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REVIEW

The $P2X_7$ receptor–pannexin connection to dye uptake and IL-1β release

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Abstract The $P2X_7$ receptor ($P2X_7R$) is uniquely associated with two distinct cellular responses: activation of a dye-permeable pathway allowing passage of molecules up to 900 Da and rapid release of the pro-inflammatory cytokine, interleukin-1β (IL-1β), from activated macrophage. How this dye uptake path forms and whether it is involved in IL-1β release has not been known. Pannexin-1 is a recently identified protein found to physically associate with the $P2X_7R$. Inhibition of pannexin-1 does not alter $P2X_7R$ ion channel activation or associated calcium flux but blocks one component of $P2X_7R$-induced dye uptake and unmasks a slower, previously undetected, dye uptake pathway. Inhibition of pannexin-1 blocks $P2X_7R$-mediated IL-1β release from macrophage as well as release mediated by other stimuli which couple to activation of capase-1 and additionally inhibits the release of interleukin-1α, a member of the IL-1 family whose processing does not require caspase-1 activation. Thus, pannexin-1 is linked to both dye uptake and IL-1β release but via distinct mechanisms.

Keywords Caspase-1 · Inflammasome · Dye uptake · Inflammation · Macrophage

Introduction

The first report of extracellular adenosine triphosphate (ATP) as a "cell-permeabilizing" agent was in 1975 when Rozengurt and Heppel [1] found that *p*-nitrophenyl phosphate entered transformed 3T3 cells during a 5-min exposure to ATP. Then, in 1979 Cockcroft and Gomperts [2] similarly found that inorganic phosphates rapidly entered mast cells upon ATP stimulation. Through the 1980s, work by the groups of Silverstein [3–7], Weisman [8–11], and Wiley [12–14] provided pharmacological, biophysical, and biochemical characterization of what became known as the P2Z receptor [15], which was particularly prominent in immune cells. The most striking cellular feature that clearly separated P2Z receptor activation from other purine receptors was the rapid uptake of higher molecular weight molecules (up to ~900 Da). This cell permeabilization process was considered to be due to the opening of a non-selective "large pore" in contrast to the cationic channels that formed the P2X receptor subtype [15]. The P2Z receptor in macrophage and lymphocytes became a potential anti-inflammatory drug target by the early 1990s with studies by the groups of Chaplin [16], Gabel [17–19], Di Virgilio [20–24], and Dubyak [12, 25–29] demonstrating that ATP, most likely acting on P2Z receptors, was the most potent physiological stimulus for the rapid release of the pro-inflammatory cytokine, interleukin-1β (IL-1β), from activated monocytes and macrophages. Soon after the Glaxo-Geneva group discovered the molecular identity of the P2Z receptor as the $P2X_7$ receptor ($P2X_7R$) in 1996/1997 [30, 31], high throughput screening on heterologously expressed, or endogenous, $P2X_7$ receptors using dye uptake assays was begun by virtually all Big Pharma companies [32, 33]. During this first decade of the twenty-first century, several highly selective and potent $P2X_7R$ antagonists have been discovered and found to be effective in animal models of neuropathic pain and other inflammatory processes, most likely through a reduced release of bioactive IL-1β and other members of the IL-1 family of pro-inflammatory cytokines (IL-1α and IL-18) [32]. Most recently, AstraZeneca have reported initial positive results from phase II clinical trials of their $P2X_7R$ antagonist in the treatment of rheumatoid arthritis [34]. Thus, the practical link between the $P2X_7R$-

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mediated "dye uptake/large pore" path and IL-1β release inflammatory processes in regard to drug discovery is clear. But is there a physiological link, and, if there is, what is the underlying mechanism by which the dye uptake path signals to IL-1 processing and release? This is the main question we will address in this review. We will focus on results obtained from studies of endogenous $P2X_7R$ in monocytes and macrophage and of heterologously expressed $P2X_7R$ in mammalian cells because few, if any, differences have been found when comparing data obtained from these systems. Although $P2X_7R$ functions in other immune cells (neutrophils, B and T lymphocytes) are generally similar to macrophage, there are some distinct differences that may suggest alternative signaling pathways and/or interacting protein complexes; these will not be considered here.

