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Species and agonist dependent zinc modulation of endogenous and recombinant ATP-gated $P2X_7$ receptors

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ABSTRACT

Zinc (Zn^{2+}) and copper (Cu^{2+}) are key signalling molecules in the immune system and regulate the activity of many ion channels. Both Zn^{2+} and Cu^{2+} potently inhibit rat $P2X_7$ receptors *via* a binding site identified by mutagenesis. Here we show that extracellular Cu^{2+} also potently inhibits mouse $P2X_7$ receptors. By contrast, the receptor expression system and agonist strongly influence the action of extracellular Zn^{2+} at mouse $P2X_7$ receptors. Consistent with previous reports, Zn^{2+} inhibits recombinant rat $P2X_7$ receptors. However, recombinant mouse $P2X_7$ receptors are potentiated by Zn^{2+} when activated by ATP^{4-} but inhibited when stimulated with the ATP analogue $BzATP^{4-}$. Endogenous murine macrophage $P2X_7$ receptors are not modulated by Zn^{2+} when stimulated by ATP^{4-} however Zn^{2+} inhibits $BzATP^{4-}$ mediated responses. In summary, these findings provide a fundamental insight into the differential actions of Zn^{2+} and Cu^{2+} between different $P2X_7$ receptor species.

1. Introduction

Zinc (Zn^{2+}) and copper (Cu^{2+}) are essential trace metal nutrients where imbalances have been observed to provoke a variety of different physiological effects. Zn^{2+} plays an important signalling role in the immune system, whereby treatment with oral Zn^{2+} has profound effects on the immune response and acts as an anti-inflammatory therapeutic [1,2]. Cu^{2+} deficiencies have been related to neurological disturbances, anaemia and hair changes. Zn^{2+} and Cu^{2+} bind directly to amino acid residues including the sulfhydral group of cysteines, the imidazole of histidines and the carboxylic acid residues of aspartate [3]. Both of these metals play a fundamental role in the maintenance of protein structure and can act as critical enzyme cofactors. These trace metals have been observed to modulate a plethora of membrane receptors and ion channels including members of the adenosine 5′-triphosphate (ATP)-gated P2X receptor family [4,5].

Estimates of Zn^{2+} concentrations in the brain range from 100 μM to 150 μM through to levels of 300 μM Zn^{2+} that have been detected in the giant boutons of hippocampal mossy fibres [6]. For monocytes and circulating immune cells, the normal plasma level of Zn^{2+} is estimated to be 13–15 μM [6]. Normal plasma copper concentrations have been observed to be around 14–18 μM [7] and are increased during inflammation [8,9]. In this study, we have investigated the action of up to 300 μM Zn^{2+} and 100 μM Cu^{2+} on both endogenous and recombinant mouse $P2X_7$ receptors.

The ionotropic $P2X_7$ receptor is expressed on cells of the immune system and proposed to play an important role in pro-inflammatory responses [10,11]. P2X receptors are thought to consist of three subunits with each subunit comprising of two putative transmembrane segments and a large extracellular loop containing the proposed ATP binding site. Several studies have reported that extracellular Zn^{2+} and Cu^{2+} inhibit $P2X_7$ receptor responses *via* a direct binding site in the extracellular loop [5,12,13]. $P2X_7$ receptors possess biophysical and pharmacological properties unique to the P2X receptor

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family. Homomeric $P2X_7$ receptors require high micromolar concentrations of extracellular ATP for receptor activation while the ATP analogue, 2′- and 3′-O-(4-benzoyl)benzoyl-ATP (BzATP), activates the receptor at lower concentrations. After rapid activation of the intrinsic $P2X_7$ receptor ion channel, the receptor also couples to the opening of pannexin 1 leading to the influx of propidium dyes [14,15]. Extracellular Cu^{2+} and Zn^{2+} are reported to block rat $P2X_7$ receptor mediated ionic currents and subsequent pannexin 1 opening [5,12,13].

There are clear species differences in the sensitivity of $P2X_7$ receptors to BzATP and ATP; mouse $P2X_7$ receptor display a markedly lower sensitivity to ATP (EC_{50} 800–935 μM) and BzATP (295 μM) compared with rat (ATP EC_{50} 115–123 μM; BzATP EC_{50} 2–3 μM) [16–18]. By contrast, human $P2X_7$ receptors have a BzATP sensitivity comparable to mouse (EC_{50} 210 μM) but a lower ATP sensitivity (EC_{50} 1.8 mM) [19]. Mutagenesis studies have identified amino acids in the ectodomain responsible for conferring differences in ATP and BzATP sensitivity between rat and mouse [16]. Differences in antagonist potency have been observed between mammalian $P2X_7$ receptor orthologs; isoquinoline derivatives, KN-62 and KN-04, potently block human (IC_{50} 44–75 nM) but are 1000-fold less sensitive at mouse [17,20]. A cyclic imide compound, AZ11645373 blocks human $P2X_7$ receptors (K_B value 5–20 nM) and has little or no action at rat $P2X_7$ receptors [19]. By contrast, low concentrations of brilliant blue G block rat $P2X_7$ receptor (IC_{50} 10 nM) but higher concentrations are required for action at human or mouse [17,21]. As there are striking species differences observed for both agonists and antagonists at the $P2X_7$ receptor, we have decided to investigate the action of Zn^{2+} and Cu^{2+} on the mouse $P2X_7$ receptor.

