$ uptake into mock-transfected HEK 293 cells compared to mock-transfected cells incubated without ATP (Fig. 3.3). In contrast, ATP significantly induced ethidium$^+$ uptake into P2X7-transfected HEK 293 cells compared to P2X7-transfected cells incubated without ATP (Fig. 3.3). Ethidium$^+$ uptake in
the absence of ATP (basal) was similar between the two treated cell lines (Fig. 3.3).

![Figure 3.2: Canine P2X7-transfected HEK 293 cells express P2X7 protein.](image)

Cell lysates of RPMI 8226 cells (positive control), and mock- or P2X7-transfected HEK 293 cells, as well as molecular weight (MW) markers, were separated by electrophoresis and proteins were transferred to a nitrocellulose membrane. Membranes were incubated with rabbit anti-P2X7 pAb (intracellular or extracellular epitope) followed by HRP-conjugated goat-anti-rabbit IgG Ab or streptactin-HRP conjugate and visualised using chemiluminescent substrate and hyperfilm.

**3.2.4 ATP induces ethidium⁺ uptake into canine P2X7-transfected HEK 293 cells in time-dependent manner**

To ensure the ATP incubation time of 5 min is suitable for further pharmacological characterisation of the recombinant P2X7 receptor, canine P2X7-transfected HEK 293 cells were incubated with ethidium⁺ in the absence
or presence of ATP for 2, 4 or 6 min. Ethidium\(^+\) uptake in the absence of ATP (basal) did not change with incubation time (Fig. 3.4). In contrast, ethidium\(^+\) uptake in the presence of ATP increased in a time-dependent manner and remained linear over 6 min (Fig. 3.4). Therefore, 5 min ATP incubations were continued for the studies below.

**Figure 3.3: ATP induces ethidium\(^+\) uptake into canine P2X7-transfected but not mock-transfected HEK 293 cells.** (A, B) Mock- or P2X7-transfected HEK293 cells were incubated with 25 \(\mu\)M ethidium\(^+\) in the absence (basal) or presence of 1 mM ATP at 37°C for 5 min. The incubations were stopped by the addition of ice-cold MgCl\(_2\) solution and centrifugation. The MFI of ethidium\(^+\) uptake was measured by flow cytometry. (A) (left panels) Viable cells were gated by forward and side scatter and (right panels) histograms show relative ethidium\(^+\) uptake into basal (shaded) and ATP-treated (solid line) cells. (B) Results are expressed as the mean ± SD (\(n = 3\)). \(* * * P < 0.001\) compared to corresponding cells incubated without ATP.
Figure 3.4: ATP induces ethidium\(^+\) uptake into canine P2X7-transfected HEK 293 cells in a time-dependent manner. P2X7-transfected HEK 293 cells were incubated with 25 \(\mu\)M ethidium\(^+\) in the absence (Basal) or presence of 1 mM ATP at 37\(^\circ\)C for 2, 4 or 6 min. The incubations were stopped by the addition of ice-cold MgCl\(_2\) solution and centrifugation, and the MFI measured by flow cytometry. Results are expressed as the mean \(\pm\) SD \((n = 3)\). *** \(P < 0.001\) compared to corresponding cells incubated without ATP.

3.2.5 P2X7 agonists induce ethidium\(^+\) uptake into canine P2X7-transfected HEK 293 cells

To characterise the pharmacology of the recombinant canine P2X7 receptor, canine P2X7-transfected HEK 293 cells were first incubated with ethidium\(^+\) in the absence or presence of various concentrations of ATP, BzATP and ATP\(_{\gamma}\)S for 5 min. All three compounds induced ethidium\(^+\) uptake into cells in a concentration-dependent manner (Fig. 3.5). The half-maximal effective concentration (EC\(_{50}\)) values for ATP, BzATP and ATP\(_{\gamma}\)S were 253 \(\mu\)M, 13 \(\mu\)M and 438 \(\mu\)M respectively. Thus, similar to human and rodent P2X7 (Donnelly-
Roberts et al., 2009), the relative agonist potency against canine P2X7 was revealed to be BzATP > ATP > ATPγS.

Canine P2X7-transfected HEK 293 cells were then incubated with ethidium+ in the absence or presence of ATP, P2Y agonists ADP or UTP, or P2X agonist α,β-meATP (all 1 mM) for 5 min. Again, ATP significantly induced ethidium+ uptake into cells compared to cells incubated without ATP (basal) (Fig. 3.6). In contrast, ADP, UTP and α,β-meATP failed to induce ethidium+ uptake into cells compared to cells incubated in the absence of these agonists (basal) (Fig. 3.6).

Figure 3.5: P2X7 agonists induce ethidium+ uptake into canine P2X7-transfected HEK 293 cells in a concentration-dependent manner. P2X7-transfected HEK 293 cells were incubated with 25 µM ethidium+ in the absence or presence of varying concentrations of BzATP, ATP or ATPγS (as indicated) at 37°C for 5 min. The incubations were stopped by the addition of ice-cold MgCl₂ solution and centrifugation. The MFI of ethidium+ uptake was measured by flow cytometry. Ethidium+ uptake was calculated as the difference between agonist and the corresponding basal treatments, and the results expressed as percent maximal ethidium+ uptake compared to 50 µM BzATP-induced ethidium+ uptake. Results are expressed as the mean ± SD (n = 3).
**Figure 3.6: ATP but not ADP, UTP or αβ-meATP induces ethidium⁺ uptake into canine P2X7-transfected HEK 293 cells.** P2X7-transfected HEK 293 cells were incubated with 25 µM ethidium⁺ in the absence (basal) or presence of ATP, ADP, UTP or α,β-meATP (all 1 mM) at 37°C for 5 min. The incubations were stopped by the addition of ice-cold MgCl₂ solution and centrifugation, and the MFI of ethidium⁺ uptake was measured by flow cytometry. Results are expressed as the mean ± SD (n = 3). *** P < 0.001 compared to corresponding cells incubated without agonist (basal).

### 3.2.6 P2X7 antagonists impair ATP-induced ethidium⁺ uptake into canine P2X7-transfected HEK 293 cells

A438079 (Nelson et al., 2006), AZ10606120 (Michel et al., 2008), AZ11645373 (Stokes et al., 2006), Brilliant Blue G (BBG) (Jiang et al., 2000) and KN-62 (Gargett and Wiley, 1997) are all well-established P2X7 antagonists (Syed and Kennedy, 2012). However, the ability of these antagonists to inhibit P2X7 varies between species (Donnelly-Roberts et al., 2009). Therefore, to determine if these antagonists inhibit canine P2X7, canine P2X7-transfected HEK 293 cells were pre-incubated in the absence or presence of varying concentrations of A438079, AZ10606120, AZ11645373, BBG and KN-62, and then incubated
with ethidium\(^{+}\) in the absence or presence of 250 µM (EC\(_{50}\) \(\pm\)) ATP. All five antagonists inhibited ATP-induced ethidium\(^{+}\) uptake into P2X7-transfected HEK 293 cells in a concentration-dependent manner (Fig. 3.7), with IC\(_{50}\) values of 190 nM, 11 nM, 7 nM, 1110 nM and 19 nM respectively. All five antagonists completely inhibited ethidium\(^{+}\) uptake into P2X7-transfected HEK 293 cells at concentrations of 0.1 µM (AZ11645373 and AZ10606120), 1 µM (KN-62) or 10 µM (A-438079 and BBG). Thus, the relative antagonist potency against canine P2X7 was revealed to be AZ11645373 = AZ10606120 > KN-62 > A438079 >> BBG.

