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ORIGINAL ARTICLE

The $P2X_7$ receptor mediates the uptake of organic cations in canine erythrocytes and mononuclear leukocytes: comparison to equivalent human cell types

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Abstract We previously demonstrated that canine erythrocytes express the $P2X_7$ receptor, and that the function and expression of this receptor is greatly increased compared with human erythrocytes. Using $^{86}Rb^+$ (K^+) and organic cation flux measurements, we further compared $P2X_7$ in erythrocytes and mononuclear leukocytes from these species. Concentration response curves of BzATP- and ATP-induced $^{86}Rb^+$ efflux demonstrated that canine $P2X_7$ was less sensitive to inhibition by extracellular Na^+ ions compared to human $P2X_7$. In contrast, canine and human $P2X_7$ showed a similar sensitivity to the $P2X_7$ antagonists KN-62 and Mg^{2+}. KN-62 and Mg^{2+} also inhibited ATP-induced $choline^+$ uptake into canine and human erythrocytes. BzATP and ATP but not ADP or NAD induced $ethidium^+$ uptake into canine monocytes, T- and B-cells. ATP-induced $ethidium^+$ uptake was twofold greater in canine T-cells compared to canine B-cells and monocytes. KN-62 inhibited the ATP-induced $ethidium^+$ uptake in each cell type. $P2X_7$-mediated uptake of organic cations was 40- and fivefold greater in canine erythrocytes and lymphocytes (T- and B-cells), respectively, compared to equivalent human cell types. In contrast, $P2X_7$ function was threefold lower in canine monocytes compared to human monocytes. Thus, $P2X_7$ activation can induce the uptake of organic cations into canine erythrocytes and mononuclear leukocytes, but the relative levels of $P2X_7$ function differ to that of equivalent human cell types.

Keywords P2X receptor · Purinergic receptor · Red blood cell · White blood cell · Dog

Abbreviations

BSA	bovine serum albumin
ADP	adenosine 5′-diphosphate
ATP	adenosine 5′-triphosphate
BzATP	2′- and 3′-0(4-benzoylbenzoyl) ATP
DMSO	dimethyl sulphoxide
FITC	fluorescein isothiocyanate
mAb	monoclonal antibody
NAD	β-nicotinamide adenine dinucleotide
SEM	standard error of the mean

Introduction

$P2X_7$ receptors are ligand-gated ion channels comprised of trimeric subunits within the plasma membrane [1] that interact with intracellular macromolecules [2] to regulate various membrane and intracellular responses [3]. Activation of $P2X_7$ by adenosine 5′-triphosphate (ATP) or the most potent agonist, 2′- and 3′-0(4-benzoylbenzoyl) ATP (BzATP) results in the movement of K^+, Ca^{2+}, Na^+ and large organic cations across the plasma membrane [4]. Moreover, $P2X_7$ activation induces various downstream events including the release of pro-inflammatory interleukin-1β and -18 [5], formation of reactive oxygen and nitrogen species [6], killing of intracellular mycobacterium and

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chlamydiae [7], and cell death [8]. $P2X_7$ has become a potential therapeutic target in a number of inflammatory, infectious, neurological and bone disorders [9–11], but progress in this field is limited by the difficulty in purifying large quantities of $P2X_7$ and the absence of a structural model. Much of the available structural data on $P2X_7$ has been derived from site-directed mutagenesis studies of recombinant $P2X_7$ and other P2X receptors [12], and comparisons of $P2X_7$ between species [11].

$P2X_7$ is predominately expressed on blood, bone and epithelial cells [13]. Studies with human peripheral blood using anti-$P2X_7$ antibodies and cation flux measurements demonstrate that $P2X_7$ is present, in increasing order of magnitude, on erythrocytes, B-cells, T-cells, natural killer cells, monocytes, dendritic cells and macrophages [14–16]. Similar studies in other species and comparisons between equivalent cell types from different species are limited. Recently, we have observed that canine erythrocytes express the $P2X_7$ and that the relative function of this receptor is at least 40-fold greater than that of human erythrocytes [17]. This difference in receptor function corresponded to increased amounts of $P2X_7$ present on the plasma membrane of canine erythrocytes compared to human erythrocytes [17]. The pharmacological properties of canine $P2X_7$ however have only been partly characterised. Canine $P2X_7$ is activated by BzATP and ATP, and to a lesser extent 2-methylthio-ATP and adenosine 5′-0-(3-thiotriphosphate), but not adenosine 5′-diphosphate (ADP) nor uridine 5′-triphosphate [17]. Moreover, canine $P2X_7$ can be inhibited by KN-62, oxidised ATP, Brilliant blue G or a phthalazinamine derivative, termed compound 18, each applied at concentrations conventionally used to impair either human or rat $P2X_7$ [17, 18]. Whether canine $P2X_7$ can be impaired by ions such as Na^+ and Mg^{2+}, as shown for other mammalian $P2X_7$ receptors [4], is unknown. Therefore, using measurements of nucleotide-induced $^{86}Rb^+$ (K^+) efflux from erythrocytes, we further characterised and compared the pharmacological properties of canine and human $P2X_7$. Moreover, we compared the relative amounts of functional $P2X_7$ on erythrocytes and mononuclear leukocytes from both species using measurements of nucleotide-induced uptake of the organic cations, choline$^+$ and ethidium$^+$, respectively.

