P2X7 receptors regulate multiple types of membrane trafficking responses and non-classical secretion pathways

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Abstract Activation of the P2X7 receptor (P2X7R) triggers a remarkably diverse array of membrane trafficking responses in leukocytes and epithelial cells. These responses result in altered profiles of cell surface lipid and protein composition that can modulate the direct interactions of P2X7R-expressing cells with other cell types in the circulation, in blood vessels, at epithelial barriers, or within sites of immune and inflammatory activation. Additionally, these responses can result in the release of bioactive proteins, lipids, and large membrane complexes into extracellular compartments for remote communication between P2X7R-expressing cells and other cells that amplify or modulate inflammation, immunity, and responses to tissue damages. This review will discuss P2X7R-mediated effects on membrane composition and trafficking in the plasma membrane (PM) and intracellular organelles, as well as actions of P2X7R in controlling various modes of non-classical secretion. It will review P2X7R regulation of: (1) phosphatidylserine distribution in the PM outer leaflet; (2) shedding of PM surface proteins; (3) release of PM-derived microvesicles or microparticles; (4) PM

blebbing; (5) cell–cell fusion resulting in formation of multinucleate cells; (6) phagosome maturation and fusion with lysosomes; (7) permeability of endosomes with internalized pathogen-associated molecular patterns; (8) permeability/integrity of mitochondria; (9) exocytosis of secretory lysosomes; and (10) release of exosomes from multivesicular bodies.

Keywords P2X7 receptor · Plasma membrane · Non-classical secretion · Membrane trafficking

Introduction

Although rapid changes in ionic fluxes and ion homeostasis are the best characterized cellular responses to activation of the P2X7 receptor (P2X7R), this adenosine triphosphate (ATP)-gated ion channel also triggers a remarkably diverse array of membrane trafficking responses in immune and inflammatory effector cells. These responses result in altered profiles of cell surface lipid and protein composition that can modulate the direct interactions of P2X7R-expressing leukocytes with other cell types in the circulation, the vasculature, or sites of immune and inflammatory activation. Additionally, these responses can result in the release of bioactive proteins, lipids, and large membrane complexes into extracellular compartments for longer range and remote communication between P2X7R-expressing leukocytes and other cells that amplify or modulate innate and acquired immunity. As an organizational framework, this review will first discuss P2X7R-mediated regulation of membrane composition and trafficking at the level of the plasma membrane, and will then survey known or likely actions of P2X7R in controlling the composition or trafficking of intracellular membranes.

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P2X7R-mediated regulation of plasma membrane composition and trafficking

P2X7R regulation of phosphatidylserine distribution in the plasma membrane

Phosphatidylserine (PS) transfer to the extracellular leaflet of the plasma membrane (PM) is a well-characterized signal that facilitates: (1) the clearance of apoptotic/senescent cells or apoptotic bodies by professional or non-professional phagocytes; and (2) the activation of platelet-mediated hemostatic responses [1–3]. The outer leaflet of eukaryotic plasma membrane bilayers predominantly contains sphingomyelin and phosphatidylcholine while aminophospholipids, such as PS and phosphatidylethanolamine, are enriched in the inner leaflet. Phospholipid distribution within these outer and inner leaflets of the PM is tightly regulated by specialized enzymatic mechanisms that include “flippases”, “floppases”, and “scramblases” [4, 5]. Loss of membrane phospholipid asymmetry, particularly PS flip from the inner to the outer leaflets, has been associated with many pathologic conditions. Although the mechanism underlying loss of PS asymmetry remains only partially understood, current models propose that two ATP-dependent transporters—“flippase” and “floppase”—actively maintain inward or outward aminophospholipid transport while a Ca^{2+} -dependent, but ATP-independent, scramblase mediates the bidirectional movement of phospholipids between the bilayer leaflets [6].

