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REVIEW

Inhibition of $P2X_7$ receptors by divalent cations: old action and new insight

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Abstract P2X family receptors form ATP-gated ion channels by assembling homo/hetero-trimers from seven receptor subunits. The homomeric $P2X_7$ receptor is extraordinary in that in addition to distinctive localization and biological functions it exhibits several hallmark properties, for example, the receptor is potently inhibited by divalent cations such as calcium, magnesium, zinc and copper. Despite the fact that this distinct feature was first described almost three decades ago, our understanding is still contentious. Recent site-directed mutagenesis studies have provided direct evidence showing that functional inhibition by zinc and copper primarily results from direct interaction with the receptor. In this short review, I will give a concise description of the major localization, biological functions, and unique properties of the $P2X_7$ receptor, and particularly discuss the evolving understanding of how divalent cations inhibit the $P2X_7$ receptor and the potential implication of such inhibition to the physiological and pathophysiological role of the $P2X_7$ receptor.

Keywords $P2X_7$ receptor · Ion channel · Pore formation · Functional inhibition · Divalent cations · ATP^{4-}

Introduction

Extracellular ATP is an important signaling molecule that mediates or regulates a wide range of biological processes via activation of P2 purinergic receptors on the cell surface (Ralevic and Burnstock 1998; Khakh 2001; North 2002; Khakh and North 2006). P2 receptors can be subdivided into P2X and P2Y families, based on their actions and signaling mechanisms: P2X operate as ligand-gated ion channels and P2Y are G-protein-coupled receptors. P2X receptor channels open upon binding of extracellular ATP and permeate cations including Ca^{2+}, resulting in membrane depolarization and/or an elevation in intracellular Ca^{2+} concentration. There are seven mammalian P2X subunits, $P2X_1$-$P2X_7$, which assemble homo/hetero-trimers to form functional P2X receptors. The mammalian P2X subunits are 384 to 595 amino acids long, and have a basic architecture that comprises intracellular N- and C-termini, two transmembrane domains (TM1 and TM2) and a large extracellular loop (Fig. 1). There is substantial sequence conservation within the P2X receptor family (North 2002; Vial et al. 2004; Khakh and North 2006). For example, the extracellular domain contains a number of conserved residues, including ten cysteine residues, which are thought to form intra-subunit disulfide bonds, and several positively charged lysine and aromatic phenylalanine residues, which are critical in receptor activation by ATP and its analogues (Fig. 1). However, except for having a common trafficking motif (Chaumont et al. 2004), the C-termini vary considerably in sequence and length, and associate with receptor specific functional properties, such as receptor desensitization (Smith et al. 1999; Fountain and North 2006), and membrane trafficking (Bobanovic et al. 2002; Denlinger et al. 2001). Studies over the past decades have demonstrated that the $P2X_7$ receptor is an extraordinary member of the P2X receptor family exhibiting quite unique localization and biological functions as well as several hallmark properties. In this short review, I will give a brief description of the major cell localization, biological

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functions and hallmark properties of the $P2X_7$ receptor, and particularly discuss the evolving understanding of how divalent cations inhibit the $P2X_7$ receptor and the potential implication of such inhibition to the physiological and pathophysiological roles of the $P2X_7$ receptor.

Localization and biological functions of $P2X_7$ receptors

Our knowledge of localization and function of $P2X_7$ receptors originated from the seminal findings made by Dahlquist and Diamant and by Cockcroft and Gomperts about three decades ago. Dahlquist and Diamant found that extracellular ATP evoked histamine secretion in mast cells (Dahlquist and Diamant 1974). Cockcroft and Gomperts among others later confirmed this finding and further demonstrated that prolonged ATP exposure resulted in cell permeabilization responsible for remarkable leakage of intracellular nucleotides and metabolites (Cockcroft and Gomperts 1979, 1980). This salient phenotype led to designation of P2Z by Gordon (Gordon 1986) to distinguish the ATP receptors expressed in mast cells and lymphocytes from the receptor counterparts identified in platelets and thrombocytes (P2T) and in smooth muscles and other cell types (P2X and P2Y) (Burnstock and Kennedy 1985; Gordon 1986). The P2Z receptor was molecularly identified 10 years later as the $P2X_7$ receptor (Surprenant et al. 1996; Rassendren et al. 1997) (and P2T as the $P2Y_{12}$ receptor: Hollopeter et al. 2001).