$P2X_7R$ ion channel and large pore

Evidence for $P2X_7R$ ion channel itself as the large pore

Until very recently, most studies of $P2X_7R$-mediated membrane currents and dye uptake had concluded that the $P2X_7R$ ion channel itself was the large pore. Several lines of evidence supported this conclusion. Initial electrophysiological studies of the membrane current in macrophage and ion substitution experiments indicated the response resulted from a non-selective increase to both cations and anions [3]. A non-selective and simultaneous increase in permeability to both cations and anions, primarily in the form of NaCl, would inevitably draw water into the cell, leading to cell swelling (an often observed sequelae of $P2X_7R$ activation) with transient plasma membrane disruption allowing passage of molecules and dyes up to 900 Da size. Subsequent ion replacement studies in the human embryonic kidney 293 cell line (HEK 293) ectopically expressing $P2X_7R$ indicated the ionic current resulted solely from a cationic (Na^+,K^+,Ca^{++}) conductance but that the channel size dilated over several seconds to minutes (depending on agonist concentration) so that larger cations were able to pass [35]. An example of this is shown in Fig. 1a, where the ATP-evoked current in the presence of the large cation, *N*-methyl-D-glucamine ($NMDG^+$, MW 195), is initially outward due to the outward flow of intracellular Na^+ but gradually becomes inward as NMDG begins to flow into the cell. Reversal potential measurements made at 2-s intervals show a progressive shift to more depolarized potentials (Fig. 1b), delineating the

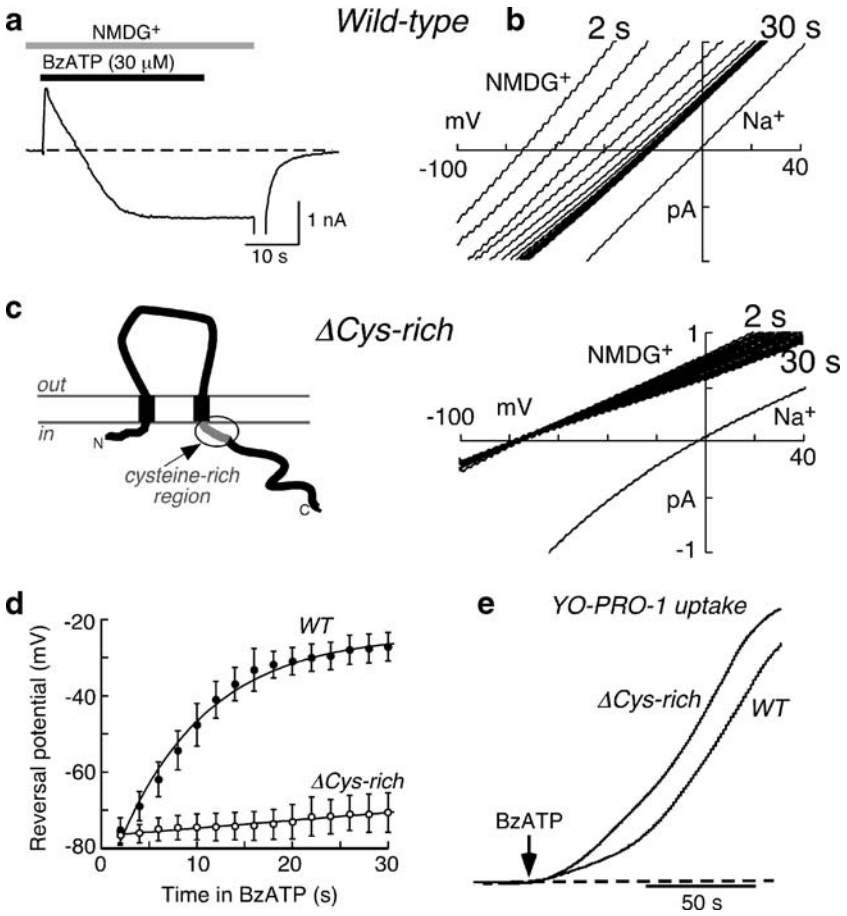

Fig. 1 Evidence for and against the $P2X_7R$ ion channel itself dilating to form the dye uptake large pore. **a** Example of membrane current recorded in response to $P2X_7R$ stimulation in extracellular solution containing the large cation, $NMDG^+$. Initial current is outward because $NMDG^+$ initially is impermeable and only intracellular sodium passes outward but with time current turns inward due to $NMDG^+$ entering the cell. **b** Example of reversal potential measurement experiments carried out at 2-s intervals during application of BzATP as shown in (**a**); reversal potential shifts to positive values which shows that $NMDG^+$ is entering the cell. **c**, **d** Examples of the same experiment but from cell expressing mutated $P2X_7R$; now there is no $NMDG^+$ shift indicating no entry of $NMDG^+$ into the cell, but the same mutated receptor shows *enhanced* dye uptake (**e**). Recording in (**a**) is from Virginio et al. [35] while results shown in (**b**)–(**e**) are from Jiang et al. [39]