2. Materials and methods

2.1. Reagents

Unless stated, all compounds were obtained from Sigma–Aldrich, Poole, UK.

2.2. Solutions

All experiments were performed in physiological salt solution containing (mM) 147 NaCl, 2 KCl, 10 HEPES, 12 glucose, 2 $CaCl_2$ and 1 $MgCl_2$ (pH 7.3 with NaOH) or a nominal Ca^{2+}/Mg^{2+}-free salt solution containing (mM) 147 NaCl, 2 KCl, 10 HEPES, and 12 glucose (pH 7.3 with NaOH). The di-sodium salt of ATP and the chloride salts of zinc and copper ($ZnCl_2$ and $CuCl_2$) were used in these experiments.

2.3. Cell culture and transfection

Murine RAW264.7 macrophage cells (American Type Culture Collection, Manassas, VA, USA) and human embryonic kidney 293 cells (HEK293; European Collection of Cell Cultures, Wiltshire, UK) were maintained in DMEM:F12 medium (1:1) containing 10% heat inactivated foetal bovine serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (all from Invitrogen, Paisley, UK) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Mechanical scrapping was used to detach macrophages from tissue culture plastic and HEK293 cells were detached using 0.05% Trypsin–EDTA (Invitrogen, Paisley, UK). For ethidium bromide (EtBr) influx measurements and lactic dehydrogenase (LDH) release assays, RAW246.7 macrophages (1.5×10^6 cells ml^{-1}) were plated overnight in either black or clear-walled 96 well plates respectively and maintained in standard culture medium for a maximum of 24 h. Human embryonic kidney 293 (HEK293) cells were used to transiently express rat or mouse $P2X_7$ receptors by transfection of cDNA with Fugene-6® (Roche Diagnostics Ltd., Burgess Hill, UK) according to the manufacturer's instructions. HEK293 cells were plated (2×10^5 cells ml^{-1}) in a 96 well black walled plate overnight before being transiently transfected using Fugene-6® and incubated for 48 h at 37 °C in standard culture medium before use.

2.4. Measurement of pore formation

Prior to the addition of the agonist, cells were washed in a salt solution containing a propidium dye, EtBr (25 μM) (Fisher Scientific, Loughborough, UK), with the test divalent cation for 5 min. EtBr fluorescence was measured at excitation/emission wavelengths of 544/590 nm at 37 °C using a multi-detection plate reader (FLUOstar OPTIMA, BMG Labtech, Aylesbury, UK). ATP was manually injected. In each assay, maximum permeabilization was determined by addition of Triton X-100 (0.2%) to account for potential quenching of EtBr fluorescence by addition of divalent cations or any differences in final cell numbers in each well. However, we did not observe quenching of cellular EtBr in the presence of divalent cations. ATP-mediated EtBr influx was not detected in mock-transfected HEK293 cells (data not shown).

2.5. Cytotoxicity

Lactate dehydrogenase activity was evaluated using the Cytotox-96 assay kit (Promega, Southampton, UK) according to the manufacturer's instructions. Total cellular LDH was determined by the addition of 0.2% Triton X-100 to untreated cells.

2.6. Data analysis

EtBr raw data was converted to percentage of total EtBr influx assessed by permeabilizing the cells with 0.2% Triton X-100. Percentage of total EtBr influx was plotted against time and linear regression (GraphPad Prism 4.0; San Diego, CA) performed to determine the rate of EtBr influx. Data was then normalised to the maximal rate of EtBr influx induced by ATP in the control solution. Dose–response curves fitted by $R = R_{min} + (R_{max} - R_{min})/(1 + 10\text{^}((\log EC_{50} - A) \times n_H))$ using Graphpad Prism 4.0 (San Diego, CA) where R is the peak response, A is the logarithm of the agonist concentration, R_{max} is the maximal peak agonist response, R_{min} is the minimal agonist response, n_H represents the hill coefficient and EC_{50} is the half maximal response. Cytotoxicity raw data was normalised against zero LDH (buffer recording) and expressed as a percentage of total cellular LDH (permeabilized cell recording).

In order to assess whether the effects of Zn^{2+} and Cu^{2+} independent of those on due to the chelation of ATP^{4-} and ATP^{4-} concentrations were calculated from total ATP concentrations using Webmaxc Standard (Stanford, USA) [22]. The K_d value of BzATP was assumed to be the same as ATP [13] for calculation of free $BzATP^{4-}$ concentrations.

Statistical analyses on experiment and control groups were performed using either Student's *t*-test or one-way ANOVA with Dunnetts post hoc analysis using a statistical software package (Prism version 4, GraphPad Software, San Diego, CA). Where appropriate, data expressed as mean ± standard error of the mean.