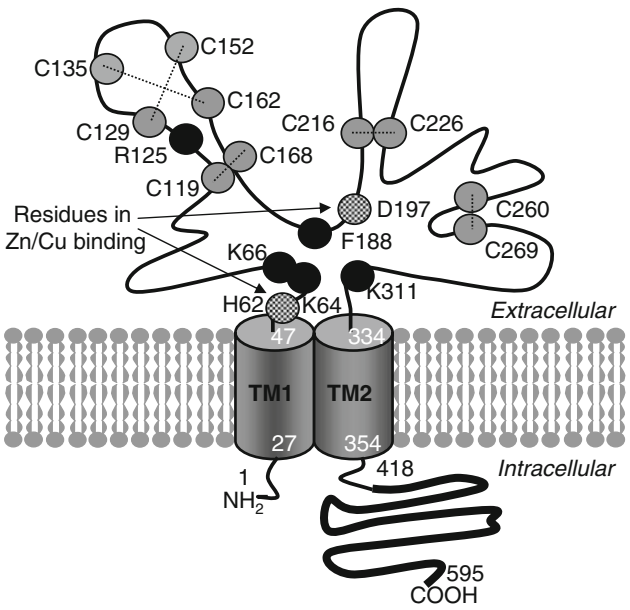

Fig. 1 Schematic representation of membrane topology of a $P2X_7$ receptor subunit and the key structure-function relationship. The $P2X_7$ receptor subunit is 595 amino acids long, and comprises intracellular short N-terminus and long C-terminus, two transmembrane domains (TM1: residues 27–47 and TM2: residues 334–354), and a large extracellular domain (residues 48–333). The extracellular domain contains ten cysteine residues that form intra-subunit disulfide. K64, K66, K311 and F188 are critical for receptor activation by ATP. R125 residue is required for activation of $P2X_7$ receptor ADP-ribosyltransferase via ADP-ribosylation. H64 and D197 residues are required for functional inhibition by zinc and copper. The long C-terminal tail (residues 418–595) is important for dye uptake pore formation and receptor trafficking

Since the first description and in particular molecular identification, extensive studies, employing electrophysiology, pharmacology, immunocytochemistry and gene knockout approaches, have revealed quite unique cellular localization and a plethora of physiological and pathological roles of the $P2X_7$ receptors (Ralevic and Burnstock 1998; North and Surprenant 2000; North 2002; Duan and Neary 2006; Ferrari et al. 2006). $P2X_7$ receptors are highly expressed in cells of hemopoietic origin such as mast cells, macrophages, monocytes, lymphocytes, leukocytes, osteoclasts and osteoblasts, where $P2X_7$ receptors serve the primary site mediating ATP-dependent immune responses, inflammation, cell proliferation, cell death, elimination of intracellular pathogens, and bone formation and reabsorption (e.g., Lammas et al. 1997; Baricordi et al. 1999; MacKenzie et al. 2001; Solle et al. 2001; Labasi et al. 2002; Ke et al. 2003; Adinolfi et al. 2005; Tsukimoto et al. 2006). Altered expression and function of $P2X_7$ receptors have been implicated in chronic lymphocytic leukemia (Adinolfi et al. 2002; Wiley et al. 2002; Cabrini et al. 2005). $P2X_7$ receptors are also expressed on satellite glial cells enwrapping the peripheral neurons (e.g., dorsal root ganglion neurons) and on microglia, astrocytes and oligodendrocytes in the brain. They mediate release of cytokines from satellite glial and microglial cells or release of neurotransmitters from astrocytes, and therefore play a crucial part in neuron-glia communications in the peripheral and central nervous systems (Duan et al. 2003; Zhang et al. 2007). Compelling evidence exists to indicate involvement of the $P2X_7$ receptors in neuronal excitoxicity that contributes to diseased states such as neuronal death (Choi et al. 2007) and multiple sclerosis (Matute et al. 2007). In addition, recent genetic linkage analysis suggests strong association of the $P2X_7$ receptor with human mood disorders such as bipolar disorder as well as major depressive disorder (Barden et al. 2006; Lucae et al. 2006; Erhardt et al. 2007). The $P2X_7$ receptor has been accordingly postulated to be part of a regenerative network that may give rise to these disorders (Bennett 2007).