increased permeability to the larger cation. This type of gradual change in permeability to larger cations is the most likely explanation for earlier reports of anion flux through $P2X_7R$ channels because reversal potentials measured at equilibrium conditions could be interpreted as indicating permeability to anions as well as cations. Parallel experiments measuring dye uptake (YO-PRO-1 fluorescence) or simultaneous measurements of dye uptake and membrane currents showed that the kinetics of dye uptake and NMDG permeability shift were the same [39]. The tight kinetic correlation between NMDG permeability shift and dye uptake was observed at all agonist concentrations and thus provided strong circumstantial evidence that the $P2X_7R$ ion channel dilated over time to allow the passage of larger cations and cationic dyes such as YO-PRO-1 (MW 629) and ethidium bromide (MW 394).

Evidence against $P2X_7R$ ion channel itself as the large pore

Certain observations were inconsistent with the hypothesis that the $P2X_7R$ ion channel dilates to allow passage of larger molecules. Firstly, very low concentrations of the calmodulin inhibitor calmidazolium were shown to inhibit the $P2X_7R$ ion channel by up to 95% yet did not decrease dye uptake [36]. Secondly, it was not clear how anionic dyes, such as Lucifer yellow (MW 457), which are well established as being taken up by cells in response to $P2X_7R$ activation [3, 6, 37, 38], could permeate the cation-selective channel. Thirdly, specific deletions or mutations in the C-terminal domain of the $P2X_7R$ were made that completely blocked pore dilatation as measured by $NMDG^+$ permeability shifts (Fig. 1c, d) yet dye uptake and membrane currents were both significantly *enhanced* [39] (Fig. 1e). Moreover, although significant YO-PRO-1 uptake was observed in normal extracellular sodium concentrations, there was no NMDG permeability increase observed, thus dissociating NMDG permeability changes from dye uptake [39]. Finally, low micromolar concentrations of the gap junction blocker carbenoxolone (CBX) markedly inhibited $P2X_7R$ dye uptake without altering membrane currents or initial calcium flux [40]. These results suggested two other possibilities: that $P2X_7R$ channel activation induces a distinct signal transduction pathway which leads to dye uptake [41], or a distinct $P2X_7R$-interacting protein is the dye uptake pathway [39].

Pannexin-1 mediates rapid dye uptake pathway

We recently identified pannexin-1 (panx1) as a $P2X_7R$-associated protein which appears to be the large pore or is responsible for activation of the large pore [40]. Panx1 and $P2X_7R$ co-immunoprecipitated in HEK 293 cells; small interfering RNA (siRNA) directed against panx1, a panx1-mimetic inhibitory peptide (^{10}panx1 peptide), and CBX all inhibited $P2X_7R$-mediated dye uptake but not membrane currents or calcium flux in HEK 293 cells and in human and murine macrophage [40]. Notably, inhibition of panx1 blocked only an initial phase of the $P2X_7R$-induced dye uptake, revealing a previously undetected slow (panx1-independent) dye uptake (Fig. 2) [42]. The mechanisms underlying the slow dye uptake, and/or whether it has any physiological significance, remain to be determined but it does not appear to be involved in the release of IL-1β (see below). Interestingly, two earlier studies had suggested the involvement of MAP kinase (MAPK) signal transduction in $P2X_7R$-mediated dye uptake in macrophage: in one study the dye uptake was found to be calcium-dependent and was not inhibited by very high concentrations (500 μM) of CBX [41]. It should be noted that all other studies on $P2X_7R$-mediated dye uptake have found the process to be calcium-independent [35, 36, 39]. In a separate study, a MAPK-dependent component of the dye uptake was independent of $P2X_7R$-mediated IL-1β release [43]. Because $P2X_7R$-induced panx1-dependent dye uptake is calcium-independent, CBX-sensitive, and associated with IL-1β release [39, 40], it is tempting to speculate that the panx1-independent slow dye uptake may result from the MAPK signaling cascade described by Jarvis and colleagues [43].