3. Results

3.1. ATP and BzATP evokes EtBr influx in RAW264.7 cells in the absence of cytolysis

Several macrophage cell lines express functional $P2X_7$ receptors including the murine macrophage RAW264.7 cell line [17,23]. Application of 3 mM ATP, in a physiological salt solution, evokes EtBr influx and an increase in fluorescence within 5 min (Fig. 1A). This increase in EtBr influx was continuous over the 30 min recording period. During the first 15 min ATP application, EtBr influx occurred in the absence of necrotic cell death where cellular LDH release was not detected (Fig. 1B). Therefore the rates of EtBr influx were measured between a 0 and 15 min agonist application in order to construct dose–response curves. ATP was established to induce EtBr influx with a pEC_{50} of 2.81 ± 0.02 ($n = 28$) (Fig. 1C). Effects of the non-physiological but more potent $P2X_7$ receptor agonist, BzATP, on EtBr influx were also established. BzATP was found to elicit EtBr influx at lower concentrations than ATP however a maximal response was not reached by up to 1 mM BzATP ($n = 3$) (Fig. 1C). This data along with previous observations [17] suggests that EtBr influx in RAW264.7 cells is mediated primarily by $P2X_7$ receptor activation.

3.2. Mouse $P2X_7$ receptors are insensitive to direct Zn^{2+} inhibition

Previous studies have reported a direct action of Zn^{2+} at recombinant rat $P2X_7$ receptors *via* a specific Zn^{2+} binding site [5,12,13]. Low concentrations of Zn^{2+} (<100 μM), potently block recombinant rat $P2X_7$ receptors independent of $BzATP^{4-}$ concentration [13] and partially block ATP-induced ionic currents [5,12]. We have extended these studies to investigate the action of Zn^{2+} on mouse $P2X_7$ receptors. In physiological salt solutions, the addition of up to 300 μM Zn^{2+} failed to block ATP^{4-} evoked EtBr influx in RAW264.7 cells (Fig. 2A) where no significant differences in pEC_{50}, maximal response or hill-slope were observed ($p > 0.05$, $n = 6$). As a full response to BzATP (≤1 mM) was not observed in physiological salt solutions, the action of extracellular Zn^{2+} was studied in nominal Ca^{2+}/Mg^{2+}-free salt solution, described in the subsequent section.

In order to rule out the lack of effect of Zn^{2+} on the mouse $P2X_7$ receptor in RAW264.7 cells being due to the expression system used we also studied the effect of Zn^{2+} on the recombinant receptor expressed in HEK293 cells. Application of 300 μM Zn^{2+} failed to block ATP^{4-} evoked EtBr influx in HEK293 cells expressing mouse $P2X_7$ receptors. Furthermore it was observed that 300 μM Zn^{2+} potentiated EtBr influx shifting

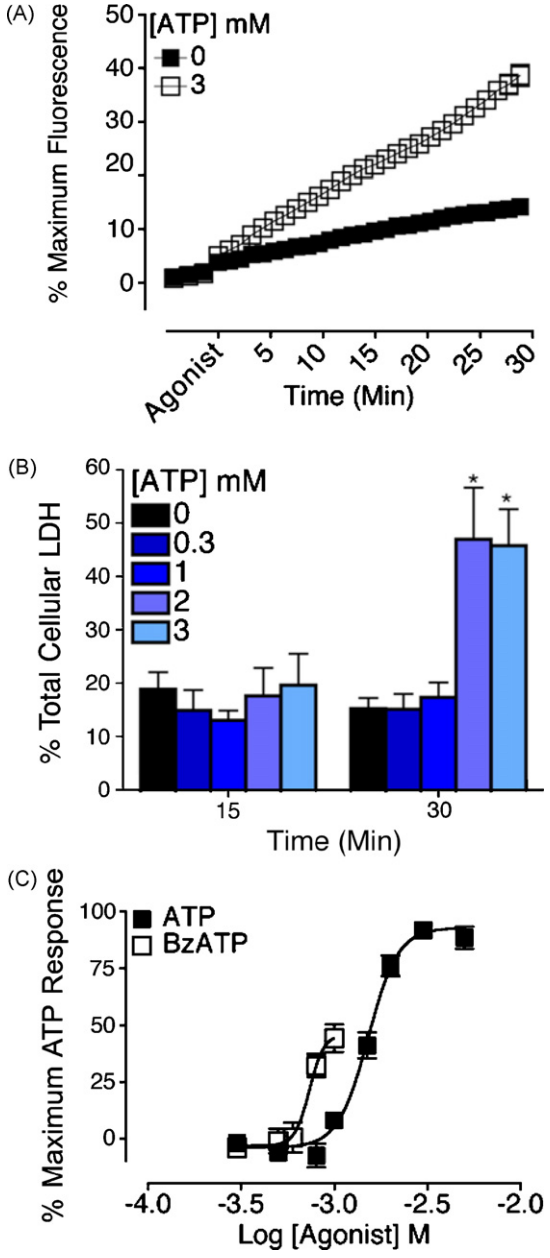

Fig. 1 – Application of ATP onto RAW264.7 cells induces EtBr influx (representative trace) (A), in the absence of significant LDH release when the application of ATP was <30 min (B). Both ATP (pEC_{50} of 2.81 ± 0.02) and BzATP-induced dose-dependent influx of EtBr (C). Cells were washed into a physiological saline solution containing 25 μM EtBr. Basal recordings were made for 5 min before manually injection of the agonist. EtBr fluorescence was measured at excitation/emission wavelengths of 544 nm/590 nm at 37 °C using a multi-detection plate reader (FLUOstar OPTIMA, BMG Labtech, Aylesbury, UK). Data: average from at least three independent experiments performed in triplicate ± S.E.M. (*$p < 0.05$ in comparison with control).