It is worth mentioning that the increasing recognition of the functional importance of $P2X_7$ receptor has sparked enormous interests over the past few years in searching for potent and selective $P2X_7$ receptor antagonists and in targeting the $P2X_7$ receptor to develop therapeutic intervention. A number of compounds have been reported to potently and selectively antagonize the $P2X_7$ receptor,

including KN-62 (Humphreys et al. 1998), Brilliant Blue G (Jiang et al. 2000), AZ11645373 (Stokes et al. 2006), A-438079 (Nelson et al. 2006) and A-740003 (Honore et al. 2006). One should bear in mind that the potency for some of these antagonists is species-dependent, that is, considerably different between rodent and human $P2X_7$ receptors. Utilization of $P2X_7$ receptor specific antagonists (and $P2X_7$ receptor deficient mice) have identified a previously unrecognized role of the $P2X_7$ receptor in pain sensation and particularly in chronic inflammatory and neuropathic pain (Chessell et al. 2005; Nelson et al. 2006; Honore et al. 2006) (more details see Donnelly-Roberts and Jarvis 2007).

Hallmark properties of $P2X_7$ receptors

The $P2X_7$ receptor exhibits several hallmark properties that separate itself from other members of the P2X receptor family (North and Surprenant 2000; North 2002). Firstly, it shows remarkable functional plasticity; in response to short stimulation, just like other P2X receptors, $P2X_7$ receptor operates as an ion channel conducting Ca^{2+} and other small cations. However, upon prolonged or repetitive activation, $P2X_7$ receptor is able to engage signaling and other proteins to form large membrane pores to activate the inflammasome (Di Virgilio 2007) or to induce rapid membrane and mitochondrial morphological changes, cytoskeletal rearrangement, and ultimate cell death (Surprenant et al. 1996; Wilson et al. 2002; Morelli et al. 2003; MacKenzie et al. 2005; Pelegrin and Surprenant 2006). The extraordinarily long C-terminal tail of the $P2X_7$ receptor (Fig. 1) is known to be critical in pore formation (Surprenant et al. 1996; Jiang et al. 2005) and protein-protein interaction (Denlinger et al. 2001; Kim et al. 2001; Wilson et al. 2002). Secondly, the receptor agonist profile is unusual. $P2X_7$ receptor is activated by ATP in concentrations of >100 μM, which are significantly higher than those required for activation of the other P2X receptors. BzATP (2′-3′-(O)-(4-benzoylbenzoyl)ATP), an ATP analogue, is more potent than ATP for the $P2X_7$ receptor, whereas the opposite is true for the other P2X receptors. Furthermore, activation of the $P2X_7$ receptor can be achieved via ADP-ribosylation of Arg^{125} residue in the extracellular domain by ADP-ribosyltransferase (Seman et al. 2003; Adriouch et al. 2008) (Fig. 1). Thirdly, the ion current, the most immediate event following $P2X_7$ receptor activation, is distinctive. The $P2X_7$ receptor mediated currents are potently inhibited by zinc and copper in submicromolar concentrations, whereas the other P2X receptors are strongly potentiated or unaffected, except $P2X_1$ receptor which is also inhibited by zinc (Nakazawa and Ohno 1997; Wildman et al. 1998, 1999a, b; Xiong et al. 1999; Acuno-Catillo et al. 2000; Clyne et al. 2002). $P2X_7$ receptor shows strong sensitivity to inhibition by calcium and magnesium, albeit with lower potency than zinc and copper (Virginio et al. 1997). The other P2X receptors can be also inhibited by calcium and magnesium, and significantly higher concentrations are however required (Virginio et al. 1998; Ding and Sachs 1999).