Figure 3.7: P2X7 antagonists inhibit ATP-induced ethidium\(^{+}\) uptake into canine P2X7-transfected HEK 293 cells in a concentration-dependent manner. P2X7-transfected HEK 293 cells were pre-incubated in the absence or presence of various concentrations of A438079, AZ10606120, AZ11645373, BBG or KN-62 at 37°C for 15 min. Cells were then incubated with 25 µM ethidium\(^{+}\) in the absence or presence of 250 µM ATP at 37°C for 5 min. The incubations were stopped by the addition of ice-cold MgCl\(_2\) solution and centrifugation. The MFI of ethidium\(^{+}\) uptake was measured by flow cytometry. Ethidium\(^{+}\) uptake was calculated as the difference between agonist and the corresponding basal treatments, and the results are expressed as percent maximal ethidium\(^{+}\) uptake compared to ethidium\(^{+}\) uptake in the absence of antagonist. Results are expressed as the mean ± SD (\(n = 3\)).
3.2.7 ATP induces ethidium\(^+\), YO-PRO-1\(^{2+}\) and propidium\(^{2+}\) uptake into canine P2X7-transfected HEK 293 cells

The P2X7 pore in other species is a non-selective cation channel (North, 2002). Therefore, to determine if canine P2X7 activation mediated the uptake of cations other than ethidium\(^+\), canine P2X7-transfected HEK 293 cells were incubated with ethidium\(^+\) (314 Da), YO-PRO-1\(^{2+}\) (375 Da) or propidium\(^{2+}\) (415 Da) in the absence or presence of ATP for 5 min. ATP induced significant uptake of all three cations compared to cells incubated without ATP (Fig. 3.8).

**Figure 3.8: ATP induces ethidium\(^+\), YO-PRO-1\(^{2+}\) and propidium\(^{2+}\) uptake into canine P2X7-transfected HEK 293 cells.** P2X7-transfected HEK 293 cells were incubated with 25 µM ethidium\(^+\), 1 µM YO-PRO-1\(^{2+}\) or 25 µM propidium\(^{2+}\) in the absence (basal) or presence of 1 mM ATP at 37\(^\circ\)C for 5 min. The incubations were stopped by the addition of ice-cold MgCl\(_2\) solution and centrifugation, and the MFI measured by flow cytometry. Results are expressed as the mean ± SD (n = 3). **P < 0.01; ***P < 0.001 compared to corresponding cells incubated without ATP (basal).
3.3 Discussion

Canine P2X7 was first cloned and pharmacologically characterised by Roman et al. (2009) from a Beagle. The current study characterised recombinant P2X7 cloned from an English Springer Spaniel due to previous data obtained on the native P2X7 receptor from the same breed within our group (Sluyter et al., 2007, Stevenson et al., 2009). The canine P2X7 construct was first sequenced and compared to the corresponding NCBI reference sequence deduced from a Boxer (Accession no: NP_001106927). The P2X7 cDNA sequence was near identical to the reference sequence, containing 595 amino acids (Fig. 3.1). Compared to human P2X7, the cloned canine P2X7 receptor in this study and that of Roman et al. (2009), as well as the reference sequence contained an additional threonine residue at position 284, while the valine residue at position 538 was lacking. Three SNPs were identified in the P2X7 receptor cloned from an English Springer Spaniel, of which two were non-synonymous (missense) SNPs. A change in the amino acid residue at position 452 from proline to serine (Pro452Ser) was identified. This SNP has been reported previously online (www.ensembl.org; rs22315462). It is not known, however, if Pro452Ser alters P2X7 function. Next, a change in the amino acid residue at position 440 from leucine to phenylalanine (Leu440Phe) was identified. This SNP has not been previously reported and it remains unknown if Leu440Phe alters P2X7 function. Lastly, a synonymous SNP was identified in amino acid position 415, changing the nucleobase from C→T; this SNP has also not been previously reported.
The size of the protein encoded by the canine P2X7 construct was examined by via immunoblotting. A major band at 68-72 kDa was detected in human RPMI 8226 cells (positive control) and canine P2X7-transfected HEK 293 cells, but not in mock-transfected HEK 293 cells (Fig. 3.2). This corresponds to the predicted size of glycosylated P2X7. A second major band at 155-160 kDa, which corresponds to the predicted size of P2X7 dimers, was detected in RPMI 8226 cells and canine P2X7-transfected HEK 293 cells, but not in mock-transfected HEK 293 cells (Fig. 3.2). A faint band at 75 kDa was detected in mock-transfected HEK 293 cells using the anti-P2X7 polyclonal Ab targeting the intracellular epitope (Fig. 3.2). This may indicate that HEK 293 cells express small amounts of P2X7. However, the band was not detected in mock-transfected HEK 293 cells using the anti-P2X7 polyclonal Ab targeting the extracellular epitope, suggesting that the band detected by the Ab against the intracellular epitope is caused by non-specific binding. It should be noted however, that despite loading equal amounts of protein onto the gel, this was not confirmed by the use of a housekeeping protein such as actin. Thus, the possibility remains that the absence of P2X7 in mock-transfected cells is due to differences in loading. Moreover, it should be noted that the current study did not employ an empty vector for mock-transfected cells, but rather cells which were treated with Lipofectamine 2000 only. Thus, the possibility remains that transfection of cDNA into HEK 293 cells causes upregulation of endogenous P2X7. However previous studies by our group (Wiley et al., 2003, Stokes et al., 2011) and others (Wilson et al., 2002) have repeatedly shown that transfection of HEK 293 cells with empty vector cDNA does not induce expression of functional P2X7 receptors.
The agonist profile deduced from P2X7 cloned from an English Springer Spaniel was similar to that of native P2X7 in red blood cells from the same breed (Sluyter et al., 2007, Stevenson et al., 2009). ATP induced ethidium\(^{+}\) uptake into canine P2X7-transfected HEK 293 cells compared to cells incubated in the absence of ATP in a time-dependent manner (Fig. 3.4). ATP, BzATP and ATP\(\gamma\)S induced ethidium\(^{+}\) uptake into P2X7-transfected cells in a concentration-dependent manner with a rank order of potency of BzATP > ATP > ATP\(\gamma\)S (Fig. 3.5). The EC\(_{50}\) value obtained for BzATP (13 \(\mu\)M) was similar to that of native P2X7 (Sluyter et al., 2007, Stevenson et al., 2009). Moreover, BzATP was a complete agonist of recombinant canine P2X7 in the current study, as previously found for native P2X7 (Sluyter et al., 2007, Stevenson et al., 2009). The EC\(_{50}\) value obtained for ATP (235 \(\mu\)M) at the recombinant canine P2X7 receptor was also comparable to that of native P2X7 (Sluyter et al., 2007, Stevenson et al., 2009). ATP\(\gamma\)S was the least potent agonist of the recombinant canine P2X7 receptor with an EC\(_{50}\) value of 438 \(\mu\)M. This result is consistent with the study by Sluyter et al. (2007) where ATP\(\gamma\)S was found to be a partial agonist of native canine P2X7 receptors. The potencies of BzATP, ATP and ATP\(\gamma\)S for P2X7 cloned from an English Springer Spaniel and to that for native canine P2X7 from the same breed (Sluyter et al., 2007) is comparable to that obtained for recombinant human P2X7 (Donnelly-Roberts et al., 2009). ADP, UTP and \(\alpha,\beta\)-meATP failed to induce ethidium\(^{+}\) uptake into P2X7-transfected HEK 293 cells (Fig. 3.6). These agonists of other P2 receptors have previously shown to be ineffective at human, rodent and canine P2X7 receptors (Donnelly-Roberts et al., 2009, Sluyter et al., 2007).
A previous study by Roman et al. (2009), which pharmacologically characterised recombinant P2X7 cloned from a boxer, showed comparable EC$_{50}$ values for BzATP (21 µM) and ATP (1 mM) compared to P2X7 cloned from an English Springer Spaniel. The study by Roman et al. (2009), however, demonstrated that BzATP is a partial agonist of canine P2X7 compared to ATP, contrasting the current study. Of note, the current study incubated P2X7-transfected HEK 293 cells with agonists for 5 min, whereas the study by Roman et al. (2009) used a 16 min incubation time. In addition, the current study cloned P2X7 from a different breed compared to the study by Roman et al. (2009). Thus, this may account for some of the differences between the action of BzATP and ATP. It should be noted however, that BzATP is also considered a partial agonist of the Guinea pig P2X7 receptor (Fonfria et al., 2008). In contrast, BzATP is a complete agonist of human P2X7 compared to ATP (Donnelly-Roberts et al., 2009). Therefore, further studies are required to determine whether or not BzATP is a true complete agonist of canine P2X7.