Experimental procedures

Reagents ATP, BzATP, ADP, β-nicotinamide adenine dinucleotide (NAD), ethidium bromide, $MgCl_2$, dimethyl sulphoxide (DMSO), Drabkin's reagent, bovine serum albumin (BSA) and other general reagent grade chemicals were from Sigma (St. Louis, MO). KN-62 was from Alexis Biochemicals (Lausen, Switzerland). Ficoll-Paque PLUS was from GE Healthcare Biosciences AB (Uppsala, Sweden). Rubidium-86 ($^{86}Rb^+$), SOLVABLE™ tissue solubilizer and Ultima Gold™ were from PerkinElmer Life Sciences (Boston, MA). [*Methyl*-^{14}C]choline Cl was from GE Healthcare UK Ltd (Little Chalfont, Buckinghamshire, UK). Di-*n*-butyl phthalate and di-isooctyl phthalate (BDH Chemicals, Poole, England) were blended 80:20 (*v*:*v*) to give an oil mixture of density 1.03 g/ml. Fluorescein isothiocyanate (FITC)-conjugated murine anti-human CD3 (clone UCHT1), CD14 (clone TUK4), which cross-reacts with canine CD14, and CD19 (clone HD37) monoclonal antibodies (mAb) were from Dako (Carpinteria, CA). Murine anti-canine CD3 (clone CA17.2A12) and B-cell (clone CA2.1D6) mAb were from AbD Serotec (Serotec, Oxford, United Kingdom). FITC-conjugated, $F(ab)_2$ fraction sheep anti-murine immunoglobulin secondary antibody was from Chemicon (Boronia, Australia).

Erythrocytes and mononuclear leukocytes This study was approved by the Westmead Hospital Animal Ethics (Westmead, Australia) and Sydney West Area Health Service Human Ethics (Penrith, Australia) Committees. Peripheral blood was collected in heparin-containing vacutainer tubes from adult English Springer spaniels and healthy adult human volunteers. Blood was centrifuged at 580×*g* for 15 min, and the plasma and platelets discarded, and leukocytes and the upper 10% of erythrocytes collected to obtain buffy coats. The remaining erythrocytes were washed twice in NaCl medium (147.5 mM NaCl, 2.5 mM KCl, 5 mM D-glucose, 0.1% BSA, 20 mM HEPES, pH 7.5) at 450×*g* for 5 min. To isolate mononuclear leukocytes, buffy coats were diluted in five volumes of NaCl medium, underlaid with Ficoll-Paque PLUS and centrifuged at 580×*g* for 30 min. Isolated mononuclear leukocytes were washed in NaCl medium at 450×*g* for 10 min.

$^{86}Rb^+$ efflux measurements Erythrocytes were loaded with $^{86}Rb^+$ (5 μCi/ml) at a haematocrit of 40% (*v*:*v*) in NaCl medium for 4 h at 37 °C. Cells were then washed three times with ice-cold NaCl medium at 4 °C (1800×*g* for 3 min) and resuspended in either NaCl medium or KCl medium (150 mM KCl, 5 mM D-glucose, 0.1% BSA, 20 mM HEPES, pH 7.5) at a final haematocrit of 5% (*v*:*v*). $^{86}Rb^+$-loaded erythrocyte suspensions were incubated in the absence or presence of ATP or BzATP at 37 °C. At 4 (canine) or 60 (human) min, 1 ml samples were overlaid on 300 μl of phthalate oil mixture and centrifuged at 8,000×*g* for 30 s. Previous studies have demonstrated that ATP-induced cation fluxes in canine and human erythrocytes at these time points are within the linear response ranges and allow for the detection of significant differences between treatments [16, 17, 19]. For studies using KN-62 or Mg^{2+}, $^{86}Rb^+$-loaded erythrocytes resuspended in NaCl medium

were pre-incubated in the presence or absence of KN-62 or an equal volume of DMSO, or $MgCl_2$ or an equal volume of H_2O (as indicated) for 5 min at 37 °C before incubation in the absence or presence of ATP. The level of radioactivity in cell lysates (lysed with an equal volume of 0.4% saponin) and supernatants was measured using a Wallac (Turku, Finland) 1480 Wizard 3″ Automatic Gamma Counter.

Choline$^+$ uptake measurements Erythrocytes were washed once with choline Cl medium (150 mM choline Cl, 5 mM D-glucose, 0.1% BSA, 20 mM HEPES, pH 7.5). Erythrocyte suspensions (2 ml) at a final haematocrit of 5% (*v*:*v*) in choline Cl medium containing [*methyl*-^{14}C]choline$^+$ (1 μCi/ml) were pre-incubated in the presence or absence of 1 μM KN-62 or an equal volume of DMSO, or 10 mM $MgCl_2$ or an equal volume of H_2O for 5 min at 37 °C, before incubation in the absence or presence of 1 mM ATP for 12 (canine) or 60 (human) min at 37 °C. Cells were then washed three times with ice-cold isotonic saline containing 1 mM choline Cl (1,800×*g* for 60 s). To determine the level of [^{14}C]choline$^+$ uptake, 50 μl aliquots of erythrocyte pellets were incubated with 1 ml SOLVABLE tissue solubilizer for 1 h at 60 °C, followed by incubation with 100 μl 100 mM ethylenediaminetetraacetic acid and 500 μl 30% H_2O_2 for 30 min at room temperature and then for a further 30 min at 60 °C, before the addition of 10 ml Ultima Gold™. The level of radioactivity was measured using a Packard (Meriden, CT) Tri-Carb 2100TR Liquid Scintillation Analyser. The haemoglobin content of erythrocyte pellets (50 μl) was measured spectrophotometrically using Drabkin's reagent according to the manufacturer's instructions. The haemoglobin content was used to determine the level of choline$^+$ uptake per ml of canine and human erythrocytes based on mean cell haemoglobin concentrations of 5.4 and 5.2 μmol Hb/ml, respectively.