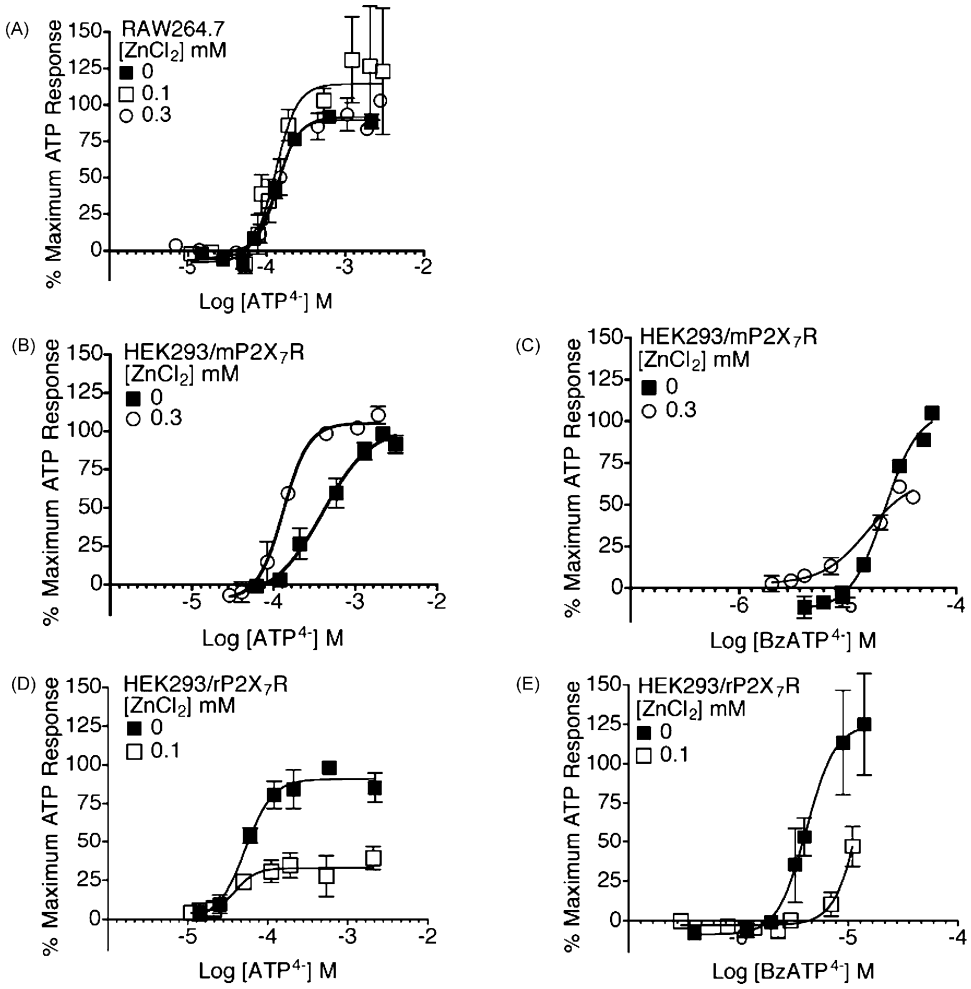

Fig. 2 – Zn^{2+} failed to significantly inhibit ATP^{4-}-induced EtBr influx in RAW 264.7 cells (A) and HEK293 cells expressing mouse $P2X_7$ receptors (HEK293/mP2X$_7$R) (B). In contrast Zn^{2+} inhibited $BzATP^{4-}$-induced EtBr influx in HEK293 cells expressing mouse $P2X_7$ receptors (C) and ATP^{4-}- (D) or $BzATP^{4-}$- (E) induced EtBr influx in HEK293 cells expressing rat $P2X_7$ receptors (HEK293/rP2X$_7$R). Cells were washed into a physiological saline solution containing 0 (closed squares), 100 μM (open squares) or 300 μM (open circles) Zn^{2+} and 25 μM EtBr. Basal recordings were made for 5 min before manually injection of the agonist. EtBr fluorescence was measured at excitation/emission wavelengths of 544 nm/590 nm at 37 °C using a multi-detection plate reader (FLUOstar OPTIMA, BMG Labtech, Aylesbury, UK). Data: average from at least three independent experiments performed in triplicate ± S.E.M.

the ATP^{4-} pEC_{50} value from 3.36 ± 0.13 ($n = 5$) to 3.96 ± 0.11 ($n = 4$, $p < 0.05$) (Fig. 2B). By contrast, addition of 300 μM Zn^{2+} reduced $BzATP^{4-}$-induced maximal EtBr influx by 48 ± 12% ($n = 4$, $p < 0.05$) (Fig. 2C).