Each of the five P2X7 antagonists studied completely impaired ATP-induced ethidium$^+$ uptake in canine P2X7-transfected HEK 293 cells with a rank order of potency of AZ11645373 = AZ10606120 > KN-62 > A438079 > BBG (Fig. 3.7). The IC$_{50}$ values obtained from AZ11645373 (7 nM), AZ10606120 (11 nM) and KN-62 (19 nM) were comparable to that previously obtained for recombinant canine and human P2X7 (Donnelly-Roberts et al., 2009, Roman et al., 2009). Moreover, the IC$_{50}$ value for KN-62 against recombinant canine P2X7 is similar to that of native P2X7 in red blood cells from English Springer Spaniels (Stevenson et al., 2009). The IC$_{50}$ value of A438079 (190 nM) for the canine P2X7 receptor has not been determined previously, however our group recently
demonstrated that A438079 impairs ATP-induced organic dye uptake into MDCK cells and canine monocytes (Jalilian, 2011). Compared to recombinant human P2X7 (Donnelly-Roberts et al., 2009), A438079 was five-fold more potent at impairing the P2X7 receptor cloned from an English Springer Spaniel. However, the study by Donnelly-Roberts et al. (2009) used a different assay medium (PBS without Mg\(^{2+}\) or Ca\(^{2+}\)), agonist (BzATP) and organic dye (YO-PRO-1\(^{2+}\)) compared to the current study. Thus, the assay variations may account for the potency differences of A438079 at human and canine P2X7 receptors. BBG was the least potent antagonist of the P2X7 receptor cloned from an English Springer Spaniel. The IC\(_{50}\) value (1110 nM) was 23-fold higher compared to the IC\(_{50}\) value for BBG blockade at the canine P2X7 receptor cloned by Roman et al. (2009). Of note, the study by Roman et al. (2009) cloned the P2X7 receptor from a different breed (Beagle), and thus may account for the higher potency of BBG at this receptor compared to that of the current study. Moreover, the IC\(_{50}\) value of BBG at the P2X7 receptor cloned from an English Springer Spaniel was comparable to that previously obtained for recombinant human P2X7 (Donnelly-Roberts et al., 2009). Despite some differences in IC\(_{50}\) values at some P2X7 antagonists, the current study confirms that canine P2X7 can be blocked by numerous P2X7 antagonists as previously observed by our group (Stevenson et al., 2009, Sluyter et al., 2007) and others (Roman et al., 2009). Thus, this suggests P2X7 drugs developed to block P2X7 in humans may also be of therapeutic value in dogs. The P2X7 pore is known to be permeable for organic cations up to 900 Da following prolonged exposure to ATP (Surprenant et al., 1996a). The current study demonstrated that ATP induced ethidium\(^{+}\) (314 Da), YO-PRO-1\(^{2+}\) (375
Da) and propidium$^{2+}$ (415 Da) uptake into P2X7-transfected HEK 293 cells (Fig. 3.8). The canine P2X7 receptor has previously been shown to be permeable to choline$^+$ (104 Da) (Sluyter et al., 2007, Stevenson et al., 2009), ethidium$^+$ (Roman et al., 2009, Stevenson et al., 2009) and YO-PRO-1$^{2+}$ (Jalilian, 2011, Peranec, 2011). ATP-induced propidium$^{2+}$ uptake however has not been previously demonstrated for the canine P2X7 receptor. Of note, P2X7 activation also mediates propidium$^{2+}$ uptake into other cell types, including HEK 293 cells expressing recombinant human P2X7 and rat bone nodule cells (Milius et al., 2007, Panupinthu et al., 2008). Thus propidium$^{2+}$ uptake is not unique to canine P2X7, and this receptor allows uptake of cations at least 415 Da in size.

In conclusion, this study sequenced and pharmacologically characterised canine recombinant P2X7 cloned from an English Springer Spaniel. Sequencing identified two non-synonymous SNPs: Leu440Phe and Pro452Ser. Moreover, the cloned receptor could form functional P2X7 receptors in transfected HEK 293 cells with characteristics similar to native canine and recombinant human P2X7.
Chapter 4: Relative P2X7 function differs amongst dogs: possible role for polymorphisms in the P2RX7 gene

4.1 Materials and Methods

4.1.1 Materials

Allophycocyanin (APC)-conjugated anti-human/canine CD14 monoclonal antibody (mAb) (clone M5E2) was from BioLegend (San Diego, CA). SPHERO Rainbow Fluorescent Particles were from Spherotech (Lake Forest, IL). Mango Taq DNA polymerase, 5X MangoTaq Colorless Reaction Buffer, MgCl₂ and dNTP master mix were from Bioline (Alexandria, Australia). Primers for genomic DNA PCRs and sequencing were from Geneworks (Hindmarsh, Australia). ExoSAP-IT PCR Clean-up Kit was from GE Healthcare (Buckinghamshire, United Kingdom). The remainder of materials are described in Chapter 2 and 3.

4.1.2 Cell lines

Madin-Darby canine kidney (MDCK) epithelial cells were purchased from the European Collection of Cell Cultures (Porton Down, UK). Cells were cultured in DMEM/F12 medium supplemented with 10% foetal calf serum (FCS), 2 mM GlutaMAX, 100 units/mL penicillin and 10 µg/mL streptomycin (complete culture medium). Cells were incubated at 37°C/5% CO₂ and were passaged two times per week by limited trypsinisation, as required.
4.1.3 Collection of blood

Peripheral blood from 30 mixed and pure breed dogs was collected into vacutainer tubes containing lithium heparin (Greiner Bio-One, Frickenhausen, Germany), from either Albion Park Veterinary Hospital (Albion Park, Australia) or Oak Flats Veterinary Clinic (Oak Flats, Australia). Written consent was obtained from informed owners with approval by the University of Wollongong animal ethics committee. Peripheral blood from five human volunteers was collected as above, with written consent and approval by the University of Wollongong human ethics committee.

4.1.4 Isolation of peripheral blood mononuclear cells

Blood from 22 dogs was centrifuged (450 x g for 15 min; with brake off) and the buffy coat was collected. Buffy coats were diluted with Dulbecco’s phosphate-buffered saline (D-PBS), underlaid with Ficoll-Paque PLUS and centrifuged (560 x g for 20 min; with brake off). The peripheral blood mononuclear cells (PBMCs) were collected and washed twice with D-PBS (450 x g for 10 min followed by 300 x g for 5 min) and once in NaCl medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 0.1% bovine serum albumin (BSA) and 5 mM glucose pH 7.4) (300 x g for 5 min). PBMCs were then suspended in NaCl medium at 2 x 10^6 cells/mL.