Ethidium$^+$ uptake measurements Mononuclear leukocytes were pre-labelled with FITC-conjugated mAb or mAb with FITC-conjugated secondary antibody, and resuspended in 1 ml KCl medium (2×10^6 cells/ml) at 37 °C. At 0 s, 25 μM ethidium$^+$ was added, followed 40 s later by the addition of ATP as indicated. In some experiments, cells were pre-incubated for 5 min at 37 °C in the presence of 1 μM KN-62 or an equal volume of DMSO. Data was collected using a Becton Dickinson (San Jose, CA) FACSCalibur flow cytometer over 6 min at 37 °C with constant stirring using a Cytek (Fremont, CA) Time Zero Module. The linear mean channel of ethidium$^+$ fluorescence intensity for each gated population over successive 5 s intervals was analysed by WinMDI 2.8 Software developed by Joseph Trotter (http://www.scripps.edu) and plotted against time. Agonist-induced ethidium$^+$ uptake was quantified as the difference in arbitrary units of area under the uptake curves in the presence and absence of agonist in the first 5 min of incubation as described [20].

Statistical analyses Results are represented as means with standard error of the mean (SEM). Differences between treatments were compared using either the unpaired Student's *t* test for single comparisons or one-way analysis of variance for multiple comparisons with Bonferroni's post test using Prism 5 for Mac OS X Version 5.0a (GraphPad Software, San Diego, CA) with $P<0.05$ considered significant. Concentration response curves were plotted, and EC_{50} values and Hill coefficients determined using Prism 5 for Mac OS X Version 5.0a.

Results

Canine $P2X_7$ is less sensitive to inhibition by extracellular Na^+ ions compared to human $P2X_7$ The relative potency of BzATP and ATP on human and rodent $P2X_7$ is reduced in the presence of extracellular Na^+ ions [21, 22], however, the effect extracellular Na^+ ions on canine $P2X_7$ is unknown. Therefore, the BzATP- and ATP-induced efflux of $^{86}Rb^+$ from canine and human erythrocytes in KCl medium (nominally free of Na^+ ions) and NaCl medium was measured (Fig. 1). The data is summarised in Table 1. As previously observed [17], the rate of BzATP- and ATP-induced $^{86}Rb^+$ efflux from canine erythrocytes was approximately 100-fold more than that from human erythrocytes. The EC_{50} values for both canine and human $P2X_7$ to either agonist were higher in the presence of extracellular Na^+ ions. Moreover, efflux rates and maximum responses to BzATP and ATP were lower in NaCl medium compared to KCl medium for both canine and human receptors. Canine $P2X_7$ however was less sensitive to the inhibitory effects of extracellular Na^+ ions. The average rates of BzATP- and ATP-induced $^{86}Rb^+$ efflux from canine erythrocytes were 5% and 28%, respectively, slower in NaCl medium compared to KCl medium; while the average rates of BzATP- and ATP-induced $^{86}Rb^+$ efflux from human erythrocytes were 29% and 56%, respectively, slower in NaCl medium compared to KCl medium. In addition, the average maximum responses for BzATP- and ATP-induced $^{86}Rb^+$ efflux from canine erythrocytes were 15% and 21%, respectively, lower in NaCl medium compared to KCl medium; while the average maximum responses for BzATP- and ATP-induced $^{86}Rb^+$ efflux from human erythrocytes were 24% and 56%, respectively, lower in NaCl medium compared to KCl medium.

Sensitivity to KN-62 and extracellular Mg^{2+} is similar between canine and human $P2X_7$ We have previously

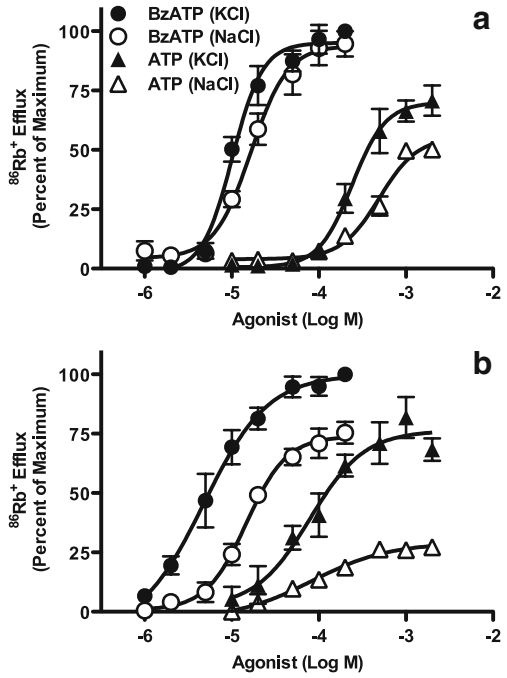

Fig. 1 BzATP and ATP induce $^{86}Rb^+$ efflux from canine and human erythrocytes in a concentration-dependent fashion. $^{86}Rb^+$ loaded (**a**) canine or (**b**) human erythrocytes were incubated at 37 °C for 4 or 60 min respectively in KCl or NaCl medium containing varying concentrations of BzATP or ATP as indicated. $^{86}Rb^+$ release, calculated as the difference in percentage release between 0 and 4 or 60 min, respectively, was used to determine the percentage of maximal response to 200 μM BzATP. Results are expressed as the mean (SEM; n=3)

determined that the human $P2X_7$ antagonist, KN-62 applied at 1 μM can inhibit ATP-induced $^{86}Rb^+$ effluxes canine erythrocytes [17], however the relative potency of this compound was not determined. Therefore, $^{86}Rb^+$-loaded canine and human erythrocytes in KCl medium were pre-incubated with varying concentrations of KN-62 for 5 min and the ATP-induced $^{86}Rb^+$ effluxes measured as above. In these experiments, canine and human erythrocytes were incubated with 250 and 100 μM ATP respectively, concentrations approximate to the EC_{50} values obtained above (Table 1). KN-62 inhibited ATP-induced $^{86}Rb^+$ efflux from canine and human erythrocytes in a concentration-dependent fashion, and was maximal at 1 μM with 100% inhibition in both species (Fig. 2a). The IC_{50} values for KN-62 for canine and human erythrocytes were similar (4±1 nM verses 19±9 nM, respectively; P=0.16).