Pannexins were originally identified in 2003/2004 by low sequence homology to invertebrate gap junction channels, the innexins [44-46]. Like innexins and the mammalian gap junction channels, connexins, the three members of the pannexin family (panx1, 2, 3) share a similar membrane topology consisting of four transmembrane domains, short extracellular segments, and intracellular C and N termini [44–46]. Pannexins do not show sequence homology to the large family of connexin proteins, although recent detailed phylogenetic analysis has convincingly placed innexins, pannexins, and connexins within the same molecular superfamily [47]. *In situ* hybridization and immunohistochemistry at both light and electron microscopy levels show that panx1 is widely expressed, particularly in immune cells, endothelia, and epithelia; panx2 is relatively neuronal-specific, while panx3 shows fairly localized expression to joints and skin [45, 46, 48]. Gap junctions are composed of 12 connexin proteins via hexameric connexin complexes (called connexons or hemichannels) on two adjacent cells coming together to form a junctional channel through which ions and small molecular weight (up to 1,000 Da) molecules can pass [47]. Initial studies in which panx1 was over-expressed in oocytes suggested panx1 could act to form gap junctions in a manner similar to that known for connexins [45, 48], but no gap junction formation has been observed when panx1 is expressed in mammalian cells [40, 49] and

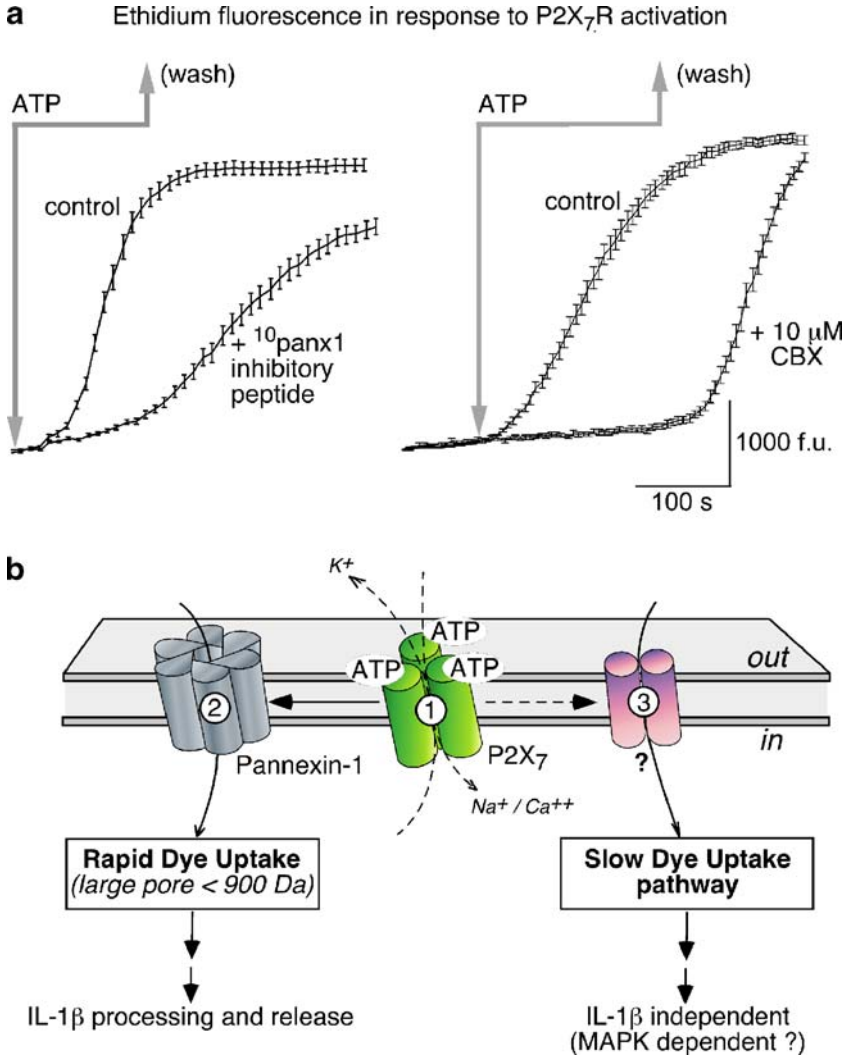

Fig. 2 Two phases to $P2X_7R$-mediated dye uptake revealed by inhibition of panx1. **a** Original traces of typical dye uptake experiments carried out on HEK 293 cells expressing rat $P2X_7R$; each trace shows average ± SEM from 20 to 30 cells; control fluorescence (in arbitrary fluorescence units) saturates the optical system in both examples. Inhibition of panx1 with CBX or ^{10}panx1-mimetic inhibitory peptide dramatically delays dye uptake; results detailed in [40, 42] suggest two independent processes. **b** Distinct roles and underlying mechanisms for the two dye uptake processes after $P2X_7R$ activation. Panx1 is involved in the initial rapid dye uptake and in IL-1β processing and release. Mechanisms underlying the slow dye uptake and its physiological significance are unknown but results from [43] suggest it may involve MAPK-dependent pathways. $P2X_7R$ cartoon is represented as a homotrimer based on studies of the mammalian $P2X_1$ and $P2X_2$ receptors [71]. Panx1 cartoon is represented as a plasma membrane hemichannel consisting of a complex of six subunits, hypothetically arranged based on analogy to connexin gap junction hemichannels

the current understanding is that panx1 does not form gap junctions [49, 50].

When panx1 is ectopically expressed in HEK 293 cells lacking $P2X_7R$, a low level of constitutive dye uptake occurs which is not otherwise observed in untransfected or vector-transfected cells nor in $P2X_7R$-expressing cells in the absence of ATP [40]. We have also noted a significant level of constitutive dye uptake in peritoneal macrophage obtained from $P2X_7R^{-/-}$ mice that is not seen in wild-type macrophage (unpublished observations). These findings may suggest that panx1 is normally under negative control when it is in association with unstimulated $P2X_7R$.