Activation of P2X7R, which induces a rapid and sustained Ca^{2+} influx, has been reported to stimulate PS translocation (as measured by fluorescein-conjugated annexin-V binding to the plasma membrane) in HEK293 cells expressing the P2X7R [7]. While prolonged activation of P2X7R results in irreversible PS flip and cell death within 6 h, brief pulses of P2X7R stimulation (≤ 10 min) trigger reversible PS flip that precedes microvesicle shedding and is independent of apoptotic cell death. Although—as noted above—PS translocation is considered a key indicator of apoptotic cell death, these observations regarding reversible PS flip during brief P2X7R activation suggest that cell surface PS redistribution can be part of normal physiological responses to developmental cues or cell stress. Indeed, this notion of physiological PS exposure is supported by an increasing number of examples. Transient expression of PS on the surface is essential for skeletal muscle cell differentiation and mediates myotube formation [8]. Likewise, exposure of sperm cells to bicarbonate results in profound architectural changes in the sperm cell plasma membrane including a PS flip that appears to be an important and early physiological event during sperm capacitation [9]. Neutrophils stimulated with chemotactic peptides, such as formylated Met-Leu-Phe

(fMLP), exhibit transient cell surface PS expression [10]. Macrophages per se undergo membrane PS flip during their recognition and phagocytosis of dead cells [11–14]. Similarly, rapid changes in cell surface PS triggered by the natively expressed P2X7R in various leukocyte types have been associated with physiological signal transduction. Elliott et al. have described a P2X7R-stimulated PS asymmetry in non-apoptotic murine T lymphocytes that is associated with altered expression of adhesion molecules, negative regulation of the CD45 transmembrane tyrosine phosphatase, and suppressed activity of P-glycoprotein [15]. Moreover, a high level of PS in the inner leaflet of the T-cell plasma membrane markedly inhibits channel activity of one naturally occurring allelic variant (Leu-451) of the murine P2X7R, but not the Pro-451 variant of this receptor. The mechanism(s) underlying P2X7R-induced changes in PS asymmetry remain incompletely defined but likely involve indirect effects of altered ionic flux on floppase/scramblase activation or, perhaps, direct protein–protein interaction between these enzymes and the P2X7R itself.

Another potential physiological role for the P2X7R-induced PS exposure observed in non-apoptotic T lymphocytes is modulation of T-cell migration, particularly extravasation and homing to inflammatory loci. This possibility is supported by several observations: (1) PS translocation has been correlated with increased cellular adhesion to endothelia [16]; and (2) activation of P2X7R triggers shedding of CD62L [15], a major homing receptor [17], whose shedding occurs immediately before extravasation at inflammatory loci [18]. Moreover, as with PS exposure, shedding of CD62L from T cells occurs within seconds of P2X7R activation. MDR1 inhibitors that block PS translocation also completely prevent P2X7R-induced CD62L shedding [15]. It is possible that the increased membrane fluidity resulting from P2X7R activation facilitates the marked changes in cell shape required for the diapedesis of leukocytes between the packed endothelial cells that comprise these barriers.

P2X7R regulation of plasma membrane protein shedding

As noted above, P2X7R activation in murine T cells triggers a rapid loss of cell surface CD62L (L-selectin), which is correlated with the increased levels of cell surface PS. This latter finding reiterates the initial observations by Wiley and colleagues who demonstrated an extracellular ATP-mediated loss of CD62L from human leukemic B cells [19, 20]. Subsequent studies of blood leukocytes from normal C57BL/6 mice versus P2X7R knockout mice verified that the ATP-induced loss of CD62L observed in murine T cells, B cells, and monocytes was absolutely dependent on P2X7R rather than other P2 receptor subtypes

[21]. Because CD62L is an intrinsic membrane protein with a single membrane-spanning domain, a reduction in cell surface CD62L can reflect: (1) protease-mediated shedding of the large CD62L ecto-domain; (2) internalization of CD62L via endocytosis; or (3) release of intact CD62L protein within membrane vesicles that evaginate and scission away from the plasma membrane (see below). The ability of matrix metalloprotease (MMP) inhibitors to markedly suppress P2X7R-dependent loss of surface CD62L from human B cells indicated that bona fide shedding of the ecto-domain is the predominant mechanism. P2X7R activation in human B cells and monocyte-derived dendritic cells has also been shown to elicit the shedding of CD23, an intrinsic plasma membrane protein that functions as a low-affinity receptor for IgE with links to chronic inflammatory diseases [22, 23]. The ATP-induced loss of surface anti-CD23 epitopes was correlated with the extracellular accumulation of soluble CD23 protein which was suppressed in the presence of MMP inhibitors. CD27, a 55-kDa member of the TNF receptor superfamily, is another cell surface protein that is rapidly shed from murine T lymphocytes by a MMP-mediated mechanism in response to P2X7R activation [24]. The ligand for CD27 is CD70, yet another intrinsic plasma membrane protein expressed on activated dendritic cells or B cells. CD27–CD70 interactions occur in the context of antigen presentation to effector or memory T cells.