Mg^{2+} ions are well known to inhibit human and rat $P2X_7$ [21, 23, 24], however the effect of these ions on canine $P2X_7$ is unknown. Therefore, using an approach similar for KN-62, $^{86}Rb^+$-loaded erythrocytes in KCl medium were pre-incubated with varying concentrations of Mg^{2+} for 5 min and the ATP-induced $^{86}Rb^+$ effluxes measured. Mg^{2+} inhibited ATP-induced $^{86}Rb^+$ efflux from both canine and human erythrocytes in a similar and concentration-dependent fashion, and was maximal at 1 mM with near 100% inhibition in both species (Fig. 2b). Again, the IC_{50} for Mg^{2+} for canine and human erythrocytes were similar (113±24 μM verses 52±22 μM respectively; P=0.13).

KN-62 and Mg^{2+} inhibit ATP-induced choline$^+$ influx into canine and human erythrocytes We have previously shown that ATP can induce the uptake of choline$^+$ into both canine and human erythrocytes [17]; however, a direct role for $P2X_7$ in this process was not demonstrated. Therefore, canine and human erythrocytes were resuspended in choline Cl medium (containing [^{14}C]choline$^+$) and incubated with 1 mM ATP for 12 and 60 min, respectively. ATP induced choline$^+$ uptake into erythrocytes from both species incubated in the absence of KN-62 and Mg^{2+} (Fig. 3). The rate of uptake was approximately 40-fold greater in canine erythrocytes compared to human erythrocytes. KN-62 (1 μM) inhibited the ATP-induced choline$^+$ influx into canine and

Table 1 BzATP- and ATP-induced $^{86}Rb^+$ efflux from canine and human erythrocytes in KCl or NaCl medium

	Canine				Human			
	Rate[a]	Maximum[b]	EC_{50}[c]	n_H[d]	Rate[a]	Maximum[b]	EC_{50}[c]	n_H[d]
BzATP/KCl[e]	13.4±0.6	100	11±1	2.5±0.4	0.14±0.06	100	6±2	1.6±0.3
BzATP/NaCl[e]	12.7±1.1	95±5	17±2	2.3±0.2	0.10±0.04	75±5	15±1	2.2±0.5
ATP/KCl[e]	9.5±1.3	71±6	256±41	2.4±0.2	0.09±0.04	82±9	82±14	1.5±0.0
ATP/NaCl[e]	6.8±0.7	50±3	485±78	2.7±0.7	0.04±0.01	27±1	91±34	1.7±0.8

The data were derived from Fig. 1 and are expressed as mean ± SEM (n=3)

[a] Percent $^{86}Rb^+$ release per min (basal subtracted)

[b] The maximum response compared to erythrocytes suspended in KCl medium containing 200 μM BzATP for each species

[c] EC_{50} values expressed as μM

[d] Hill coefficient

[e] Canine or human erythrocytes were incubated with BzATP or ATP in KCl or NaCl medium as indicated

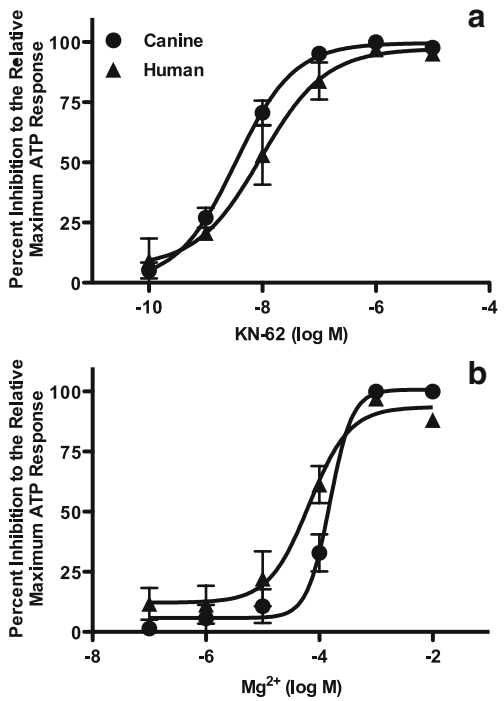

Fig. 2 KN-62 and Mg^{2+} inhibit ATP-induced $^{86}Rb^{+}$ efflux from canine and human erythrocytes in a concentration-dependent fashion. $^{86}Rb^{+}$ loaded canine or human erythrocytes in KCl medium were pre-incubated at 37 °C for 5 min with varying concentrations of **a** KN-62 and **b** Mg^{2+} as indicated. The canine and human erythrocytes were then incubated at 37 °C for 4 min with 250 μM ATP or 60 min with 100 μM ATP, respectively. $^{86}Rb^{+}$ release, calculated as the difference in percentage release between 0 and 4 or 60 min, respectively, in the absence and presence of either antagonist was used to determine the percent inhibition relative to the maximum ATP response. Results are expressed as the mean (SEM; n=3)

human erythrocytes by 94±0% and 84±8%, respectively (Fig. 3a, c). Similarly, Mg^{2+} (10 mM) inhibited the ATP-induced choline^{+} influx into canine and human erythrocytes by 100±0% and 84±3%, respectively (Fig. 3b, d). Both KN-62 and Mg^{2+} had minimal effect on the basal choline^{+} uptake.