4.1.5 Detection of P2X7 function in peripheral blood monocytes by flow cytometry

An ATP-induced YO-PRO-1^{2+} uptake assay to measure P2X7 function was
performed as previously described (Peranec, 2011, Jalilian, 2011). PBMCs (in duplicate) were pre-incubated at 37°C for 5 min, then with 1 µM YO-PRO-1²⁺ in the absence or presence of 1 mM ATP for 5 min at 37°C. Incubations were stopped by adding an equal volume of ice-cold MgCl₂ solution (NaCl medium containing 20 mM MgCl₂) and centrifugation (300 x g for 5 min). PBMCs were washed once in NaCl medium (300 x g for 5 min) and incubated with the APC-conjugated anti-CD14 mAb in the dark at room temperature for 15 min. PBMCs were then washed once in NaCl medium (300 x g for 5 min) and the data was acquired by flow cytometry, using a LSR II flow cytometer and FACSDiva software (BD Bioscience, San Diego, CA). A total of 1 x 10⁵ events were collected using emission windows of 515/20 and 660/20 nm for YO-PRO-1²⁺ and APC, respectively. For each sample, the 515/20 nm voltage was adjusted using SPHERO Rainbow Fluorescent Particles to a mean fluorescent intensity of 10000. FlowJo 8.7.1 software (Tree Star Inc., Ashland, CA) was used to analyse collected data. Relative P2X7 function was determined as the difference in YO-PRO-1²⁺ uptake into CD14⁺ cells in the presence and absence of ATP, and is expressed as the mean of duplicate samples.

4.1.6 Amplification of the canine P2RX7 gene

Genomic DNA was isolated from peripheral blood from 30 dogs or MDCK cells using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer’s instructions. The quality of the DNA was assessed by electrophoresis, using a 1% agarose gel at 100 V for 60 min in Tris
acetate-EDTA (TAE) buffer (20 mM acetic acid, 1 mM EDTA, 40 mM Tris, pH 8), and staining with ethidium bromide.

Primers for exons 3, 9, 10 (forward), 11 (reverse), 12 and 13a of the canine P2XR7 gene were obtained from Michelle Peranec (University of Wollongong, Australia). The remaining exon sequences of P2RX7 from Canis familiaris were retrieved from the Ensembl database (http://www.ensembl.org, accession number NP_001106927), together with the flanking intronic regions. These sequences were submitted into the online primer design program Primer3 (http://frodo.wi.mit.edu/ primer3/). Candidate primers were then analyzed for their ability to form hairpins, self- and hetero-dimers using the online OligoAnalyzer 3.1 program (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). The resulting primer sequences are shown in Table 4.1.

Table 4.1 Primer pairs used to amplify and sequence canine P2RX7 gene exons.

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<th>Reverse Primer 5'–3'</th>
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<th>Annealing Temperature</th>
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Each PCR reaction contained 1X Mango Taq Colourless Reaction Buffer, 3.5 mM MgCl$_2$, 100 µM dNTP, 1 µM forward and reverse primer, 1 U Mango Taq DNA Polymerase, 1 µL DNA template and sterile deionised H$_2$O to a final volume of 20 µL. The PCR was performed using a Mastercycler Pro S thermocycler (Eppendorf, Germany) with an initial denaturation at 94 ºC for 1 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 58.6-64.5 ºC (Table 4.1) for 30 sec, and extension at 72ºC for 1 min. After the cycling was completed, a final extension at 72ºC for 5 min was performed, and the reactions returned to room temperature (30ºC for 1 min). PCR products were separated via electrophoresis using a 1.5% agarose gel at 100 V for 1 hour in TAE buffer and ethidium bromide staining.

4.1.7 Sequencing of exons 1-13 of the canine P2RX7 gene

PCR products were purified using 1 µL of diluted (1:4) ExoSAP-IT enzyme per 2.5 µL of PCR product, and the samples incubated using a Mastercycler Pro S thermocycler at 37ºC for 15 min, and then 80ºC for 15 min. Sequencing reactions were performed as described in section 2.1.6, using the primers described in Table 4.1. Purification of ExoSAP-IT treated PCR products was performed as described in section 2.1.6. Resulting sequences were compared to the NCBI reference sequence (Accession no: NM_001113456.1) using Geneious Pro Software (Biomatters, Auckland, New Zealand). Identified single nucleotide polymorphisms (SNPs) were confirmed by repeating PCRs.
4.1.8 Data presentation and statistical analysis

Data is expressed as mean ± SD (and ranges where indicated). All graphs were prepared using GraphPad Prism 5 (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 Tukey’s post hoc test) using GraphPad Prism 5 software with $P < 0.05$ considered significant.
4.2 Results

4.2.1 Relative P2X7 function of monocytes varies between and within dog breeds

To determine whether P2X7 function varies between and within breeds the relative P2X7 function of canine monocytes from 43 dogs has been measured previously within our laboratory (Jalilian, 2011, Peranec, 2011). Using the same flow cytometric assay, ATP-induced YO-PRO-1\textsuperscript{2+} uptake into canine PBMCs was assessed in a further 22 dogs. The gating strategy used to study YO-PRO-1\textsuperscript{2+} uptake into CD14\textsuperscript{+} cells (monocytes) from two Siberian Huskies with either high or low P2X7 function is shown in Figure 4.1. Figure 4.2 illustrates the relative P2X7 function in monocytes from all 65 dogs studied. ATP induced YO-PRO-1\textsuperscript{2+} uptake into monocytes from all dogs examined with an average MFI of 154 ± 86 (range 21-466) and an average coefficient of variation (CV) of 0.20 ± 0.22 (range 0.01-1.41). Relative P2X7 function varied between dog breeds. The highest P2X7 function was observed in monocytes from a Siberian Husky (MFI of 466), which was 22-fold greater than the lowest value obtained from a Labrador (MFI of 21). Relative P2X7 function varied among cross breed dogs, with approximately 13-fold difference between the highest and lowest relative P2X7 function. The relative P2X7 function also varied within some dog breeds. Amongst the pure breed dogs, the highest variation in relative P2X7 function occurred in Siberian Huskies and Labradors, with an 18- and 13-fold difference between the respective highest and lowest values. Furthermore, the relative P2X7 function in Miniature Dachshunds differed six-fold between the respective highest and lowest values.
Differences in relative P2X7 function were also found between five Staffordshire Bull Terriers, with a four-fold difference between the highest and lowest P2X7 function. In contrast, some dog breeds showed less variation in monocyte P2X7 function, such as the American Staffordshire Terrier (MFI of 77 and 85), Border Collie (MFI of 149 and 191), Bull Terrier (MFI of 152 and 193), Golden Retriever (MFI of 153, 159 and 188) and Miniature Fox Terrier (MFI of 80 and 123).

**Figure 4.1: Gating strategy used for determining the relative canine monocyte P2X7 function.** Canine PBMCs from two Siberian Huskies were isolated using Ficoll-Paque density centrifugation and suspended in NaCl medium containing 1 µM YO-PRO-1. PBMCs were then incubated in the absence or presence of 1 mM ATP at 37°C for 5 min. Incubations were stopped by adding ice-cold MgCl₂ solution and centrifugation. Monocytes were incubated with APC-conjugated anti-CD14 mAb and analysed by flow cytometry. (Left panels) PBMCs were gated by forward and side scatter. (Centre panels) Monocytes were gated by relative CD14 expression. (Right panels) Histograms showing basal (shaded) or ATP-induced (solid line) YO-PRO-1²⁺ uptake into monocytes from two Siberian Huskies with low (top) or high (bottom) P2X7 function.
Figure 4.2: Relative P2X7 function of monocytes varies between and within dog breeds. Canine PBMCs from various dog breeds were isolated using Ficoll-Paque density centrifugation and suspended in NaCl medium containing 1 µM YO-PRO-1^2+. PBMCs were then incubated in the absence or presence of 1 mM ATP at 37°C for 5 min. Incubations were stopped by adding ice-cold MgCl2 solution and centrifugation. Monocytes were incubated with APC-conjugated anti-CD14 mAb and analysed by flow cytometry. Relative P2X7 function was determined by the difference in the YO-PRO-1^2+ uptake in the presence or absence of ATP. Results are expressed as the mean of duplicate samples for each dog (n = 65). Broken line represents mean of all samples; solid lines represent mean for each group.