Thus, each of the three membrane proteins known to be shed during P2X7R stimulation is involved in a temporally or spatially distinct phase of the immune response: homing of leukocytes to immune/inflammatory loci (CD62L); direct interactions between antigen-presenting and antigen-responsive subsets of leukocytes (CD27); and activation of leukocytes by soluble immunoregulatory molecules that accumulate during particular immunological states (CD23). This suggests that local P2X7R signaling can fine-tune the duration or intensity of these diverse phases of innate or acquired immunity. It is likely that other leukocyte surface proteins can be shed via MMP-mediated pathways that are entrained by transient P2X7R stimulation. Major unresolved issues include the mechanism(s) by P2X7R activation is coupled to MMP activation and identification of the particular MMP subtypes that are targeted by this ATP-gated ion channel.

P2X7R regulation of plasma membrane microvesicle release

The ability of P2X7R activation to rapidly modulate the composition of the cell surface proteome extends beyond the MMP-mediated shedding of specific membrane proteins described above. Stimulus-induced shedding of plasma membrane-derived microvesicles—which bear distinctive

complements of membrane markers—has been described in both hematopoietic cell types (platelets, DCs, and neutrophils) and non-hematopoietic cell types (epithelial cells, cancer cells, and neurons) [25–29]. The size of these released vesicles ranges between 100 nm and 1 μ m in diameter which distinguishes them from the 1–4- μ m apoptotic bodies containing fragmented DNA derived from damaged cells, and from the 30–80-nm exosomes (discussed below) derived from the intraluminal vesicles of endosomal multivesicular bodies (MVB). Depending on their cellular origin and the stimulus which triggers their release, microvesicles can differ with regard to intravesicular contents, membrane lipid and protein compositions, biological functions, and nomenclature. Small membrane vesicles released into the circulation from activated platelets are described in the literature as microparticles (MP); these have major roles in blood coagulation by providing a large surface area for the assembly of clotting factors that bind to the exposed phosphatidylserine of the released MPs [30, 31]. Activated human neutrophils release “ectosomes” that exhibit anti-inflammatory activity and mediate inhibition of DC maturation from cultured blood monocytes [26, 27, 32]. Particles secreted from the basolateral membranes of various epithelial cells during tissue development are called “argosomes” due to their involvement in the transfer of developmental morphogens between cells and the maintenance of morphogen gradients [33].

Of particular relevance to this review, stimulation of P2X7R in human DC [34], murine microglial lines [35], and THP-1 human monocytes [36] triggers rapid release of plasma membrane-derived microvesicles (MV) which have been associated with the non-classical secretion of IL-1 β and IL-18 in response to extracellular ATP. This ATP-induced microvesicle shedding is correlated with decreased plasma membrane capacitance [36] and is markedly inhibited by the removal of extracellular Ca²⁺ [28] or the pretreatment of cells with P2X7R antagonists such as oxidized ATP or KN-62 [34]. The shed microvesicles can contain: (1) soluble proteins including unprocessed proIL-1 β , caspase-1 processed mature IL-1 β , and the caspase-1 itself; (2) intrinsic membrane proteins including MHC-II, P2X7R itself, P2Y2 receptors, CD63, CD39, and LAMP-1 [35, 37–40]; and (3) an increased level of surface active phospholipids such as PS [36]. Therefore, released MV have been proposed, or might be considered, as: (1) effective mechanisms for extracellular delivery of leaderless proteins such as bioactive cytokines (IL-1 β and IL-18), the so-called danger molecules such as the high mobility group box 1 protein [41, 42], and normal intracellular thiol-reducing agents [41, 42]; or (2) a novel route for the long range intercellular transfer of membrane proteins and lipids (MHC-II, P2X7R, P2Y2, CD39, PS).