ATP-induced ethidium^{+} uptake differs between canine and human monocytes and lymphocytes We have previously demonstrated that the ATP-induced ethidium^{+} uptake, via activation of $P2X_7$, is approximately fivefold greater in human monocytes compared to human T- and B-cells [15, 20]. In addition, we have previously shown that the ATP-induced ethidium^{+} uptake is approximately threefold lower in canine monocytes compared to human monocytes [17]. Whether canine T- and B-cells express $P2X_7$, and how the function of this receptor on these cells compares to canine monocytes or human leukocytes are unknown. Therefore, the ATP-induced ethidium^{+} uptake into canine and human mononuclear leukocytes was measured as described [20]. ATP (1 mM) induced ethidium^{+} uptake into all three canine and human leukocyte subpopulations (Fig. 4; Table 2). ATP-induced ethidium^{+} uptake into canine T-cells was approximately twofold greater than that into canine monocytes and B-cells. In contrast, but similar to our previous observations [15, 20], ATP-induced ethidium^{+} uptake was approximately eightfold greater in human monocytes compared to human T- and B-cells, with the ATP-induced ethidium^{+} uptake partially greater in human T-cells than in human B-cells. Comparisons between the species demonstrated that ATP-induced ethidium^{+} uptake in canine monocytes was approximately threefold lower compared to human monocytes. Conversely, ATP-induced ethidium^{+} uptake was approximately fivefold higher in canine T- and B-cells compared to human T- and B-cells.

$P2X_7$ activation mediates ATP-induced ethidium^{+} uptake into canine leukocytes To confirm that $P2X_7$ activation mediates ATP-induced ethidium^{+} uptake into canine leukocytes, cells were incubated with 200 μM BzATP, as well as 1 mM ADP, which does not stimulate $P2X_7$ [4]. BzATP induced ethidium^{+} uptake into canine monocytes, T- and B-cells with values (20,574±2,056, 28,426±624 and 15,478±630 arbitrary units of uptake respectively; Fig. 5) similar to that of ATP-induced ethidium^{+} uptake (Table 2). In contrast, the ability of ADP to induce ethidium^{+} uptake into canine monocytes, T- and B-cells was negligible (221±138, 82±23 and 33±17 arbitrary units of uptake, respectively; Fig. 5).

Previous studies by Seman et al. have demonstrated that NAD, at a maximum concentration of 300 μM, can induce cation fluxes in murine T-cells via ADP-ribosylation of $P2X_7$ [25]. Therefore, the ability of NAD to induce ethidium^{+} uptake in canine leukocytes was also examined. In contrast to ATP and BzATP, the ability of 300 μM NAD to induce ethidium^{+} uptake into canine monocytes, T- and B-cells was negligible (32±26, 10±12 and 38±2 arbitrary units of uptake respectively; Fig. 5).

Finally, to confirm that $P2X_7$ activation mediates ATP-induced ethidium^{+} uptake into canine leukocytes, cells were pre-incubated with 1 μM KN-62 and the ATP-induced ethidium^{+} uptake measured. KN-62 inhibited the ATP-induced ethidium^{+} uptake into canine monocytes, T- and B-cells by 99±0%, 98±1% and 95±1%, respectively (Fig. 6). KN-62 had no effect on basal ethidium^{+} uptake (results not shown).

Discussion

This study confirms previous reports that $P2X_7$ is present on canine and human erythrocytes [16–19, 26, 27], and extends our knowledge regarding the pharmacological characteristics of canine $P2X_7$. Initially, we used $^{86}Rb^{+}$

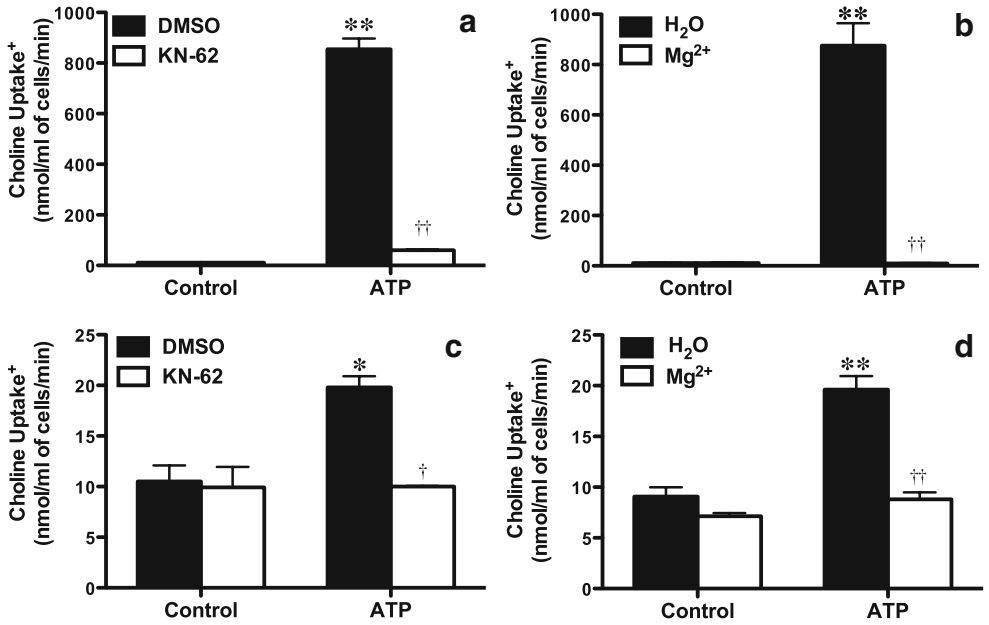

Fig. 3 KN-62 and Mg^{2+} inhibit ATP-induced choline$^+$ uptake into canine and human erythrocytes. **a**, **b** Canine and **c**, **d** human erythrocytes resuspended in 150 mM choline Cl medium containing [*methyl*-^{14}C]choline$^+$ (1 μCi/ml) were pre-incubated at 37 °C for 5 min with **a**, **c** DMSO or 1 μM KN-62, or **b**, **d** H_2O or 10 mM Mg^{2+}. The canine and human erythrocytes were then incubated at 37 °C for 12 or 60 min, respectively, in the absence or presence of 1 mM ATP. The level of choline$^+$ uptake at each time point was determined as nanomole choline$^+$ per millilitre of cells per minute. Results are expressed as the mean (SEM; n=3). *P<0.01, **P<0.001 to Control; $^{\dagger}P$ < 0.01, $^{\dagger\dagger}P$<0.001 to corresponding ATP-treated sample. Results are expressed as the mean (SEM; n=3)