How panx1 mediates dye uptake has not been resolved although it is currently assumed that panx1 forms a large conductance hemichannel in the plasma membrane through which cationic and anionic molecules of up to 800–900 Da can pass [46, 49, 50]. This hypothesis is largely based on analogy to gap junction channels rather than to direct experimental evidence. The main evidence that panx1 acts as a large conductance hemichannel is that when panx1 is heterologously expressed (in the absence of $P2X_7R$), a cation/anion non-selective membrane current can be recorded [40, 45]. However, there are critical questions that must be resolved before panx1 can be considered a plasma membrane hemichannel through which marker dyes directly pass. (1) Single channel recordings showing large conductance unitary opening are critically required. We have consistently failed to record large conductance single channels from panx1-expressing, or $P2X_7R$/panx1 over-expressed, mammalian cells; there is one report of large conductance single channels (>200 pS) being observed in oocytes over-expressing panx1 [50], but this does not appear to be a consistent observation. (2) Site-directed mutagenesis of residues within the panx1 protein that result in altered voltage dependence, ion permeability, and/or unitary conductance are required to provide direct demonstration that panx1 is, itself, an ion channel. (3) If a large conductance pannexin channel is activated by $P2X_7R$

stimulation, then one would expect considerable current inhibition in the ATP-induced current in response to $P2X_7R$ stimulation. But, although CBX and ^{10}panx1 inhibitory peptide completely block the membrane current recorded when panx1 is ectopically expressed (in the absence of $P2X_7R$), they have no effect or slightly enhance the membrane current activated by $P2X_7R$ stimulation in cells expressing both panx1 and $P2X_7R$ [40]. (4) It remains unclear whether endogenous panx1 is a plasma membrane protein or an endoplasmic reticulum (ER) membrane protein or both. There is evidence that panx1 may be an ER membrane protein involved in ER calcium regulation [51]. There are currently no studies showing high-resolution subcellular localization of endogenous panx1 in macrophage using antibodies that are highly specific for panx1. (5) Can endogenous panx1-like currents be recorded from macrophage? There are no reports of membrane currents recorded from monocytes or macrophage (or any neuronal or non-neuronal cell) having properties similar to those observed when panx1 is over-expressed in HEK 293 or other mammalian cells. Thus, until these questions are answered, alternative possibilities must be considered, in particular the possibility that panx1 may recruit, or activate, transporter proteins which provide the direct route for entry and exit of dyes and other small molecules.