4.2.2 Variation in dog P2X7 function may be due to experimental factors

To determine if sex or age differences are responsible for the variation in
relative P2X7 function between dogs, the function data obtained in Figure 4.2 were grouped according to sex or age. Female dogs showed significantly lower relative P2X7 function compared to male dogs (Fig. 4.3A). However, it should be noted that this difference was largely attributed to four male dogs with relatively high P2X7 function. In contrast, the relative P2X7 function did not significantly alter between groups of animals separated by age (Fig. 4.3B).

Figure 4.3: Relative P2X7 function varies with sex but not with age. Dogs were grouped according to (A) sex or (B) age and compared to the relative monocyte P2X7 function (obtained from Fig. 4.2) compared. Solid line represent mean for each group. * $P < 0.05$ compared to male dogs.
Due to practical difficulties (including ethical restrictions) it was not possible to obtain more than one sample from the same dog. Thus, it is also possible that the variation in relative P2X7 function is due to interassay variation. Therefore, ATP-induced YO-PRO-1$^{2+}$ uptake into human monocytes from five individuals was assessed on four separate occasions over 72-81 days. ATP induced YO-PRO-1$^{2+}$ uptake into monocytes from all subjects investigated with an average MFI of $554 \pm 403$ (range 104-1415) (Fig 4.4). However, similar to dogs, the relative P2X7 function varied between human subjects, with a 14-fold difference between the highest value observed in subject 4 on day 0 (MFI of 1415) and the lowest value observed in subject 3 on day 75 (MFI of 104). Subject 5 showed the greatest variation with time, with a five-fold difference between the highest and lowest relative P2X7 function. Subjects 2 and 3 showed a three-fold difference in relative P2X7 function between the highest and lowest values. Subjects 1 and 4 both showed a two-fold difference in relative P2X7 function between the highest and lowest values. The interassay variations from each individual subject are shown in Table 4.2.

4.2.3 Determination of relative quality and quantity of canine genomic DNA

Genomic DNA was isolated from 120 dogs during the course of two previous studies (Jalilian, 2011, Peranec, 2011) and the current study. To determine the relative quality and quantity of the DNA, samples were separated by agarose gel electrophoresis and stained with ethidium bromide. All samples contained high molecular weight DNA, which indicates intact genomic DNA (Fig. 4.5; data
not shown). Smeared bands were found in most samples, including the examples from a Jack Russel Terrier, Staffordshire Bull Terrier and Sharpei, indicating relatively high concentrations of DNA. Less smeared bands such as examples from a Maltese cross Poodle and Maltese cross, indicate lower relative DNA concentrations.

Figure 4.4: Relative P2X7 function varies between monocytes from human subjects and within the same subject over time. PBMCs were isolated from five human subjects, approximately every 3-4 weeks over a period of 72-81 days (on four separate occasions as indicated), using Ficoll-Paque density centrifugation and suspended in NaCl medium containing 1 μM YO-PRO-1²⁺. PBMCs were then incubated in the absence or presence of 1 mM ATP at 37°C for 5 min. Incubations were stopped by adding ice-cold MgCl₂ solution and centrifugation. Monocytes were incubated with APC-conjugated anti-CD14 mAb, and analysed by flow cytometry. Relative P2X7 function was determined by the difference in the YO-PRO-1²⁺ uptake in the presence or absence of ATP. Results are expressed as the mean of duplicate samples for each individual (n = 5).
Table 4.2: Relative P2X7 function from human monocytes: interassay variations.
The average and range mean fluorescent intensity (MFI) of ATP-induced YO-PRO-1\(^{2+}\) uptake into monocytes from each subject was obtained from Figure 4.4. CV represents coefficient of variation; SD represents standard deviation.

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Figure 4.5: Relative quality and quantity of canine genomic DNA. Genomic DNA was isolated from dog peripheral blood and separated using a 1% agarose gel electrophoresis and stained using ethidium bromide. Results are representative of 120 samples. X represents cross breed; H\(_2\)O represents lane loaded with H\(_2\)O and loading buffer only; MW represents base pair (Bp) markers.

4.2.4 Amplification and sequencing of the canine P2RX7 gene

Variation in relative P2X7 function between humans is largely attributed to
SNPs in the human \textit{P2RX7} gene (Sluyter and Stokes, 2011). To determine if the variation in relative P2X7 function between dogs is caused by SNPs, sequencing of the \textit{P2RX7} gene was carried out in 11 dogs with the highest monocyte P2X7 function, 8 dogs with the lowest monocyte P2X7 function, as well as MDCK cells which express low amounts of functional P2X7 (Jalilian, 2011). Each of the 13 exons were amplified by PCR, separated by agarose gel electrophoresis and stained with ethidium bromide. Due to the short intron between exons 10 and 11 these exons were amplified and sequenced as a single product. The resulting products corresponded to the predicted size of each exon (Fig. 4.6). The amplified DNA was then purified with ExoSAP-IT enzyme, sequenced and compared to the NCBI reference sequence (accession no: NM_001113456.1). A complete sequence was obtained for each exon, except for exon 13, for which the sequence of the last 48 base pairs (or the final 16 amino acid residues of the C-terminus) could not be determined due to poor signal to noise ratio. Four non-synonymous (missense) SNPs were identified in the canine \textit{P2RX7} gene. Firstly, a T→C substitution in either heterozygous or homozygous dosage was identified in exon 3 of eight dogs, which causes a change in the amino acid residue at position 103 from phenylalanine to leucine (Phe103Leu) (Fig 4.7). This SNP corresponded to the previously reported SNP, rs23314713. Second, a C→T substitution in heterozygous dosage was identified in exon 8 of one dog and in MDCK cells, which causes a change in the amino acid residue at position 270 from arginine to cysteine (Arg270Cys) (Fig. 4.8). Third, a G→A substitution in heterozygous dosage was identified in exon 11 of one dog, causing a change in the amino acid residue at position 365 from arginine to glutamine (Arg365Gln) (Fig. 4.9). Fourth, a C→T substitution
in either heterozygous or homozygous dosage was identified in exon 13 of 13 dogs and in MDCK cells, which causes a change in the amino acid residue at position 452 from proline to serine (Pro452Ser) (Fig. 4.10). This SNP corresponded to the previously reported SNP, rs22315462. Finally, a T→C substitution was identified in either heterozygous or homozygous dosage in exon 4 of 12 dogs and in MDCK cells, resulting in a synonymous SNP at Asn131 (Fig. 4.11). This SNP corresponded to the previously reported SNP, rs8660017. The four non-synonymous SNPs are summarised in Fig. 4.12 and the genotypes of all 20 samples examined are summarised in Table 4.3.

**Figure 4.6:** PCR amplification of canine P2RX7 gene exons. Each exon was amplified from genomic DNA (isolated from MDCK cells) by PCR, and the products were separated using a 1.5% agarose gel electrophoresis and stained using ethidium bromide. Similar results were observed for the 19 blood genomic DNA samples examined.
Figure 4.7: Partial sequence from canine P2RX7 exon 3 showing the Phe103Leu SNP. Exon 3 from the canine P2RX7 gene was amplified and sequenced from MDCK cells and 19 dogs with high or low monocyte P2X7 receptor function. A non-synonymous SNP (T→C) in exon 3 resulting in a change from phenylalanine to leucine (Phe103Leu) at amino acid position 103 was identified. Representative partial sequences are shown from three animals with wild type (WT), homozygous (Phe103Leu) and heterozygous (WT/Phe103Leu) genotype.