Finally, we examined the action of extracellular Zn^{2+} at recombinant rat $P2X_7$ receptors where extracellular Zn^{2+} is reported to have a binding site in the receptor ectodomain [5,12]. Application of 100 μM Zn^{2+} led to a 63 ± 10% reduction in ATP^{4-} evoked maximal EtBr influx in HEK293 cells expressing rat $P2X_7$ receptors ($n = 7$, $p < 0.01$) (Fig. 2D) and potently reduced $BzATP^{4-}$ evoked responses (Fig. 2E). These data confirm that Zn^{2+} inhibits rat $P2X_7$ receptors independent of ATP^{4-} concentration and agrees with data demonstrating a direct Zn^{2+} site at the rat $P2X_7$ receptor. Overall we have demonstrated that mouse $P2X_7$ receptors expressed in HEK293 cells are potentiated by Zn^{2+} while endogenous $P2X_7$ receptors are unaffected by Zn^{2+} when activated with the physiological agonist ATP^{4-}.

3.3. *Effect of Zn^{2+} on $P2X_7$ receptors in the absence of Ca^{2+} and Mg^{2+}*

External salt solutions lacking Ca^{2+} and/or Mg^{2+} are commonly used to study the pharmacology of the $P2X_7$ receptor [16,24]. Therefore we evaluated the action of Zn^{2+} in a nominal Ca^{2+}/Mg^{2+}-free salt solution. This enabled us to study the action of Zn^{2+} on the endogenous mouse $P2X_7$ receptors activated with BzATP (Fig. 3B). In RAW246.7 macrophages, application of up to 300 μM Zn^{2+} failed to inhibit ATP^{4-} evoked EtBr influx (Fig. 3A). No significant differences in pEC_{50}, maximal response or hill-slope were observed ($p > 0.05$, $n = 3$). By contrast extracellular Zn^{2+} was observed to inhibit $BzATP^{4-}$ evoked