The molecular basis for P2X7R-dependent microvesicle shedding remains poorly characterized. This shedding may reflect direct effects of altered ion homeostasis, particularly increased cytosolic Ca^{2+} on both proteases that regulate association of the plasma membrane with the submembrane cytoskeleton and on phospholipases that induce localized changes in lipid composition and fluidity with consequent perturbation of bilayer lipid packing and geometry. It is also possible that danger signals other than extracellular ATP can act synergistically with gated P2X7R to modulate the rate, extent, and composition of microvesicle release. For example, lipopolysaccharide (LPS) per se has been shown to induce a slow microvesicle release from human monocytes [43]. However, the rapid ATP-induced release of P2X7R-positive microvesicle membranes from murine macrophages can occur independently of LPS priming and before IL-1 β maturation and secretion [40].

P2X7R regulation of plasma membrane blebbing

Membrane blebbing, which can be related to, but also distinguished from, microvesicle shedding, represents a phenomenon wherein sections of plasma membrane reversibly protrude and retract at the cell surface. A variety of stimuli, including the activation of P2X7R, have been shown to trigger membrane blebbing. However, neither the intracellular mechanisms that regulate bleb formation nor the physiological or pathological roles of these blebs are entirely understood. This dynamic and reversible blebbing triggered by P2X7R may be involved in directed migration of leukocytes through extracellular matrices to sites of infection or tissue damage, and/or the membrane trafficking responses that culminate in scission of the extended blebs away from the plasma membrane surface to produce the released microvesicles/microparticles discussed in the previous section. Studies with different cell types have indicated that P2X7R-stimulated membrane blebbing appears to involve several common signaling pathways [44, 45]. ATP-induced membrane blebbing was found to be concurrent with actin reorganization in murine macrophages [46] and could be dissociated from the non-classical release of mature IL-1 β that is also stimulated in parallel by P2X7R in such macrophages [47]. Disruption of actin filaments by cytochalasin D, which inhibits actin polymerization and depolymerization, diminished the P2X7R-induced blebbing without affecting the receptor's ionotropic function. Activation of Rho and p38 MAPKs occurred concurrently with P2X7R-induced membrane blebbing [46]. Likewise, pharmacological inhibitors of p38, Rho, and Rho kinases all reduced P2X7R-stimulated actin reorganization as well as the blebbing [44, 46, 47]. Deletion of the C terminus of P2X7R completely abrogated its interaction with epithelia membrane proteins (EMPs)/

tetraspanins in engineered HEK293 cells. This was correlated with a marked attenuation of blebbing suggesting that P2X7R–EMP interaction is crucial for induction of rapid blebbing [45]. A recent analysis by Dixon and colleagues of P2X7R-dependent membrane blebbing in osteoblasts found that extracellular ATP activated phospholipases D (PLD) and A_2 leading to production and release of lysophosphatidic acid (LPA) [48]. Antagonism or desensitization of G-protein-coupled LPA receptors in these osteoblasts suppressed blebbing in response to either LPA or P2X7R agonists but did not affect the ionotropic actions of P2X7R. LPA triggered a rapid Rho kinase-dependent membrane blebbing in osteoblasts from wild-type or P2X7R knockout mice while ATP induced blebbing only in the wild-type osteoblasts. Thus, LPA functions as an autocrine mediator downstream of P2X7R to induce signaling cascades that control osteoblast membrane blebbing in response to ATP. These authors speculate that this autocrine loop may be involved in P2X7R-stimulated osteogenesis during skeletal development and mechanotransduction. Although previous studies have verified the ability of P2X7R to stimulate PLD and PLA_2 activity via both Ca^{2+} -dependent and Ca^{2+} -independent signaling cascades in several cell types, the study by the Dixon group provides a novel link of these latter P2X7R-initiated responses to the dynamic regulation of the actin cytoskeleton which is critical for many integrative leukocyte functions, such as transient cell–cell contact during homing or antigen presentation, direct cell–cell fusion during granuloma formation, and phagocytosis of pathogens or host debris during infection or inflammation. Notably, activation of P2X7R has also been linked to several of these responses including membrane fusion events such as formation of multinucleated giant cells and the killing of intracellular mycobacteria [49, 50].