efflux measurements from canine and human erythrocytes to characterise and compare $P2X_7$ in these two species. It is worth noting however, that this assay most likely examines the large permeability state (pore) of $P2X_7$ rather than the channel, as ATP-induced $^{86}Rb^+$ efflux is impaired in human erythrocytes from subjects coding the Glu^{496}Ala polymorphism [16] which impairs $P2X_7$ pore formation [28], but not the ion channel properties of the receptor [29]. Consistent with our previous observations [17], the rate of BzATP- and ATP-induced $^{86}Rb^+$ efflux from canine erythrocytes was approximately 100-fold greater than from human erythrocytes. Moreover, as previously observed for human and rat $P2X_7$ [23, 30], ATP was a partial agonist compared to BzATP. This phenomenon however is not common to all mammalian species. Both BzATP- and ATP-induced responses for murine $P2X_7$ are similar in magnitude [31], while BzATP is a partial agonist of guinea pig $P2X_7$ compared to ATP [32]. The Hill coefficients, on average, were higher for canine erythrocytes compared to human erythrocytes indicating a greater degree of cooperativity for multiple agonist-binding sites for canine $P2X_7$ compared to human $P2X_7$.

On average, EC_{50} values were one order of magnitude lower for BzATP compared to ATP for both canine and human $P2X_7$, consistent with our previous observations [16, 17]. The EC_{50} values for BzATP-induced $^{86}Rb^+$ effluxes were less than twofold different between species. In contrast, the EC_{50} values for ATP-induced $^{86}Rb^+$ efflux were, on average, fourfold greater for canine $P2X_7$ than for human $P2X_7$. A recent study by Young et al. has identified asparagine^{284} as the residue responsible for the increased ATP sensitivity of rat $P2X_7$ compared to mouse $P2X_7$ [33]. Comparison of published sequences of canine and human $P2X_7$ [23, 34] demonstrate that these receptors contain a threonine and asparagine at residue 284 respectively. Although we have not directly sequenced the $P2X_7$ of the canine donors used in our study, we suggest that the residue difference at position 284 in these two species accounts for the observed differences in EC_{50} values for ATP.

Examination of concentration response curves for ATP and BzATP for canine and human $P2X_7$ allowed us to compare the receptor activation in the presence or absence of extracellular Na^+ ions. Inhibition of $P2X_7$ by extracellular Na^+ ions is a well-described event [21, 22], which is thought to occur via a regulatory Na^+ binding site on the extracellular loop of the receptor within the electrical field of the membrane [35]. EC_{50} values for both canine and human $P2X_7$ to either agonist were higher in the presence of extracellular Na^+. Moreover, efflux rates and maximum responses to ATP and BzATP were lower in NaCl medium compared to KCl medium for both canine and human receptors. Canine $P2X_7$ however was less sensitive to the inhibitory effects of extracellular Na^+ ions, as the percentage decreases in efflux rates and maximum responses in the

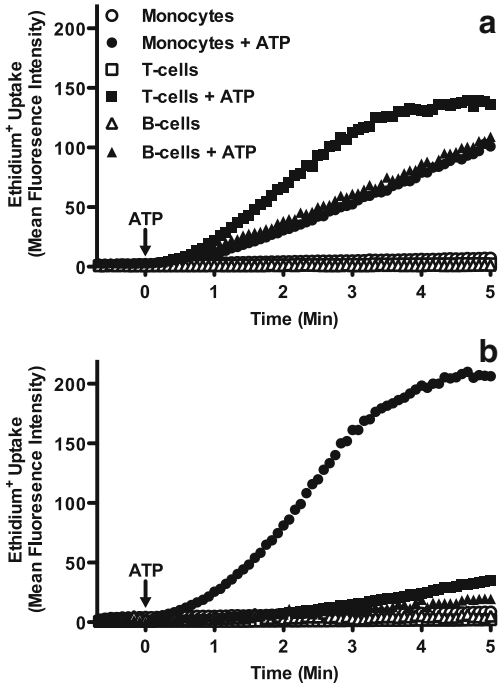

Fig. 4 ATP induces ethidium$^+$ uptake into canine and human mononuclear leukocytes. **a** Canine or **b** human mononuclear leukocytes were pre-labelled with cell-specific mAb and resuspended in KCl medium at 37 °C. Ethidium$^+$ (25 μM) was added, followed 40 s later by the addition of 1 mM ATP (*arrow*). Mean channel of cell-associated fluorescence was measured by time-resolved flow cytometry for monocytes, T- and B-cells incubated in the absence or presence of ATP. Representative results from three experiments are shown

presence of extracellular Na^+ ions were smaller for canine $P2X_7$ compared to human $P2X_7$. We cannot exclude the possibility that the increased $^{86}Rb^+$ (K^+) efflux in KCl medium compared to NaCl medium was due to increased cytosolic Ca^{2+} via KCl-induced activation of N-type Ca^{2+} channels and the subsequent activation of the Gardos channel. Although the KCl medium used in our study was nominally free of Ca^{2+}, it would be of interest to assess ATP-induced $^{86}Rb^+$ efflux from erythrocytes in the presence and absence of a N-type Ca^{2+} channel blocker such as ω-conotoxin GVIA.