Mechanisms for inhibition of $P2X_7$ receptor function by divalent cations

Dahlquist and Diamant were the first to suggest that the free acid form of ATP (or ATP^{4-}) is the actual agonist evoking histamine secretion in mast cells (Dahlquist and Diamant 1974), based on the assumption that the effect of altering the divalent cation concentrations was to change in the concentrations of the various ATP forms. By examining the concentration dependence on ATP of histamine secretion from mast cells in a series of calcium and magnesium concentrations, Cockcroft and Gomperts concluded ATP^{4-} as the active form of agonist and the term of ATP^{4-} receptor came to existence (Cockcroft and Gomperts 1979, 1980). Intriguingly, although it has never been supported by direct evidence, such concept has prevailed in the literature over the past decades since its conception.

The functional inhibition by divalent cations was nicely demonstrated in heterologous expression cells expressing the $P2X_7$ receptor immediately after it was cloned (Surprenant et al. 1996). Specifically, in HEK293 cells stably expressing the $P2X_7$ receptor, both the cationic channel and the cell permeabilizing pore, by measuring BzATP-evoked currents and uptake of a propidium dye (YO-PRO), were sensitive to inhibition by calcium (Surprenant et al. 1996). A detailed examination of the functional inhibition by divalent cations was conducted by Virginio et al. (1997), showing that calcium, magnesium, zinc and copper all strongly inhibited both $P2X_7$ receptor mediated currents and YO-PRO dye uptake. A number of other divalent cations were also subject to scrutiny. Overall, all the divalent cations studied show the same rank order of potency of inhibiting the $P2X_7$ receptor mediated currents and dye uptake: $Cu^{2+} > Cd^{2+} \sim Zn^{2+} > Ni^{2+} \gg Mg^{2+} \sim Co^{2+} > Mn^{2+} > Ca^{2+} = Ba^{2+} \gg Sr^{2+}$. For each of these divalent cations, the half-maximal inhibitory concentrations (IC_{50}) and/or the degree of current and dye uptake inhibition were similar. The inhibition was in any case voltage-independent, suggesting that divalent cations are very unlikely to cause the functional inhibition by blocking the ion conductance of the $P2X_7$ receptor channel. Furthermore, it was shown that the major effect of increasing the divalent cations in relatively low concentrations (e.g., increasing magnesium concentrations from 1 to 3 mM) was a rightward shift in the agonist concentration response

curve. These findings taken together led to proposal of a fundamentally different interpretation of the inhibition of $P2X_7$ receptor: divalent cations primarily alter the affinity of ATP binding to the $P2X_7$ receptor in an allosteric manner (Virginio et al. 1997).

We have recently revisited the functional inhibition of $P2X_7$ receptor by zinc and copper (Liu et al. 2008), following the interesting observations illustrated in Fig. 2. In this set of experiments measuring BzATP-evoked rat $P2X_7$ receptor currents in HEK293 cells, rebound currents were consistently seen upon simultaneous washing of zinc and agonist BzATP. The rebound currents were particularly prominent when the currents were strongly inhibited by relatively high concentrations of zinc (Fig. 2a). However, such rebound currents were never detected when BzATP was washed in the persistent presence of zinc (Fig. 2b). It is difficult to explain such an astonishing twist in the BzATP-evoked currents if the action of zinc is simply to alter the agonist form. However, it seems straightforward to understand the rebound currents if zinc binds and subsequently dissociates from the receptor faster than does BzATP.