P2X7R regulation of cell–cell fusion and formation of multinucleate giant cells

Formation of multinucleated giant cells (MGC) has been used as a hallmark of chronic inflammatory reactions found in foreign body reactions, sterile inflammation, and infective granulomas [51]. Additionally, the fusion of monocyte-related progenitors into the multinucleate osteoclasts is a significant example of physiological cell–cell fusion that is critical for normal bone homeostasis. Several lines of evidence implicate a role for P2X7R in MGC formation in cells of hematopoietic origin including macrophages, monocytes, and osteoclasts, wherein P2X7R is highly expressed [49, 52, 53]. MGCs within granuloma are short-lived cells which begin to die several hours after fusion, and this apoptotic death is followed by cytoplasmic fragmentation. The ability of cells grown to confluence in

culture to spontaneously form MGCs has been correlated with the expression level of P2X7R [49]. This is consistent with observations in human macrophages wherein the fusion triggered by Con A and interferon- γ can be inhibited by the P2X7R antagonist, oxidized ATP. However, expression of P2X7R per se is not obligatory for this process because mock-transfected HEK293 cells and osteoclasts derived from P2X7R-deficient mice still retain the capacity to form MGC, albeit to a lesser degree [53–56]. Notably, the C-terminal domain of P2X7R appears to be required for MGC formation based on the fusion indices of HEK293 cells engineered to express full-length versus C-terminally truncated P2X7R. This raises the issue as to which candidate proteins might interact with P2X7R to regulate this dramatic change of membrane fluidity and cytoskeletal rearrangements. It is unclear whether P2X7R-mediated cell–cell fusion is an outcome or a cause of pathologic cell–cell interactions in disease states. Likewise, more studies are needed to assess whether cell–cell fusion shares common or parallel signaling pathways observed for other P2X7R-dependent membrane trafficking responses such as PS flip, microvesicle shedding, and membrane blebbing.

P2X7R-mediated regulation of intracellular membrane traffic and organelle function

P2X7R regulation of phagosome maturation and microbial killing

Several pathogenic microbes, including mycobacteria and *Chlamydia*, are able to survive in host macrophages by subverting the normal bacteriocidal processes that sequentially involve microbe internalization within phagosomes, maturation of these phagosomes, and efficient fusion of the mature phagosomes with lysosomes to result in bacterial killing and clearance. P2X7R activation has been shown to promote the killing of both mycobacteria [50, 57, 58] and *Chlamydia* [59, 60] in two ways: enhanced maturation of the microbe-containing phagosomes and enhanced killing of the microbe-infected host cells. The first mechanism involves a P2X7R-mediated activation of phospholipase D that facilitates maturation and fusion of the mycobacteria/chlamydia-containing phagosomes with lysosomes [50]. As noted previously, P2X7R-dependent activation of phospholipase D and A₂ has been described in several cell types including macrophages [48, 58, 61–63]. Although this P2X7R→PLD pathway clearly contributes to the phagosomal maturation and microbiocidal effects stimulated by extracellular ATP, additional purinergic receptors are involved given the differential microbiocidal actions of ATP versus BzATP [58]. Moreover, a P2X7R-independent but ATP-dependent killing of intracellular *Mycobacterium*

tuberculosis is supported by studies of mycobacterial killing in macrophages from P2X7R knockout mice [64]. The particular contributions of, and possible crosstalk between, the P2X7R-dependent and P2X7R-independent components of ATP-dependent microbial killing are important areas for additional investigation. Recent studies by Kusner and colleagues [65–67] have demonstrated that—in addition to PLD—there are critical signaling roles for increased Ca²⁺, calmodulin-dependent protein kinases, and sphingosine kinase, in the maturation of microbe-loaded phagosomes. Given the ability of various P2Y family receptors to regulate Ca²⁺ and lipid signaling, it seems likely that P2X7 and various P2Y receptors act cooperatively to mediate the actions of extracellular ATP on killing of internalized mycobacteria or chlamydia. Such an interaction between P2X7R and another P2 receptor subtype(s) could explain the disparate associations of particular P2X7R polymorphisms with altered progression of tubercular phenotypes within different subgroups of human populations or mouse models [64, 68–75].