KN-62 inhibited canine $P2X_7$ in a concentration-dependent fashion, as previously observed for human $P2X_7$ in B-cells [36]. The IC_{50} for canine $P2X_7$ was relatively similar to that of human $P2X_7$ in comparison to the IC_{50} values for mouse and rat $P2X_7$ which are ten- and 100-fold greater than for human $P2X_7$, respectively [37]. Sensitivity to KN-62 between the human and rat $P2X_7$ can be largely explained by differences at residue 95 in the receptor, which contain phenylalanine and lysine, respectively [38]. Canine $P2X_7$ also contains a phenylalanine at this position [34], consistent with the KN-62 sensitivity of this receptor.

Mg^{2+} ions inhibited canine $P2X_7$ in a concentration-dependent fashion, as previously observed for recombinant human and rat $P2X_7$ [21, 23, 24] and similar to that for human erythrocyte $P2X_7$. The ability of Mg^{2+} to impair $P2X_7$ is a well-established phenomenon [39], although its mechanism of action remains unknown. It has long been considered that Mg^{2+} chelates ATP^{4-}, the active form of the agonist, thereby inhibiting $P2X_7$ [39]. More recent data, however, demonstrates that Mg^{2+} binds to the positively charged residues, histidine^{130} and histidine^{201} of rat $P2X_7$ to impair receptor function [40]. In this regard, histidine^{201} is present in canine $P2X_7$, as well as human $P2X_7$, while an uncharged serine exists at position 130 in $P2X_7$ from these species [23, 34]. Thus, Mg^{2+} may also impair canine and human $P2X_7$ by directly binding to this conserved histidine, and may explain the similarity in IC_{50} values for Mg^{2+} between these species. Direct comparison between the two species, however, is complicated by the use of different ATP concentrations, and hence differences in the concentrations of available Mg^{2+} and ATP^{4-} to inhibit and activate the receptor, respectively.

Table 2 ATP-induced ethidium$^+$ uptake into canine and human leukocytes

	ATP-induced ethidium$^+$ uptake (arbitrary units of uptake)	
	Canine	Human
Monocytes	13,185±427[a]	34,139±470[b,c]
T-cells	25,104±1065	5,036±1976[d]
B-cells	15,937±316	3,203±1172[e]

The data were derived from Fig. 4 and are expressed as mean ± SEM (n=3)

[a] $P<0.05$ compared to canine T-cells

[b] $P<0.001$ compared to human T- and B-cells

[c] $P<0.001$ compared to canine monocytes

[d] $P<0.001$ compared to canine T-cells

[e] $P<0.05$ compared to canine B-cells

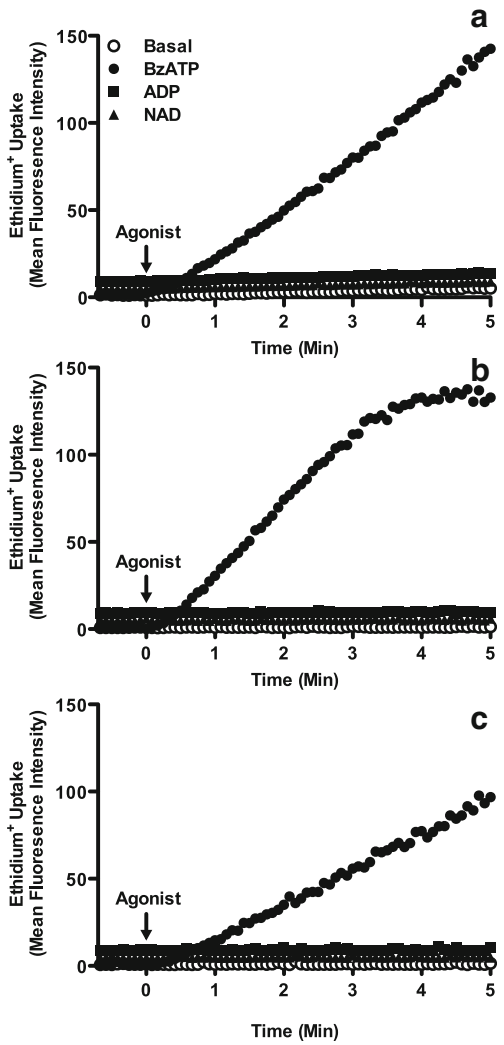

Fig. 5 BzATP but not ADP nor NAD induce ethidium$^+$ uptake into canine mononuclear leukocytes. Canine **a** monocytes, **b** T- and **c** B-cells were pre-labelled with cell-specific mAb and resuspended in KCl medium at 37 °C. Ethidium$^+$ (25 μM) was added, followed 40 s later by the addition of 200 μM BzATP, 1 mM ADP or 300 μM NAD (*arrow*). Mean channel of cell-associated fluorescence was measured by time-resolved flow cytometry for monocytes, T- and B-cells incubated in the absence or presence of agonist. Representative results from three experiments are shown

KN-62 and Mg^{2+} also inhibited ATP-induced choline$^+$ uptake in canine and human erythrocytes. Previous studies have demonstrated that ATP or BzATP can induce choline$^+$ uptake into murine macrophages [41], human lymphocytes [42], and canine and human erythrocytes [17], however, a direct role for $P2X_7$ in these processes has never been formally demonstrated. The observation that KN-62 and Mg^{2+} impair this process in erythrocytes provides the first direct evidence that $P2X_7$ activation is responsible for ATP-induced choline$^+$ uptake in cells. Choline$^+$ is an important nutrient in *Plasmodium*-infected erythrocytes, and the mechanisms that mediate its uptake are unknown but are potential therapeutic targets in malaria [43]. $P2Y_1$, via the autocrine release of ATP, is involved in the induction of an osmolyte permeability pathway in *Plasmodium*-infected erythrocytes [44], and thus the autocrine-induced activation of $P2X_7$ may play a similar role in choline$^+$ uptake in *Plasmodium*-infected erythrocytes.