We went on to search for the potential residues in the $P2X_7$ receptor that mediate the inhibition of zinc and copper, since we reasoned that identification of the key residues forming the metal binding site(s), if achievable, should ultimately provide direct evidence supporting the idea of direct interaction between divalent cations and

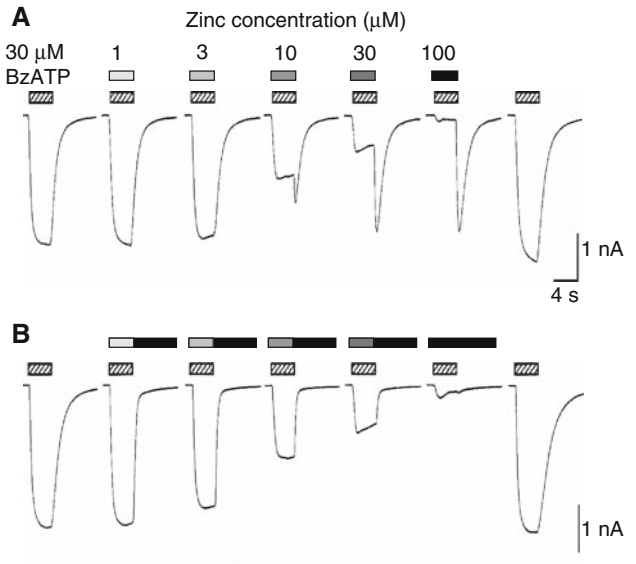

Fig. 2 Inhibition of $P2X_7$ receptor currents by zinc and rebound currents. Shown are BzATP-evoked current examples in HEK293 cells expressing rat $P2X_7$ receptor in the presence of increasing concentrations of zinc. Note that there are robust rebound currents accompanying simultaneous washing of zinc and BzATP (**a**), but not upon washing of BzATP in the persistent presence of zinc (**b**). Refer to Liu et al. (2008) for experimental details

$P2X_7$ receptor. Histidine, cysteine, lysine, aspartic acid and glutamic acid residues are known to be able to coordinate zinc binding to a number of different ion channels, including $P2X_2$ receptors (Clyne et al. 2002; Lorca et al. 2005); $GABA_C$ receptors (Wang et al. 1995), glycine receptors (Laube et al. 2000), NMDA receptors (Paoletti et al. 2000), acid-sensing ion channels (Chu et al. 2004) and voltage-gated Na^+, K^+ and Ca^{2+} channels (Mathie et al. 2006). Likewise, copper can directly bind to histidine, cysteine and glutamatic acid residues. We focused on 14 potential residues (five histidines, three glutamic acids, two aspartic acids and four lysines) in the extracellular domain that are conserved in the $P2X_7$ receptors. Each of these residues was changed to alanine in the rat $P2X_7$ receptor by site-directed mutagenesis, and the effects of zinc and copper on BzATP/ATP-evoked currents were examined upon expression of the mutant receptors in HEK293 cells. Except Lys^{145} residue at which alanine substitution had a modest ~4-fold decrease in the agonist sensitivity, mutation of any other select residues to alanine resulted in no significant change in the agonist sensitivity (Liu et al. 2008). We found that alanine replacement of His^{62} and Asp^{179} residues singly or in combination led to dramatic reduction in or nearly complete loss of the inhibition by zinc and copper, indicating that His^{62} and Asp^{179} residues are critical in the inhibition by zinc and copper. His^{201} and His^{267} may have a minor role as there was modest yet significant reduction in inhibition by zinc and copper when ATP was used as agonist to evoke $P2X_7$ receptor currents (Liu et al. 2008). The agonist dependence may relate to the fact that overlapping but distinctive parts of the $P2X_7$ receptor are engaged in the binding of BzATP and ATP (Young et al. 2007). These findings provide clear evidence indicating that the functional inhibition primarily results from direct interaction of zinc and copper with the $P2X_7$ receptor.