The second pathway by which P2X7R activation can attenuate mycobacterial survival is through direct killing of the infected macrophages [57, 76]. This acts to reduce the intracellular niches wherein these microbial pathogens survive and eventually proliferate. Previous studies have demonstrated that *M. tuberculosis* can inhibit apoptosis of infected macrophages by preventing TNF- α -mediated autocrine signaling pathways [77]. However, treatment of such infected cells with ATP or other P2X7R agonists can overcome this blockade in cell death signaling. It remains to be established whether this P2X7R-induced killing of mycobacteria-infected macrophages reflects apoptosis, necrosis, pyroptosis, or a combination of these mechanistically distinct cell death pathways.

P2X7R-induced transfer of endosomal contents to the cytosolic compartment

One of the best characterized physiological responses to P2X7R stimulation is the maturation and secretion of the proinflammatory cytokine IL-1 β [78]. This requires the activation of the cytokine processing enzyme, caspase-1. ATP and other host-derived small molecules, such as uric acid crystals [79], have been identified as “danger signals” [80] that act to amplify certain phases of the innate immune or inflammatory responses entrained by pathogen-derived molecular patterns (PAMPs) such as flagellin, bacterial and viral RNA, CpG-DNA, and muramyl dipeptide [81–84]. These are best characterized as agonistic ligands for the various Toll-like receptors (TLR) that are intrinsic membrane proteins localized to the plasma membrane or endosomal membranes. PAMP binding to TLRs triggers NF κ B- and MAPK-based signaling cascades that culminate

in transcriptional activation of many proinflammatory genes, including the precursor form of IL-1 β . However, certain PAMPs also act as direct ligands or indirect regulators for the soluble, cytosolic nucleotide oligomerization domain (NOD) family receptors. Some NOD proteins, including NALP1, NALP3/cyropyrin, and IPAF, function as central adapter molecules for the assembly of the cytosolic “inflammasome” protein complexes that facilitate the rapid proteolytic activation of caspase-1 required for maturation of IL-1 β . The efficient assembly of certain caspase-1 inflammasomes, particularly those involving NALP1 and NALP3, requires both coordinated delivery of the cognate PAMPs or PAMP metabolites into the cytosol and presence of additional endogenous signals generated in response to the host danger molecules, such as extracellular ATP or uric acid. A rapid decrease in cytosolic K⁺ concentration appears to be one such endogenous signal [85–87].

Recent reports from several labs suggest that P2X7R may be involved in both the delivery of PAMPs to the cytosol and the generation of the endogenous signal(s) required for rapid inflammasome assembly. Pelegrin and Surprenant [88, 89] first demonstrated that pannexin-1, a gap junction-like protein, can functionally interact with the P2X7R to elicit the secondary changes in plasma membrane permeabilization (as measured by organic dye influx) that typify P2X7R activation. Significantly, treatment of macrophages with pannexin-1 inhibitors also strongly attenuated the P2X7R-induced activation of caspase-1 and IL-1 β maturation/secretion. Subsequent studies by Nunez and colleagues [90, 91] indicated that this role for pannexin-1 involves a delivery of PAMPs (LPS or muramyl dipeptide) from endosomal compartments into the cytosol to initiate assembly of Nalp3/cyropyrin inflammasome. These authors propose that extracellular PAMPs are first internalized within recycling endosomes but—in the absence of P2X7R activation—are only slowly released from this compartment into the cytosol. ATP stimulation of P2X7R triggers conformational changes in the pannexin-1 pools of both the plasma membrane and endosomal membranes with consequent efflux of PAMPs from the endosomal lumen into the cytosol. Consistent with this scenario, inhibition of endosomal maturation by chloroquine completely abrogated ATP-induced caspase-1 activation in macrophages.