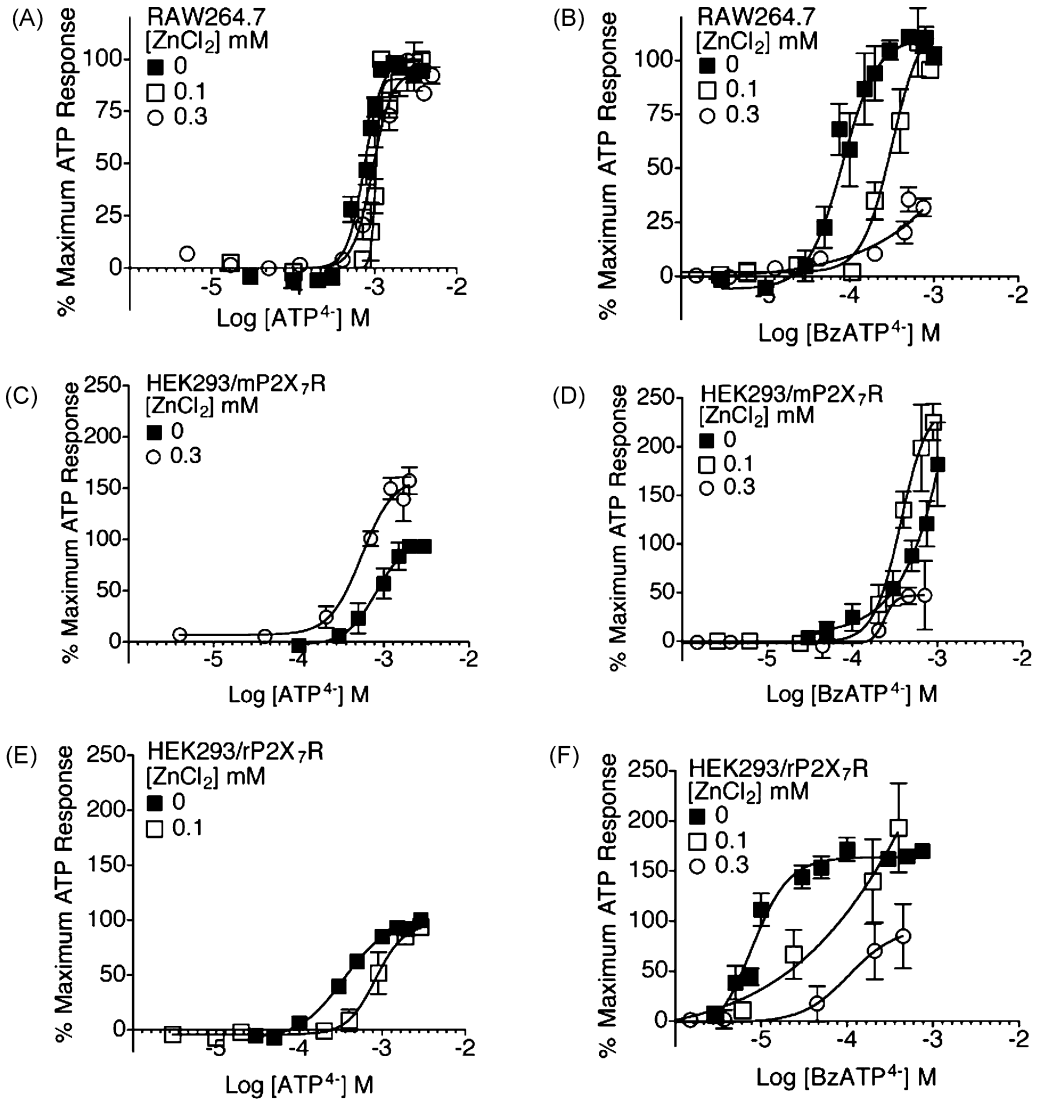

Fig. 3 – Zn^{2+} in the absence of Ca^{2+} and Mg^{2+} failed to significantly inhibit ATP^{4-}-induced EtBr influx in RAW 264.7 cells (A) and HEK293 cells expressing mouse $P2X_7$ receptors (HEK293/mP2X7R) (C). In contrast Zn^{2+} in the absence of Ca^{2+} and Mg^{2+} inhibited $BzATP^{4-}$ evoked EtBr influx in RAW 264.7 cells (B) and HEK293 cells expressing mouse $P2X_7$ receptors (D) and ATP^{4-} (E) and $BzATP^{4-}$ (F) in HEK293 cells expressing rat $P2X_7$ receptors (HEK293/rP2X7R). Cells were washed into a nominal Ca^{2+}/Mg^{2+}-free solution containing 0 (closed squares), 100 μM (open squares) or 300 μM (open circles) Zn^{2+} and 25 μM EtBr. Basal recordings were made for 5 min before manually injection of the agonist. EtBr fluorescence was measured at excitation/emission wavelengths of 544 nm/590 nm at 37 °C using a multi-detection plate reader (FLUOstar OPTIMA, BMG Labtech, Aylesbury, UK). Data: average from at least three independent experiments performed in triplicate ± S.E.M.

EtBr influx with 100 μM Zn^{2+} shifting the pEC_{50} from 4.00 ± 0.09 to 3.06 ± 0.41 ($p < 0.05$, $n = 3$) (Fig. 3B).

We next investigated the Zn^{2+} sensitivity of the recombinant mouse and rat $P2X_7$ receptors in the absence of Ca^{2+} and Mg^{2+}. Application of 300 μM Zn^{2+} was observed to significantly potentiate ATP^{4-} evoked maximal EtBr influx in HEK293 cells expressing mouse $P2X_7$ receptors ($p < 0.05$, $n = 3$) (Fig. 3C). In contrast an application of 300 μM Zn^{2+} was observed to reduce maximal EtBr influx evoked by $BzATP^{4-}$ ($n = 3$) (Fig. 3D). For recombinant rat $P2X_7$ receptors, the addition of 100 μM Zn^{2+} was observed to significantly inhibit ATP^{4-}-induced EtBr influx with a rightward shift in dose–response curve where the pEC_{50} value decreased from 3.47 ± 0.05 to 3.17 ± 0.10 ($p < 0.01$, $n = 3$) (Fig. 3E). Similar findings were observed with $BzATP^{4-}$ evoked EtBr uptake where the pEC_{50} value decreased from 4.88 ± 0.07 to 3.90 ± 0.12 ($n = 3$, $p < 0.01$) (Fig. 3F).

In summary, responses evoked by $BzATP^{4-}$ at both the endogenous and recombinant mouse $P2X_7$ receptors are inhibited by the application of Zn^{2+} suggesting an 'agonist-specific' action of Zn^{2+}.

3.4. *Mouse and rat $P2X_7$ receptors are potently blocked by Cu^{2+}*

Previous studies have reported that Cu^{2+} is able to directly inhibit several species of $P2X_7$ receptor. In rat $P2X_7$ receptors, common

or overlapping Cu^{2+} and Zn^{2+}-binding sites have been reported in the receptor ectodomain [5,12]. Functionally, extracellular Cu^{2+} is reported to potently block (IC_{50} = 0.3–5 μM) both ionic currents and pore formation mediated by rat $P2X_7$ receptors [5,12,13]. Human $P2X_7$ receptor mediated ionic currents are blocked by Cu^{2+} with an IC_{50} of approximately 1 μM where similar results were observed with putative $P2X_7$ receptors endogenously expressed by mouse acinar and ductal cells [25–27]. We have extended these studies to investigate the action of Cu^{2+} at both endogenous and recombinant mouse $P2X_7$ receptors (Fig. 4).