Acunna-Castillo et al. (2007) examined the role of extracellular histidine residues in the inhibition of ATP-evoked rat $P2X_7$ receptor currents by copper, zinc and magnesium, following expression of alanine mutant receptors in *Xenopus* oocytes. They found no significant effects of alanine mutation on the agonist sensitivity. Their results also support that the functional inhibition results from direction interaction between divalent cations and the $P2X_7$ receptor. However, contribution of the histidine residues was quite different. Mutation of His^{267} conferred complete loss of and His^{201} reduction in copper inhibition. Mutation of His^{267} or His^{219} resulted in no inhibition by zinc (100 μM), and mutation of His^{130} or His^{201} abolished the inhibition by magnesium (1 mM). Surprisingly, in their study there was no significance of His^{62} in the inhibition by zinc and copper (and Asp^{197} not examined). The reasons for the discrepancy are not clearly understood, but the

different expression cells used (HEK293 cell versus oocyte) could be a significant factor. Conflicting results were reported in previous studies on the $P2X_7$ receptor heterologously expressed in mammalian cells and oocytes. The most noticeable difference is that expression of the $P2X_7$ receptor persistently forms the large dye uptake pore in HEK293 cells (e.g., Surprenant et al. 1996), but not in oocytes (Petrou et al. 1997; Klapperstuck et al. 2000). In addition, as mentioned above, the major effect of increasing magnesium concentrations in HEK293 cells is a rightward shift in the agonist dose-response curve, that is, a reduction in the agonist sensitivity without reducing the maximal responses (Virginio et al. 1997). In contrast, when in oocytes, the primary effect is suppression of the maximal responses without significant change in the agonist sensitivity (Acuno-Castillo et al. 2007).

Identification of the binding residues in the $P2X_7$ receptor evidently indicates that the inhibition by zinc and copper is mainly due to an interaction with the receptor (Acuno-Castillo et al. 2007; Liu et al. 2008). Thus, a unified principle can account for the actions of zinc and copper on all the P2X receptors, regardless of the functional consequences being facilitating or inhibitory. The actions of calcium, magnesium and other divalent cations may also result from similar interactions with the $P2X_7$ receptor, although the key residues remain to be identified. There is some evidence indicating that this is the case for magnesium (Acuno-Castillo et al. 2007). His^{120} and His^{213} residues in the extracellular domain are critical in zinc-evoked facilitation of the $P2X_2$ receptor (Clyne et al. 2002; Lorca et al. 2005). These two histidine residues have been shown to be in close apposition and form an inter-subunit zinc binding site (Nagaya et al. 2005). A recent study by histidine scanning of the residues next to His^{120} and His^{213}, has provided further insight into how His^{120} and His^{213} residues may coordinate zinc binding to the $P2X_2$ receptor (Tittle et al. 2007). Currently, there is no information regarding how close His^{62} and Asp^{197} residues are in the $P2X_7$ receptor. The approaches used to probe the zinc binding site in the $P2X_2$ receptor (Nagaya et al. 2005; Tittle et al. 2007) should be informative to establish a better understanding of how the two key residues mediate the interaction of zinc/copper with the $P2X_7$ receptor.

The structural biology of P2X receptors is currently still lacking. Unfortunately, there is also no very strong sequence similarity between P2X receptors and any ATP binding proteins with known structures. Site-directed mutational studies in the past 5 years or so have provided considerable information that helps us greatly to relate the key functional properties to particular parts or domains within the P2X receptors (North 2002; Vial et al. 2004). However, by large there is lack of definitive evidence as for which parts form the agonist binding site and in particular how the receptor undergoes conformational changes leading to the channel opening. Thus, it still remains challenging to infer the mechanisms governing the functional modulation of P2X receptors by divalent cations including zinc and copper. In the $P2X_7$ receptor, His^{62} is located closely to Lys^{64}, the residue that is absolutely critical for activation of the $P2X_7$ receptor by BzATP and ATP (Wilkinson et al. 2006; Cao et al. 2007) and possibly engaged in interacting with the negatively charged phosphate group. Asp^{197} is a few residues away from the conserved Phe^{188} residue ($P2X_7$ receptor numbering), which is suggested to be important in coordinating binding of the adenine ring (Vial et al. 2004). Thus, one possibility is that zinc and copper bind to the $P2X_7$ receptor and introduce allosteric modulation of the BzATP/ATP binding, as was previously proposed (Virginio et al. 1997). A tempting alternative is that His^{62} and Asp^{197} residues are closely positioned and involved in coupling the agonist binding and the channel opening, given that His^{62} is positioned between Lys^{64} and the first transmembrane domain, part of the P2X receptors that is thought to undergo conformational changes during the channel gating (Jiang et al. 2001; Silberberg et al. 2007). Interestingly, our study showed that single alanine substitution of His^{62} and Asp^{179} residues had no substantial change (a tendency to decrease if any) in the agonist sensitivity, but double substitution caused an approximately 4-fold increase in the agonist sensitivity (Liu et al. 2008). The simple explanation could be that replacement of both residues with alanine (containing a small side chain) may facilitate the channel gating. Conceivably, occupation of His^{62} and Asp^{197} by zinc and copper (and other inhibitory divalent cations) would hinder the channel gating. This could also explain the observations that divalent cations cause the rightward shift and suppression of maximal receptor responses in the agonist concentration response curve (Virginio et al. 1997). Further studies are needed to test these candidate mechanisms.