Figure 4.8: Partial sequence from canine P2RX7 exon 8 showing the Arg270Cys SNP. Exon 8 from the canine P2RX7 gene was amplified and sequenced from MDCK cells and 19 dogs with high or low monocyte P2X7 receptor function. A non-synonymous SNP (C→T) in exon 8 resulting in a change from arginine to cysteine (Arg270Cys) at amino acid position 270 was identified. Representative partial sequences are shown from two animals with wild type (WT) and heterozygous (WT/Arg270Cys) genotype.
Figure 4.9: Partial sequence from canine P2RX7 exon 11 showing the Arg365Gln SNP. Exon 11 from the canine P2RX7 gene was amplified and sequenced from MDCK cells and 19 dogs with high or low monocyte P2X7 receptor function. A non-synonymous SNP (G→A) in exon 11 resulting in a change from arginine to glutamine (Arg365Gln) at amino acid position 365 was identified. Representative partial sequences are shown from two animals with wild type (WT) and heterozygous (WT/Arg365Gln) genotype.

Figure 4.10: Partial sequence from canine P2RX7 exon 13 showing the Pro452Ser SNP. Exon 13 from the canine P2RX7 gene was amplified and sequenced from MDCK cells and 19 dogs with high or low monocyte P2X7 receptor function. A non-synonymous SNP (C→T) in exon 13 resulting in a change from proline to serine (Pro452Ser) at amino acid position 452 was identified. Representative partial sequences are shown from three animals with wild type (WT), homozygous (Pro452Ser) and heterozygous (WT/Pro452Ser) genotype.
Figure 4.11: Partial sequence from canine P2RX7 exon 4. Exon 4 from the canine P2RX7 gene was amplified and sequenced from MDCK cells 19 dogs with high or low monocyte P2X7 receptor function. A synonymous SNP (T→C) in exon 4, corresponding to amino acid position 131, was identified. Representative partial sequences are shown from two animals with wild type (WT) and homozygous (Asn131) genotype.

Figure 4.12: Summary of the non-synonymous SNPs identified in the canine P2RX7 gene. Each exon of the canine P2RX7 gene from MDCK cells and 19 dogs was amplified by PCR and sequenced. Four non-synonymous SNPs were identified: Phe103Leu in exon 3; Arg270Cys in exon 8; Arg365Gln in exon 11; and Pro452Ser in exon 13.

4.2.5 Complete sequencing of exon 3, 8, 11 and 13 of the canine P2RX7 gene

To determine if the identified SNPs in the canine P2XR7 gene alter monocyte P2X7 function, 62 dogs for which we had both relative P2X7 function data and genomic DNA, were screened for the Phe103Leu, Arg270Cys, Arg365Gln and Pro452Ser SNPs. Genotypes were then compared to relative P2X7 function. The Arg270Cys SNP had a prevalence of 2% and an allele frequency of 1% (61 wild
type and one heterozygote). Although a statistical comparison could not be performed due to identifying this SNP in only one dog, this SNP was observed in a Cavalier King Charles Spaniel cross Cocker Spaniel with relatively low monocyte P2X7 function (Fig. 4.13A), as well as MDCK cells (Table 4.3), which express low amounts of functional P2X7 (Jalilian, 2011). The Arg365Gln SNP had a prevalence of 6% and an allele frequency of 3% (58 wild type and four heterozygotes). This SNP was exclusive to Labradors (pure or cross breed), however not all Labradors contained the SNP. A small but non-significant decrease in relative P2X7 function was observed in dogs heterozygous for the SNP compared to wild type dogs (Fig. 4.13B). The Phe103Leu SNP had a prevalence of 50% and an allele frequency of 25% (31 wild type, 13 heterozygotes and 18 homozygotes). A small but non-significant decrease in relative P2X7 function was observed in dogs homozygous for the SNP compared to wild type dogs (Fig. 4.13C). The Pro452Ser SNP had a prevalence of 61% and an allele frequency of 40% (24 wild type, 27 heterozygotes and 11 homozygotes). Again a small but non-significant decrease in relative P2X7 function was observed in dogs homozygous for this SNP compared to wild type dogs (Fig. 4.13D).
Table 4.3: Summary of P2X7 genotypes from dogs with high and low monocyte P2X7 function and MDCK cells. Genotypes from high and low monocyte P2X7 function dogs (obtained from Fig. 4.2) and MDCK cells. X represents cross breed; WT represents wild type; 103 represents the Phe103Leu SNP; 131 represents the Asn131 SNP; 270 represents the Arg270Cys SNP; 365 represents the Arg365Gln SNP; 452 represents the Pro452Ser SNP.

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<td>WT</td>
<td>WT</td>
<td>WT</td>
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<tr>
<td>Golden Retriever X Labrador</td>
<td>31</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>103</td>
<td>WT</td>
<td>WT</td>
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<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT/365</td>
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<tr>
<td>Miniature Dachshund</td>
<td>30</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>103</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
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<td>WT</td>
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<td>WT</td>
<td>WT</td>
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<td>WT</td>
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</table>

| MDCK cells | Breeds                  | Exon 1 | Exon 2 | Exon 3 | Exon 4 | Exon 5 | Exon 6 | Exon 7 | Exon 8 | Exon 9 | Exon 10 | Exon 11 | Exon 12 | Exon 13 |
|------------|-------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Cocker Spaniel |                       | WT    | WT    | WT | 131 | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT/452 |
Figure 4.13: Canine P2RX7 SNPs and relative monocyte P2X7 function. Exons (A) 8, (B) 11, (C) 3, and (D) 13 from 62 dogs were sequenced as described (Fig. 4.7 and 4.10) and the genotype compared to the relative P2X7 function (obtained in Fig. 4.2). WT represents wild type; 103 represents the Phe103Leu SNP; 131 represents the Asn131 SNP; 270 represents the Arg270Cys SNP; 365 represents the Arg365Gln SNP; 452 represents the Pro452Ser SNP. Solid lines represent mean for each group.
4.3 Discussion

The current study combined with previous investigations in our laboratory (Jalilian, 2011, Peranec, 2011) demonstrate that ATP induced YO-PRO-1^{2+} uptake into peripheral blood monocytes from 21 pure breeds and a range of cross breeds (Fig. 4.2). Moreover, this data shows that the relative P2X7 function varied between breeds. Relative P2X7 function also varied within the majority of pure breed dogs; however some breeds showed less variation, such as American Staffordshire Terriers and Golden Retrievers. The reduced variation in this latter group may reflect a sampling error as all dog breeds that had four or more samples showed greater variation in P2X7 function.

The cause of the variation in P2X7 function between and within dog breeds remains unknown. Sequencing of the canine P2RX7 gene identified four non-synonymous SNPs. Three of these SNPs, Arg365Gln, Phe103Leu and Pro452Ser, did not correspond with a gain- or loss-of P2X7 function (Fig. 4.13). The remaining SNP, Arg270Cys, was only observed in a single dog (Cavalier King Charles Spaniel cross Cocker Spaniel) with low P2X7 function (Fig. 4.13) as well as MDCK cells (Table 4.3), which express low amounts of functional P2X7 (Jalilian, 2011). Thus, the Arg270Cys SNP may be a loss-of-function SNP but does not account for the low P2X7 function in the other dogs.