In physiological salt solutions, endogenous and recombinant mouse $P2X_7$ receptors are inhibited by the addition of 30 μM Cu^{2+} where the maximal EtBr influx evoked by 3 mM ATP^{4-} was significantly reduced by 67 ± 18% ($n = 4$, $p < 0.001$) and 93 ± 11% ($n = 5$, $p < 0.001$) respectively (Fig. 4A and C). Comparable results were observed with recombinant rat $P2X_7$ receptors where the maximal EtBr influx was reduced by 62 ± 7% ($n = 3$, $p < 0.001$) (Fig. 4E). In nominally Ca^{2+} and Mg^{2+}-free solution, addition of 30 μM Cu^{2+} elicited a similar inhibition at mouse and rat $P2X_7$ receptors (Fig. 4B, D and F). Responses evoked by 1 mM ATP^{4-} were significantly reduced by 79 ± 4% ($n = 3$, $p < 0.001$), 59 ± 14% ($n = 3$, $p < 0.01$) and 80 ± 6% ($n = 3$, $p < 0.001$) in RAW264.7, recombinant mouse and recombinant rat $P2X_7$ receptors respectively. To conclude, these data demonstrate that both rat and mouse $P2X_7$ receptors are sensitive to inhibition by extracellular Cu^{2+}.

4. Discussion

Zn^{2+}, Cu^{2+} and protons are reported to act as allosteric modulators of a range of ligand gated ion channels including

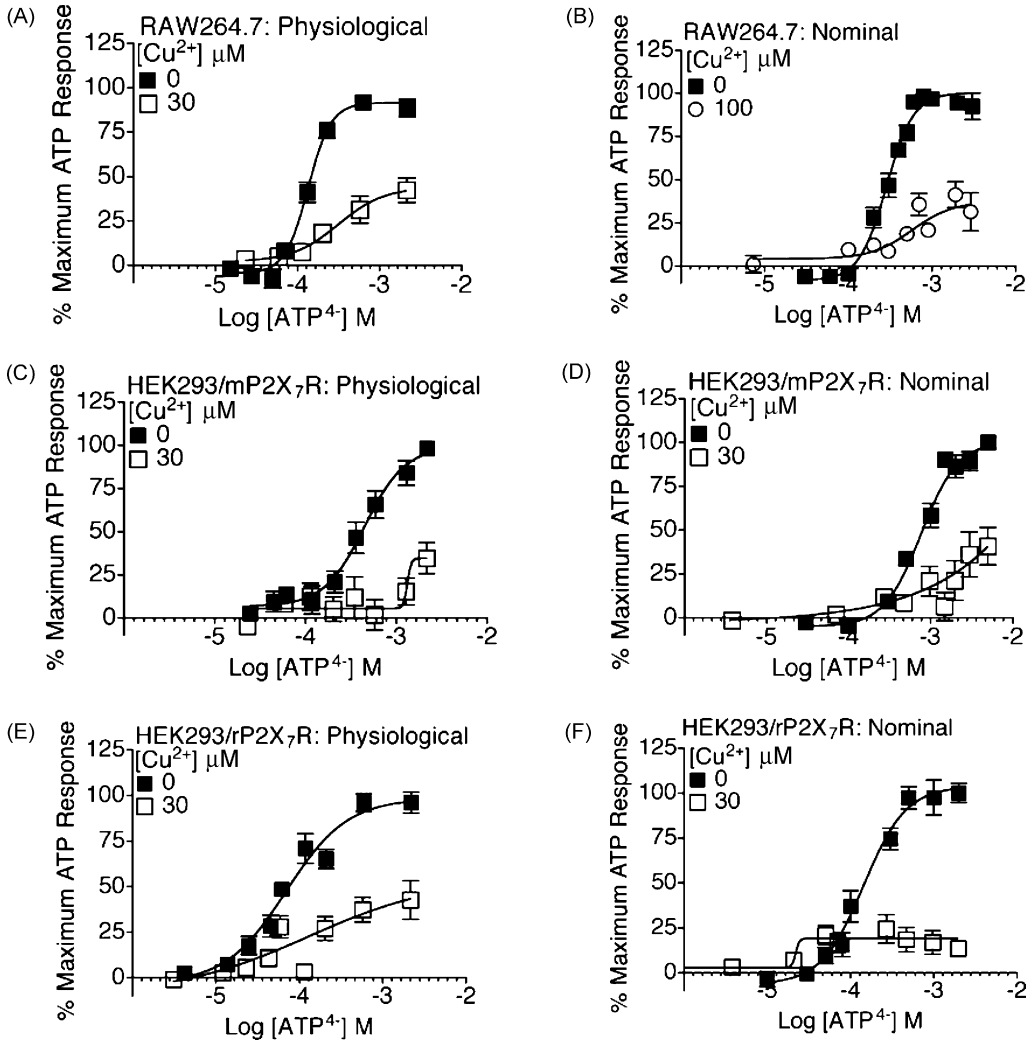

Fig. 4 – Cu^{2+} significantly inhibited ATP^{4-} evoked EtBr influx in RAW 264.7 (A) and HEK293 cells expressing either mouse $P2X_7$ receptors (HEK293/mP2X7R) (C) or rat $P2X_7$ receptors (HEK293/rP2X7R) (E). Furthermore Cu^{2+} in the absence of Ca^{2+} and Mg^{2+} also inhibited ATP^{4-} evoked EtBr influx in RAW 264.7 (B) and HEK293 cells expressing either mouse $P2X_7$ receptors (D) or rat $P2X_7$ receptors (F). Cells were washed into a nominal Ca^{2+}/Mg^{2+}-free solution containing 0 (closed squares), 30 μM (open squares) or 100 μM (open circles) Cu^{2+} and 25 μM EtBr. Basal recordings were made for 5 min before manually injection of the agonist. EtBr fluorescence was measured at excitation/emission wavelengths of 544 nm/590 nm at 37 °C using a multi-detection plate reader (FLUOstar OPTIMA, BMG Labtech, Aylesbury, UK). Data: average from at least three independent experiments performed in triplicate ± S.E.M.