P2X7R-induced changes in mitochondrial permeability and function

A growing body of data indicates that P2X7R activation not only regulates permeability of the plasma membrane but also the permeability/integrity of intracellular membrane compartments. In addition to endosomes, Adinolfi et al. have shown that overexpression of P2X7R—even in the

absence of maximal activation by exogenous ATP—in HEK293 cells hyperpolarizes mitochondrial membrane potential, increases basal intramitochondrial Ca²⁺, increases total cellular ATP content, and confers an ability to grow in the absence of serum [92]. In contrast, maximal activation of these overexpressed P2X7R by millimolar extracellular ATP triggered a rapid depolarization of the mitochondria which was associated with a very large increase in mitochondrial Ca²⁺ accumulation, mitochondrial fragmentation, and cell death. These studies indicated that low-level autocrine activation of the overexpressed P2X7R by released endogenous ATP was responsible for the increased mitochondrial potential and growth advantage, while the massive influx of extracellular Ca²⁺ induced by maximally activated receptors triggered the collapse of mitochondrial integrity and reduced viability. Similar studies by Mackenzie et al. [7] noted that the massive collapse of mitochondrial ion homeostasis (leading to cell death) induced by sustained P2X7R activation was dependent on increased Rho-dependent kinase activity but was independent of Ca²⁺. Analysis of natively expressed P2X7R in rat submandibular gland cells revealed that ATP-induced mitochondrial depolarization was additionally dependent on the influx of extracellular Na⁺ that accompanies P2X7R channel gating [93]. Although P2X7R has traditionally been associated with the early phases of inflammation, cytotoxicity, and proapoptotic induction, the additional ability of low-level P2X7R activation to increase mitochondrial energy metabolism and actually confer growth advantage may be germane to recently identified roles for altered P2X7 expression/function in several cancers [94–97]. The apparent “double-edged sword” role of P2X7R in the regulation of mitochondrial integrity and function may underlie its seemingly paradoxical actions as a trigger of cell death in some contexts but an inducer of cell survival and growth in other settings.

P2X7R-induced exocytosis of secretory lysosomes

As noted in the previous section, activation of P2X7R can trigger the rapid release or secretion of several proinflammatory cytokines compartmentalized within microvesicles that originate from evaginated blebs of plasma membrane which scission away from the cell body. However, multiple lines of data indicate that P2X7R can also stimulate the release of various intracellular macromolecules via the more classical exocytotic fusion of intracellular storage granules or organelles with the plasma membrane. Indeed, the studies of Gomperts and colleagues [98–101] on the ability of extracellular ATP to trigger degranulation of mast cells were among the earliest examples of “P2z” (now P2X7) receptor action on secretory cell types. Similarly, activation of the P2X7R in rat submandibular gland cells

elicits a rapid release of granules containing proteases such as kallikrein [102]. Another example of P2X7R-stimulated exocytosis is the fusion of the so-called secretory lysosomes with the plasma membrane triggered by extracellular ATP which occurs concurrently with the release of cytokine IL-1 β and its converting enzyme caspase-1 in murine macrophages [40] and human monocytes [39]. Secretory lysosomes represent a specialized subtype of lysosomes that predominantly are expressed in hematopoietic cells [103]. These include the histamine- and serotonin-containing dense granules of mast cells, the perforin- and granzyme-containing granules of cytolytic T lymphocytes, and the major basic protein granules of eosinophils. Additionally, macrophages and dendritic cells can release lysosomes that contain many of the proteases (e.g., cathepsins) and other degradative enzymes that are classically considered as lysosomal marker proteins. Thus, the exocytotic mobilization of these various secretory lysosomes/granules results in the export of critical mediators of diverse immune and inflammatory responses. As for other secretory granules that are released via regulated exocytosis, the fusion of the various secretory lysosomes with the plasma membrane is regulated by characteristic t- and v-SNARE proteins and Ca²⁺-dependent synaptotagmins [104–106].

Overlap between the P2X7R-triggered signaling pathways that regulate lysosome exocytosis and those required for IL-1 β secretion has suggested that these lysosomes may comprise a mechanism for non-classical IL-1 β secretion [107]. In human monocytes, both events can be blocked by inhibitors that target Ca²⁺-dependent and Ca²⁺-independent phospholipase A₂ and phosphatidylcholine-specific phospholipase C [37]. Perturbation of microtubule-directed movements of secretory lysosomes also attenuates the ATP-triggered export of IL-1 β [39, 107]. Moreover, studies performed in P2X7R knockout murine macrophages suggest that IP₃-dependent Ca²⁺ mobilization and protein kinase C activation mediated by G-protein-coupled P2Ys are not sufficient for either secretory lysosome exocytosis or IL-1 β release [40]. This latter report also indicated that both the P2X7R-dependent mobilization of secretory lysosomes and the secretion of IL-1 β are strongly potentiated by the LPS–NF κ B signaling pathway and requires de novo protein synthesis. Despite these multiple correlations between IL-1 β release and exocytosis of secretory lysosomes, removal of extracellular Ca²⁺ completely abrogates ATP-induced lysosome exocytosis from murine macrophages without affecting IL-1 β secretion, which unequivocally dissociates lysosome exocytosis from the non-classical IL-1 β secretion machinery regulated by P2X7R [40]. Additionally, immunocytochemical analyses of intact murine macrophages indicate no significant redistribution of cytosolic IL-1 β into secretory lysosomes during P2X7R activation [108]. The wide range of