The variation in P2X7 function between dogs may be partly explained by sex. Female dogs showed significantly lower relative P2X7 function compared to male dogs (Fig. 4.3A). However, this difference was largely attributed to four male dogs with relatively high P2X7 function. It has been shown that the oestrogen β-oestradiol impairs P2X7-evoked currents in P2X7-transfected cells.
(Cario-Toumaniantz et al., 1998), suggesting that the difference between dog sexes may be attributed to the absence or presence of specific sex hormones. In contrast to sex, age did not appear to affect relative P2X7 function between dogs (Fig. 4.3B). A previous study has shown, however, that there is a significant increase of P2X7 mRNA levels in mouse microglia from the animal between the ages of 3 days old through to 21 days old, with steady levels after 21 days (Crain et al., 2009). A separate study shows a decline in P2X7 receptor levels in mouse striatum from the age of 3 months through to 10 months (Díaz-Hernández et al., 2009). The current study relied on information given by the dog owners, thus the date of birth may be inaccurate in some instances. Therefore, it cannot be ruled out that age affects relative P2X7 function from canine monocytes.

The variation in P2X7 function between dogs may also be associated with experimental errors. Due to ethical constraints in getting multiple blood samples from the same dogs, the relative P2X7 function of human monocytes was assessed at four separate occasions over a period of 72-81 days using the same assay as for dog monocytes to assess the interassay variation. Similar to dogs, the relative P2X7 function of human monocytes varied between subjects (Fig. 4.4). The relative P2X7 function also varied with time for each subject. A study using ATP-induced ethidium⁺ uptake measurements into human monocytes and in PBMCs over 42 days showed similar variations with time, with approximately a two-fold difference between the highest and lowest values within all three subjects (Jursik et al., 2007). Thus, the variations in relative P2X7 function of human monocytes may indicate natural fluctuations in P2X7 function over time. A second study however using BzATP-induced YO-PRO-1²⁺
uptake into human monocytes in whole blood over 3 months showed less than a two-fold difference between the highest and lowest values within all eight subjects (Korpi-Steiner et al., 2008). Thus it is possible that the variation in P2X7 function in monocytes within canine PBMCs may be due to variation in monocytes obtained following density centrifugation. Ideally, relative P2X7 function from monocytes of each dog should have been investigated at several time-points. However, as noted above it was not possible to obtain more than one sample from the same dog.

Other reasons for variation in P2X7 function between dogs may be attributed to other influences. The medical history of the dogs were not recorded, thus disease status, most notably diseases with an inflammatory component, may have modulated P2X7 function. P2X7 expression, which correlates to P2X7 function (Gu et al., 2000) is known to be altered by inflammatory cytokines (Gadeock et al., 2010, Humphreys and Dubyak, 1998). Alternatively, medications may have also altered relative P2X7 function. Our group has shown that the gout drug probenecid can impair P2X7 function in human leukemic cells (Gadeock et al., 2012). Finally, it is also possible that other P2X receptors such as P2X4 and 5 contribute to the ATP-induced YO-PRO-1²⁺ uptake in canine monocytes. P2X4 and 5 have previously been found in human myeloid cells (Berchtold et al., 1999). However, a previous study in our laboratory demonstrated that the P2X7 antagonists KN-62 and A43879 near-completely impaired ATP-induced YO-PRO-1²⁺ uptake into canine monocytes (Jalilian, 2011), suggesting that P2X7 is predominantly responsible for the dye uptake into these cells.
As discussed above genomic DNA sequencing identified four non-synonymous SNPs in the canine \textit{P2RX7} gene. Thus, compared to the human \textit{P2RX7} gene, in which over 100 non-synonymous SNPs have been identified (http://www.ncbi.nlm.nih.gov/snp), the dog \textit{P2RX7} gene is remarkably non-polymorphic. However this difference is most likely over represented at present, as the \textit{P2RX7} gene has been sequenced and recorded for far more human than canine subjects. The Arg270Cys SNP has not been previously reported, although a similar SNP has been identified at the corresponding position in the human \textit{P2RX7} gene, with a change in the amino acid residue from arginine to histidine (Arg270His, rs7958311). Of note, this SNP results in partial loss of human P2X7 function (Stokes \textit{et al.}, 2010). Thus, the Arg270Cys SNP, which changes a positively charged arginine to the neutral cysteine in canine P2X7, may also potentially reduce P2X7 function. In fact this SNP was found only in one dog and in MDCK cells, both of which had low P2X7 function (Fig. 4.13A; Table 4.3). The Arg365Gln SNP has also not been previously reported in dogs. The Arg365Gln SNP, which changes a positively charged arginine to the neutral glutamine, is located in the intracellular C-terminal domain. In humans, the closest identified SNP to Arg365Gln is the partial loss-of-function SNP Thr357Ser (rs2230911), which results in decreased ATP-induced inward currents, dye uptake and intracellular mycobacteria killing (Shemon \textit{et al.}, 2006). The Arg365Gln SNP was only found in heterozygous dosage and was restricted to Labradors (pure or cross breed) with varying P2X7 function. However, the Arg365Gln SNP did not correspond with altered P2X7 function in canine monocytes (Fig. 4.13B). Of note, the three dogs containing the Arg365Gln SNP with highest P2X7 function also contained the Pro452Ser SNP.
Thus, it is possible that any potential loss-of-function of the Arg365Gln SNP may have been neutralised by a potential gain-of-function of the Pro452Ser SNP. However, the ability of Arg365Gln to alter P2X7 function remains unknown. The Phe103Leu SNP is reported in the Ensembl database (rs23314713), however no functional characterisation of this SNP has been reported. The SNP is located in the extracellular loop of the P2X7 subunit, and thus may have importance for agonist or antagonist binding. However, both phenylalanine and leucine are non-polar amino acids, thus the substitution may be of little biological significance and may explain why P2X7 function was similar between wild-type dogs and dogs heterozygous or homozygous for this SNP (Fig 4.13C). The Pro452Ser SNP is also reported in the Ensembl database (rs22315462), however no functional characterisation has been done. The SNP is located in the C-terminal domain of the P2X7 subunit, and thus may have importance for pore formation. In mouse P2X7, a SNP (Pro451Leu; rs48804829) has been identified in position 451 (which corresponds to position 452 in dog P2X7). This SNP causes a decrease in ATP-sensitivity and impaired P2X7 pore-formation compared to wild type P2X7 (Adriouch et al., 2002). However, the Pro452Ser SNP did not correspond with altered P2X7 function in canine monocytes (Fig 4.13D). This difference in effect between Pro452Ser in the dog and Pro451Leu in the mouse may reflect differences in the side chains of serine and leucine, which contain a hydroxyl (hydrophilic) group and aliphatic (hydrophobic) side chain, respectively.

As noted above, the Arg270Cys and Pro452Ser SNPs were also identified in MDCK cells (originally derived from a Cocker Spaniel) (Table 4.3). Both of these were non-synonymous SNPs present in heterozygous dosage. Our group
previously found that MDCK cells expressed low amounts of functional P2X7 (Jalilian, 2011). Thus, this is consistent with the Arg270Cys SNP being associated with a loss-of P2X7 function. In addition, a synonymous SNP (T→C) was identified in position 131 (Asn131) in MDCK cells. This SNP was present in heterozygous dosage in MDCK cells, as well as in heterozygous or homozygous dosage in 13 of 19 dogs. The biological relevance of this SNP is unknown, but a recent review highlights the potential importance of synonymous mutations to human disease and in particular to translation of proteins (Sauna and Kimchi-Sarfaty, 2011). However, given the presence of Asn131 in both dogs with high and low P2X7 function, it appears unlikely this SNP is responsible for the variation in P2X7 function between dogs.

Our group previously attempted to develop a protocol for amplification and sequencing of exons 3 and 9 of the canine P2RX7 gene (Peranec, 2011, Bakhsh and Sluyter, unpublished). However, only partial sequences (<15% of the total exon) were obtained due to a low signal to background noise ratio. The current study developed protocols for the amplification and sequencing of each of the exons of the canine P2RX7 gene. In addition, a number of experiments were conducted to optimise the sequencing conditions for each exon in order to get high quality sequence data (results not shown). The developed protocols allowed the sequencing of each exon of the P2RX7 gene, and will be of value further sequencing of additional dogs.