$P2X_7$ receptors [5,12,13,27,28]. The majority of studies have been performed using rat $P2X_7$ receptors in heterologous systems where divalent cations and protons potently inhibit ATP and BzATP-mediated receptor activation [5,12,13]. We have extended these studies to investigate the action of Zn^{2+} and Cu^{2+} at mouse $P2X_7$ receptors. Cu^{2+} is reported to inhibit $P2X_7$ and $P2X_4$ receptors as well as the recently characterized *Dictyostelium discoideum* P2X receptor [4,5,12,29]. We have demonstrated that copper blocks both rat and mouse $P2X_7$ receptors in the presence and absence of other divalent cations, calcium and magnesium (Fig. 4). In contrast, Zn^{2+} did not simply block mouse $P2X_7$ receptors and a range of effects were observed according to the expression system or the activating ligand (Figs. 2 and 3). For the ligand, ATP^{4-}, Zn^{2+} had no effect on endogenous murine macrophage $P2X_7$ receptors but markedly potentiated mouse $P2X_7$ receptors expressed in HEK293 cells, the opposite effect is observed for rat $P2X_7$ receptors. The simplest explanation for differences between the two systems might be the interaction with endogenous macrophage $P2X_4$ receptors that might alter the $P2X_7$ Zn^{2+}-binding site [30]. However, differences might also be explained by interaction with another non-P2X receptor protein. Further studies are required to determine why differences are observed between the two expression systems. Many studies of $P2X_7$ receptors utilize a non-physiological agonist BzATP. Zn^{2+} was found to block $BzATP^{4-}$ responses in both mouse macrophages and mouse $P2X_7$ receptors expressed in HEK293 cells (Figs. 2 and 3). These results would suggest that Zn^{2+} binds to more than one site on mouse $P2X_7$ receptors leading to either ligand dependent potentiation or inhibition.

Zn^{2+} and Cu^{2+} binding sites have been identified in a number of ligand gated ion channels including an inter-subunit site in NMDA receptors and $P2X_2$ receptors [28,31]. Recently, the binding site(s) for Cu^{2+} and Zn^{2+} in rat $P2X_7$ receptors has been investigated using site directed mutagenesis. The involvement of histidine, glutamate, lysine and aspartate has been studied by mutation to alanine [5,12]. However, the amino acid contribution to the Zn^{2+}- and Cu^{2+}-binding site differs according to the expression system. Expression of rat $P2X_7$ receptors in HEK293 cells identified a common binding site for Cu^{2+} and Zn^{2+} at H62 and D197 when activated with BzATP or ATP [12]. Activation of receptors with ATP leads to added contribution from H267 and H201. Interestingly, different residues are reported to contribute the Cu^{2+}/Zn^{2+} binding sites when rat $P2X_7$ is expressed in *Xenopus* oocyte [5]. In the second study, the ATP evoked currents mutant H267A was unaffected by Zn^{2+} or Cu^{2+} inhibition suggesting a common site of action. H201A and H130A also displayed reduced copper sensitivity while H219A had reduced Zn^{2+} sensitivity. In this study, H62A had no effect on Cu^{2+} or Zn^{2+} inhibition. Overall, both studies report involvement of H201 and H267 in the copper-binding site though there is so far no agreement on the rat $P2X_7$ Zn^{2+}-binding site.

As we are studying the properties of rat and mouse $P2X_7$ receptors expressed in HEK293 cells, direct comparison with the mutagenesis data using a HEK293 cell expression system demonstrates a common binding site at H62/D197/H201/H267 that modulates ATP responses [12]. All these residues are conserved in mouse $P2X_7$ receptors, except D197 that is replaced by a histidine. It is not clear how this single substitution could differentially alter the action of copper and Zn^{2+} at mouse $P2X_7$ receptors. The simplest explanation would be the involvement of additional, as yet unidentified residues, within distinct Cu^{2+} and Zn^{2+} binding sites. Alternatively alanine substitutions may structurally alter the ectodomain resulting in an indirect loss of the metal ion binding sites. Further mutagenesis studies are required to understand these differences between mouse and rat $P2X_7$ receptors. In summary, we have demonstrated a striking difference in the Zn^{2+} sensitivity of mouse versus rat $P2X_7$ receptors. It is noteworthy that differences in Zn^{2+} sensitivity were observed between ATP and the analogue, BzATP, highlighting the importance of using BzATP with caution in pharmacological studies.

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