$P2X_7R$–pannexin-1 and IL-1β processing and release

The release of bioactive pro-inflammatory cytokine IL-1β by macrophages is an essential element of innate immunity and is a multi-step process tightly regulated by the activation of caspase-1 [52]. Initial inflammatory stimuli by bacterial pathogen-associated molecular pattern (PAMP) molecules, such as endotoxin challenge, activate the NFκB cascade leading to increased protein synthesis, particularly of pro-IL-1β, as well as $P2X_7R$ and panx1 [40, 52–55], but a secondary stimulus is required for efficient processing and release of bioactive IL-1β [16–19, 53]. ATP is a key secondary stimulus. Activation of $P2X_7R$ by the nucleotide leads to the assembly of a multi-protein complex called the NLRP3 inflammasome that recruits pro-caspase-1 and induces its proteolytic activation (Fig. 3) [53–55]. Panx1 appears to be a critical component not only in $P2X_7R$-mediated IL-1β release but also in all caspase-1 associated signaling through the NLRP3 inflammasome [40, 42, 56].

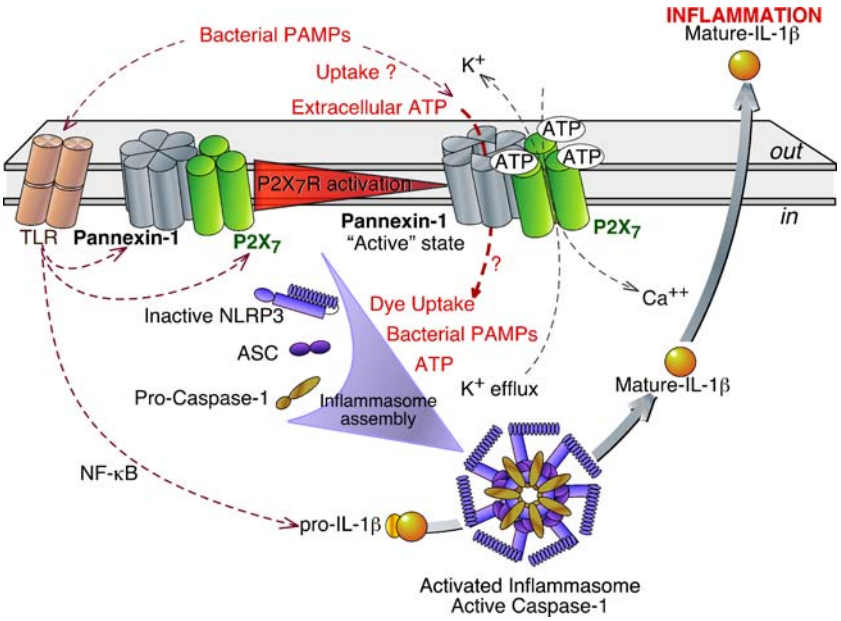

Fig. 3 Schematic depicting distinct models of the activation of caspase-1 and IL-1β release induced by $P2X_7R$ and panx1 activation. Toll-like receptor (*TLR*) activation by bacterial PAMPs activates the production of pro-IL-1β as well as enhances the expression and activity of $P2X_7R$ and panx1. Under resting conditions, $P2X_7R$ may keep panx1 under negative regulation via direct interaction. High concentrations of extracellular ATP activates $P2X_7R$ ion channel resulting in a cation permeability (Na^+ influx/K^+ efflux and intracellular Ca^{++} rise) and the activation of panx1. Panx1 activation allows a rapid dye uptake permeabilization pathway and potentially the entry of either bacterial PAMPs and/or extracellular ATP that will directly activate NLRP3 (nucleotide-binding domain and leucine-rich repeat containing a pyrin domain) molecule using the adaptor molecule ASC (apoptosis-associated speck-like protein containing a CARD). Activated NLRP3 with ASC aggregates pro-caspase-1 molecules through the formation of a macromolecular inflammasome complex that facilitates subsequent pro-caspase-1 activation and pro-IL-1β processing to its bioactive state. NLRP3 inflammasome complex cartoon is based on studies on the NLRP1 inflammasome and is probably comprised of five to seven subunits, each subunit consisting of NLRP3 and ASC with the ability to recruit five to seven pro-caspase-1 molecules [68]. Inflammasome pictorial adapted from Ting et al. [72]