In conclusion, this study confirmed that P2X7 function varies between dogs. Moreover, four non-synonymous SNPs were identified in the canine P2RX7 gene, one of which may result in a loss-of P2X7 function. Future studies are
planned using mutant canine P2X7 receptors to investigate if any of these SNPs alter P2X7 function.
Chapter 5: General Discussion

This thesis forms a part of an ongoing study aiming to investigate the role of the canine P2X7 receptor in inflammation and immunity. First, the expression of TLR4, P2X7, NALP3 and caspase-1 was confirmed in Madin-Darby canine kidney (MDCK) cells (Chapter 2). Second, these cells were found to express IL-1β and IL-18 (Chapter 2). Despite various attempts, however, IL-1β release from MDCK cells could not be detected (Chapter 2). In contrast, LPS and ATP but not LTA induced IL-1β release from canine whole blood, although a role for P2X7 in this process could not be established (Chapter 2). Third, recombinant P2X7 cloned from an English Springer Spaniel was sequenced and characterised pharmacologically (Chapter 3). Two non-synonymous (missense) SNPs were identified within this cloned receptor; Leu440Phe and Pro452Ser (Chapter 3). Fourth, relative P2X7 function of monocytes was assessed in 22 dogs, and when combined with previous obtained data from another 43 dogs (Jalilian, 2011, Peranec, 2011) showed that P2X7 function varied between and within breeds. Fifth, sequencing of the canine P2RX7 gene identified four non-synonymous SNPs (Phe103Leu, Arg270Cys, Arg365Gln and Pro452Ser) (Chapter 4). Two of which were also found in MDCK cells: Arg270Cys and Pro452Ser (Chapter 4). Neither the Phe103Leu, Arg365Gln or Pro452Ser SNPs were associated with change of P2X7 function; in contrast, the Arg270Cys SNP was associated with a loss of P2X7 function (Chapter 4).

The identification of TLR4, P2X7, NALP3, caspase-1, IL-1β and IL-18 mRNA in MDCK cells suggests that MDCK cells may provide a useful model cell line to study these molecules in canine epithelial cells. The presence of NALP3,
caspase-1, IL-1β and IL-18 in MDCK cells, however, needs to be confirmed at the protein level. The current study attempted to detect IL-1β release from MDCK cells unsuccessfully. The positive control nigericin, which is known to induce IL-1β release independently of P2X7 activation (Sluyter et al., 2004b), also failed to induce IL-1β release from MDCK cells, indicating that the experimental conditions were insufficient to induce detectable IL-1β release from MDCK cells. Further development of the procedure needs to be performed in order to determine if MDCK cells can release IL-1β or IL-18.

The P2X7 function in MDCK cells has been shown to be relatively low (Jalilian, 2011). This was attributed to low expression of P2X7 protein; however, it is possible that SNPs in the P2RX7 gene contributes to the low function. Two non-synonymous SNPs were identified in MDCK cells: Arg270Cys and Pro452Ser. These SNPs were also found in a King Charles Cavalier cross Cocker Spaniel with low relative monocyte P2X7 function (Chapter 4). However, a comparison between relative P2X7 function of canine monocytes and genotype showed that the Pro452Ser SNP did not correlate with a change in P2X7 function. Moreover, this SNP was also present in the cloned canine P2X7 receptor which formed functional receptors in HEK 293 cells (Chapter 3), thus it is unlikely this SNP codes for a loss of P2X7 function. In contrast, given that the Arg270Cys SNP is associated with low P2X7 function in one dog and MDCK cells, this SNP may result in a loss-of-function in P2X7. Furthermore, given that this dog (a Cocker Spaniel cross) and MDCK cells (originally derived from a Cocker Spaniel) both encode the Arg270Cys SNP it will be of future importance to see if this SNP is confined to Cocker Spaniels and whether it is associated with any specific disorders in this breed.
The dog P2X7 receptor contains 86% sequence identity with the human P2X7 receptor, making the dog a possible future model for studying pharmacokinetics and side effects of P2X7 antagonists. The current study pharmacologically characterised a recombinant P2X7 receptor cloned from an English Springer Spaniel (Chapter 3). Generally, the recombinant P2X7 receptor exhibited a similar pharmacological profile to that of human P2X7 (Michel et al., 2009, Roman et al., 2009, Donnelly-Roberts et al., 2009), and previously studied native and recombinant canine P2X7 (Roman et al., 2009, Sluyter et al., 2007, Stevenson et al., 2009). Although differences between the action of ATP and BzATP as full or partial agonists between the studies of our group and that of Roman et al. (2009) remains to be resolved. P2X7 is an important therapeutic target due to its role in inflammatory responses and pain sensitivity, and its association with numerous diseases including autoinflammatory, neurological, renal disorders and cancer (Jelassi et al., 2011, Goncalves et al., 2006, Skaper et al., 2010, Parvathenani et al., 2003). The current study will help contribute to broader understanding of drug interactions with the canine P2X7 receptor.

The current study showed that relative P2X7 function of monocytes varied between dogs (Chapter 4) consistent with prior data from our group with the same assay (Peranec, 2011, Jalilian, 2011). Of note, the Leu440Phe SNP present in the cloned canine P2X7 receptor was not seen in 62 dog samples. Whether this SNP represents a rare mutation, perhaps confined to English Springer Spaniels, or a mutation arising from the cloning process remains to be determined. Four non-synonymous SNPs were identified in the P2RX7 gene of dogs by sequencing of genomic DNA (Phe103Leu, Arg270Cys, Arg365Gln
and Pro452Ser; Chapter 4). In addition, ATP-induced IL-1β release in whole blood varied between dogs, although a role for P2X7 in this process remains to be established. P2X7-mediated IL-1β release in humans varies due to SNPs in the \( P2RX7 \) gene (Sluyter 2004b, Stokes, 2011). Thus, these differences in canine P2X7 activity may also be due to SNPs in the \( P2RX7 \) gene of dogs. A summary of the five identified SNPs in the canine P2X7 receptor is illustrated in Figure 5.1.

**Figure 5.1: Non-synonymous single nucleotide polymorphisms of the canine \( P2RX7 \) gene.** Five non-synonymous SNPs were identified in the canine \( P2RX7 \) gene encoding the following changes: Phe103Leu (exon 3), Arg270Cys (exon 8), Arg365Gln (exon 11), Leu440Phe (exon 13) and Pro452Ser (exon 13).
Future studies should include the generation of mutant P2X7 receptors by site-directed mutagenesis to explore if any of these SNPs alter P2X7 function. Moreover, future studies of canine monocytes and other cell types investigating the possible contribution of P2X2,4 or 5 to ATP-induced organic dye uptake should also be conducted to determine if these receptors contribute to the variation in ATP-induced YO-PRO-12+ uptake into canine monocytes. Finally, given the emergence of P2X7 splice variants in humans (Feng et al., 2006, Adinolfi et al., 2010, Cheewatrakoolpong et al., 2005) and rodents (Masin et al., 2012, Nicke et al., 2009), the presence of potential P2X7 splice variants in dogs and their potential influence on P2X7-induced pore formation needs to be examined.
References:


NICKE, A. 2008. HomotrimERIC complexes are the dominant assembly state of native P2X7 subunits. *Biochemical and Biophysical Research Communications*, 377, 803-808.


PUREN, A. J., FANTUZZI, G. & DINARELLO, C. A. 1999. Gene expression, synthesis, and secretion of interleukin 18 and interleukin 1β are differentially regulated in