This study demonstrated the presence of functional $P2X_7$ on canine monocytes, T- and B-cells. Both ATP and BzATP induced ethidium$^+$ uptake into to each of these leukocyte subpopulations, while ADP, which does not activate $P2X_7$ [4], did not. Moreover, KN-62 almost completely blocked

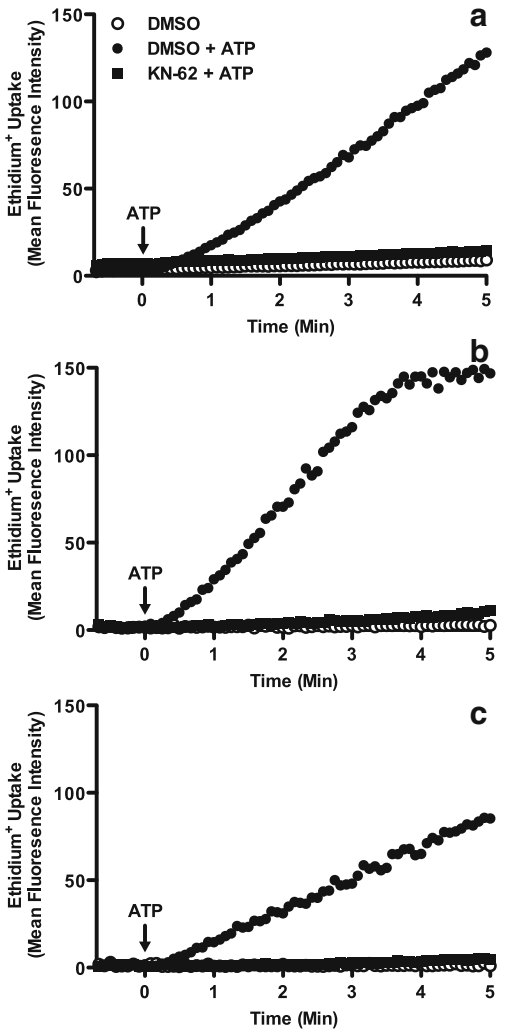

Fig. 6 KN-62 inhibits ATP-induced ethidium$^+$ uptake into canine mononuclear leukocytes. Canine **a** monocytes, **b** T- and **c** B-cells were pre-labelled with cell-specific mAb and resuspended in KCl medium at 37 °C. Cells were pre-incubated at 37 °C for 5 min with DMSO or 1 μM KN-62. Ethidium$^+$ (25 μM) was added, followed 40 s later by the addition of 1 mM ATP (*arrow*). Mean channel of cell-associated fluorescence was measured by time-resolved flow cytometry for monocytes, T- and B-cells incubated in the absence or presence of ATP. Representative results from three experiments are shown

ATP-induced ethidium$^+$ uptake into canine monocytes, T- and B-cells. $P2X_7$ function, as determined by measurements of ATP-induced ethidium$^+$ uptake, was approximately twofold greater in canine T-cells compared to canine monocytes and B-cells. This contrasts the relative differences in $P2X_7$ function in the equivalent human leukocyte subpopulations, where $P2X_7$ function is at least fivefold greater in human monocytes compared to human T- and B-cells [15, 20]. Moreover, $P2X_7$ function was approximately threefold lower in canine monocytes compared to human monocytes, but fivefold higher in canine T- and B-cells compared to human T- and B-cells. In comparison, $P2X_7$ function, as assessed by choline$^+$ uptake, was 40-fold greater in canine erythrocytes compared to human erythrocytes. Although the possibility remains that ethidium$^+$ and choline$^+$ uptake are mediated by different permeability pathways [45], the results nevertheless show striking differences in the amounts of $P2X_7$-mediated uptake of organic cations in equivalent cell subsets between the two species. Differences in $P2X_7$ expression in canine monocytes, T- and B-cells are most likely responsible for the observed differences in $P2X_7$ functions in these cells. As stated above, the relative differences in $P2X_7$ function between canine and human erythrocytes corresponds with differences in $P2X_7$ expression [17], while in human leukocytes $P2X_7$ function corresponds to $P2X_7$ expression [15, 46].

NAD was unable to activate $P2X_7$ in canine leukocytes despite being used at a concentration (300 μM) maximal for murine $P2X_7$ activation [25] and being able to stimulate ethidium$^+$ uptake into lymph node T-cells from C57Bl/6 mice (unpublished observations). NAD can induce cation fluxes in murine T-cells via ADP-ribosylation of $P2X_7$, a process that requires the co-expression of ADP-ribosyltransferases on the cell surface [25]. Thus, the most likely explanation for the inability of NAD to stimulate ethidium$^+$ uptake in canine leukocytes is the absence of this ecto-enzyme on canine monocytes, T- and B-cells. The inability of NAD to activate canine $P2X_7$ was not due to the absence of the ADP-ribosylation site as canine $P2X_7$ also contains arginine^{125} [34], which is the same residue ribosylated by ADP in murine $P2X_7$ [47].

Our data demonstrates pharmacological similarities and differences between the canine and human $P2X_7$. Thus, future studies aimed at identifying residues and domains mediating pharmacological similarities and differences between these two species may contribute to our understanding of the structure and function of this receptor. Moreover, $P2X_7$ is attracting much interest as a potential therapeutic target in a number of inflammatory, infectious, neurological and bone disorders in humans [9–11]. Thus, identification of functional $P2X_7$ in canines and in particular in various canine leukocyte subpopulations offers a potential therapeutic target in canine disorders equivalent to those in humans.

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