CBX, siRNA gene silencing of panx1 protein, and ^{10}panx1 inhibitory peptide all inhibit $P2X_7R$-mediated IL-1β release from endotoxin-primed macrophage at similar concentrations to those that inhibit the initial $P2X_7R$-mediated dye uptake [40]. This strong correlation led to the initial hypothesis that opening of dye-permeable panx1 hemichannels via $P2X_7R$ activation directly leads to inflammasome assembly and activation [40]. While this hypothesis cannot yet be ruled out in the specific case of $P2X_7R$ function, it quickly became apparent that this cannot be a general mechanism by which panx1 signals to inflammasome activation when it was found that panx1 inhibition (via CBX, panx1 gene silencing, and ^{10}panx1 inhibitory peptide) also inhibits processing and release of bioactive IL-1β via routes that are not associated with dye uptake [42]. For example, nigericin does not evoke any dye uptake but is well known to activate NLRP3 inflammasome release of IL-1β and maitotoxin evokes only a panx1-independent slow onset of dye uptake, but in both cases, panx1 inhibition prevents IL-1β processing and release [42].

How might panx1 signal to activate the NLRP3 inflammasome in response to $P2X_7R$ stimulation, or to dye uptake independent stimuli? The classic hypothesis for activation of caspase-1 via the NLRP3 inflammasome is the K^+ efflux model [17]. This model is based on three main observations: (a) activation of $P2X_7R$ in macrophages induces K^+ depletion [17, 40, 53, 57] as does stimulation with nigericin or maitotoxin; (b) activation of caspase-1 by these stimuli does not occur in high extracellular K^+ solution [40, 57–59]; and (c) the in vitro assembly of the inflammasome is dependent on K^+ concentrations lower than 70 mM [58]. Initially, panx1 was expected to be a conduit for the high K^+ efflux subsequent to $P2X_7R$ activation, but inhibition of panx1 using ^{10}panx1 inhibitory peptide abolished caspase-1 activation without altering $P2X_7R$-mediated K^+ efflux [40]. This result implies that panx1 signals downstream to K^+ efflux and that K^+ efflux is independent of panx1-mediated macrophage permeabilization and dye uptake. It is also possible that the panx1 inhibitory peptide may block downstream panx1 signaling without altering direct K^+ permeability through panx1 channels. However, panx1 inhibition does not alter membrane currents or calcium influx in response to $P2X_7R$ activation (see above). Taken together, it seems most likely that K^+ efflux occurs through the $P2X_7R$ ion channel itself and that panx1 acts on the inflammasome downstream of ion flux. This idea that panx1 is unlikely to be directly involved with ion flux in macrophage is further supported by a recent study where inhibition of panx1 by CBX prevented ATP-mediated IL-1β release from macrophage, as expected, but did not alter concurrent release of cathepsin B, this latter process resulting from a Ca^{++}-dependent exocytosis of secretory lysosomes [60].

If K^+ efflux is not the link between panx1 and inflammasome activation, an alternative hypothesis comes from the group of Nuñez who have suggested that panx1 activation by $P2X_7R$ may act to deliver PAMPs directly into the cytosol where they can then directly bind to leucine-rich repeats present in Nod-like receptors (i.e., NLRP3) to directly activate the inflammasome [61, 62]. This is an attractive idea whereby PAMPs would be not only responsible for the initial inflammatory stimulus by activating the TLR–NFκB synthetic pathway but also for the secondary stimulus leading to processing and release of IL-1β via panx1-mediated delivery into the cell interior. This novel hypothesis is based on observations that cytosolic delivery of the bacterial PAMP lysosomic muramyl dipeptide (MDP) activated the inflammasome in a manner similar to $P2X_7R$ and that $P2X_7R$ activation induced a panx1-dependent lysosome-to-cytosol translocation of MDP [61, 62]. However, there are three major difficulties in this hypothesis as a generalized mechanism for inflammasome activation. Firstly, it is not clear how a $P2X_7R$/panx1 permeabilization pore that is presumably limited to molecules <900 Da can transfer larger PAMPs. That is, while PAMPs like MDP (MW 492 Da) may be expected to pass, other PAMPs are too large, particularly the classically used lipopolysaccharide (LPS) whose active fragment, lipid A, has a molecular mass of 1,700–1,800 Da (depending on the number and identity of fatty acid chains present) [63]. Secondly, most IL-1 cytokine release experiments are performed by initial incubation with LPS or other PAMPs followed by washing away these PAMPs and applying ATP to activate $P2X_7R$ [40, 58, 60]. Therefore, minimal levels of PAMPs are likely to be present in the ATP incubation media for delivery into the cell. Thirdly, the studies using different PAMPs to activate the inflammasome [61, 62] have been carried out in the presence of ATP, thus release of bioactive IL-1β release must be in part—or in toto—due to $P2X_7R$ activation.