proteases, phospholipases, and heat shock proteins contained within the secretory lysosomes [109, 110] of macrophages and other immune effector cells may be critical for additional regulatory roles of P2X7R in extracellular matrix remodeling and release of other bioactive mediators.

P2X7R-induced release of multivesicular body (MVB)-derived exosomes

In addition to plasma membrane-derived microvesicles, immune and inflammatory cells can release another pool of membrane-delimited vesicles termed exosomes [111, 112]. These are derived from the intraluminal vesicles (ILV) contained within the MVB generated by invagination of the limit membranes of recycling endosomes. These ILVs are often enriched in plasma membrane proteins, such as ligand-occupied receptors, which are internalized on the endosomes. While many MVBs fuse with lysosomes to deliver the ILVs and their component proteins for degradation/down-regulation, some MVBs can be redirected for fusion with the plasma membranes to release the ILVs into extracellular compartment as exosomes. Significantly, exosomes released from macrophages, dendritic cells, or B lymphocytes contain intrinsic membrane proteins, such as the type II major histocompatibility complex (MHCII) that play critical roles in immune recognition and antigen presentation [113, 114]. When released from leukocytes that have internalized and processed microbial or other foreign proteins, exosomes will contain MHCII loaded with antigenic peptide (pMHCII exosomes) [115]. A growing literature indicates that these released pMHCII exosomes can activate remote T lymphocytes either by direct antigen presentation from the pMHCII displayed on the exosome surface, or an indirect pathway wherein the pMHCII exosomes are taken up by remote naive dendritic cells for DC-mediated antigen presentation [116–118]. In addition to this model of “remote” antigen presentation, released exosomes may be used for other novel types of intercellular communication including the cell-to-cell transfer of: (1) intact mRNAs and microRNAs [119]; and (2) the direct transfer of oncogenic growth factor receptors from tumor cells to remote non-transformed cells [120].

We have recently reported observations that support an involvement of exosomes as yet another non-classical pathway for the P2X7R-stimulated secretion of IL-1 β [40]. These data suggest that both plasma membrane-derived microvesicles and MVB-derived exosomes can be used to “encapsulate” small volumes of cytosol containing caspase-1 inflammasome complexes and their procytokine substrates. In other studies (Qu et al., unpublished data), we have found that P2X7R activation in murine macrophages and dendritic cells triggers the rapid extracellular release of

two pools of membranes containing MHCII. In macrophages primed with both interferon- γ and LPS or DC primed with LPS, extracellular ATP stimulated the export of ~15% of the total cellular MHCII pool within 90 min. The released MHCII was present within two morphologically and biochemically distinct populations of membrane vesicles: (1) plasma membrane-derived microvesicles (100–500 nm diameter) that sediment at 10,000 $\times$ g and also contain P2X7R protein, actin, and the LAMP1 lysosomal membrane protein; and (2) multivesicular body-derived exosomes (30–80 nm diameter) that sediment at 100,000 $\times$ g and lack the P2X7R, actin, and LAMP1 markers. Significantly, both pools of released, purified MHCII membranes were capable of binding antigenic peptide and activating T-cell receptor-dependent IL-2 production in antigen-specific T-cell hybridoma cells. These observations link the well-characterized actions of the P2X7 receptor on innate immune responses within highly localized regions of microbial infection to a possible non-juxtacrine intercellular communication pathway for delivery of microbial antigen to T cells, with consequent long-range engagement of the adaptive immune response.

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