Physiological and pathophysiological relevance

The potent inhibition by divalent cations bear potential implication to the $P2X_7$ receptor mediated functions under both physiological and pathophysiological conditions. The IC_{50} values for calcium (2–3 mM) and magnesium (0.5–1 mM) (Virginio et al. 1997) are within the extracellular concentrations of these two divalent cations and thus it seems reasonable to believe that the $P2X_7$ receptors are considerably suppressed under physiological conditions. Such tonic inhibition can be beneficial as it may prevent or reduce unnecessary cell permeabilization while the physiological functions are maintained. For instance, it is known

that very low level of basal $P2X_7$ receptor activation facilitate cell proliferation while the high level of $P2X_7$ receptor activation induces cell apoptosis (Baricordi et al. 1999; Adinolfi et al. 2005). On the infection and/or inflammatory sites, the local extracellular concentration of calcium and magnesium could be reduced as a result of release of the cytosolic fluids of the surrounding damaged or dying cells, and the inhibition be alleviated to enhance the $P2X_7$ receptor mediated immune responses. Potent inhibition of $P2X_7$ receptors by zinc and copper also has significant physiological relevance, considering the IC_{50} values of 5–20 and 2–6 μM for zinc and copper, respectively (Virginio et al. 1997; Liu et al. 2008). As discussed above, $P2X_7$ receptors are expressed in astrocytes, oligodendrocytes and microglia, and satellite glial cells and play an important role in mediating neuron-glia communications as well as neuronal excitoxicity. Zinc and copper released from nerve terminals can reach 10–100 μM concentrations (Kardos et al. 1989; Li et al. 2001) and thus easily subject the astrocytes and glial cells in direct contact with or in close vicinity of these nerves to the concentrations that can effectively inhibit the $P2X_7$ receptors. Again, such inhibition can provide a potential protective or negative feedback mechanism by which neuronal excitoxicity is minimized during physiological neuron-glia interaction. Copper is found at micromolar concentrations and is essential for T cell proliferation and the ability of neutrophils to generate reactive oxygen species to eliminate ingested pathogens under conditions of infection and/or inflammation (Percival 1998). $P2X_7$ receptor based signaling pathway has been shown to contribute to T cell death (Tsukimoto et al. 2006). Functional suppression of the $P2X_7$ receptor by copper can conceivably promote T cell proliferation and reduce cell death during infection/or inflammation. It is also known that the $P2X_7$ receptor is expressed in neutrophils and mediated superoxide generation (Suh et al. 2001). Obviously, more studies are needed to investigate the regulation of $P2X_7$ receptor mediated biological processes by physiological concentrations of divalent cations calcium, magnesium, zinc and copper. Increase in our knowledge of how divalent cations inhibit the $P2X_7$ receptor at the molecular level will surely assist us to gain a better understanding of the cell and systems physiology and pathophysiology of $P2X_7$ receptor.

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