A third hypothesis regarding panx1-mediated activation of the inflammasome is that extracellular ATP activates the $P2X_7R$, which in turn activates panx1 hemichannel (or transporter) activity to directly transport ATP *into* the cell. Evidence has been presented to suggest panx1 can act as a conduit for ATP release from erythrocytes [64]. If panx1 is a conduit for ATP, it must be a conduit for either entry or exit of ATP depending on the concentration gradient. Concentrations of ATP required to activate $P2X_7R$ (usually >100 μM) may be higher than free intracellular ATP in localized regions near the plasma membrane, especially as the vast majority of intracellular ATP (~90%) is produced in, and bound to, mitochondria [65]. Importantly, ATP can directly activate capase-1 in cell-free systems [66], and elevations of intracellular ATP levels have been associated with, and required for, caspase-1 activation and IL-1β

release [67, 68]. This is an attractive model for $P2X_7R$-mediated inflammasome activation although there is currently no direct or indirect experimental evidence for or against this hypothesis. However, this hypothesis also cannot be a generalized mechanism for panx1 involvement in caspase-1-dependent IL-1β processing and release because other stimuli, such as nigericin and maitotoxin, that are equally panx1-dependent, do not require extracellular ATP.

Recent work in our lab suggests panx1 may have a wider role in pro-inflammatory cytokine release beyond initial involvement in caspase-1 activation. In an investigation of the kinetics of action of ^{10}panx1-mimetic inhibitory peptide, we found that this peptide was able to stop further IL-1β release from macrophage after caspase-1/inflammasome activation had already been instigated by $P2X_7R$ stimulation. The ^{10}panx1 peptide did not inhibit caspase-1 activity per se in cell-free assays, so ruling out a direct chemical or enzymatic interaction with caspase-1. Moreover, inhibition of panx1 not only inhibited caspase-1-dependent processing and release of both IL-1β and IL-18 but also IL-1α which does not directly require caspase-1 inflammasome activation for its processing [56]. Additionally, a significant practical finding was that inhibition lasted for >1 h after removal of the inhibitory peptide, thus indicating tight, long-lasting binding and/or prolonged inhibition of unknown processes. In this regard, it is important to note that prolonged incubation (>2–3 h) with the ^{10}panx1 inhibitory peptide is toxic to macrophage [56]. Although the mechanism of ^{10}panx1-mimetic toxicity during prolonged application has not been investigated, it clearly differs from the rapid (non-toxic) inhibition of caspase-1 activation that occurs with brief (<30 min) applications of this inhibitor [40, 42, 56]. These results make it clear that this compound should be used with caution, and investigators should ensure that appropriate optimization protocols (concentration and time dependence) are carried out when employing this inhibitor. This is particularly true in view of the well-known variability in potency/purity of commercially synthesized peptides and proteins.

Concluding remarks

$P2X_7R$ activation leads to numerous downstream signaling events: the most upstream event subsequent to initial ion channel opening is induction of the large pore/dye uptake path that occurs approximately 2–3 s after receptor activation [35]. The most physiologically relevant downstream event is the release of the pro-inflammatory group of IL-1 cytokines, IL-1α, IL-1β, and IL-18; cytokine release can be measured by ELISA or Western blot techniques within 5–15 min of receptor activation [60, 69, 70] although it is likely that release occurs even sooner. Recent studies summarized in this review have identified panx1 as a critical component in both these events, but it appears that the panx1-dependent dye uptake path is not the causative event underlying cytokine release. Is this dye uptake event that has been so extensively utilized since the original studies in the mid-1970s simply a functionally meaningless by-product of $P2X_7R$–panx1 activation? If so, it has been an enormously practical by-product by providing a highly sensitive and selective screening assay that has resulted in the identification of potent $P2X_7R$ antagonists that are now of promising therapeutic value in the treatment of inflammatory diseases [32–34]. Or, if the dye uptake event does represent a functionally relevant mechanism underlying $P2X_7R$ function, we have still to discover what this is. Speculative hypotheses have been discussed in this review and are depicted in Fig. 3. It is likely that some, or all, of these hypotheses will be revised, discarded, or perhaps even shown to be valid, but certainly, they provide exciting impetus for much further exploration of the $P2X_7R$–pannexin connection.

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