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Roles of extracellular nucleotides and P2 receptors in ectodomain shedding

Aleta Pupovac
University of Wollongong, Monash University, ap251@uowmail.edu.au

Ronald Sluyter
University of Wollongong, rsluyter@uow.edu.au

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Ectodomain shedding of integral membrane receptors results in the release of soluble molecules and modification of the transmembrane portions to mediate or modulate extracellular and intracellular signalling. Ectodomain shedding is stimulated by a variety of mechanisms, including the activation of P2 receptors by extracellular nucleotides. This review describes in detail the roles of extracellular nucleotides and P2 receptors in the shedding of various cell surface molecules, including amyloid precursor protein, CD23, CD62L, and members of the epidermal growth factor, immunoglobulin and tumour necrosis factor families. This review discusses the mechanisms involved in P2 receptor-mediated shedding, demonstrating central roles for the P2 receptors, P2X7 and P2Y2, and the sheddases, ADAM10 and ADAM17, in this process in a number of cell types.

Keywords

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Roles of extracellular nucleotides and P2 receptors in ectodomain shedding

Aleta Pupovac[1,2,3,4], Ronald Sluyter[1,2,3]

[1]School of Biological Sciences, University of Wollongong, Wollongong, NSW 2522, Australia, [2]Centre for Medical and Molecular Bioscience, University of Wollongong, Wollongong, NSW 2522, Australia, [3]Illawarra Health and Medical Research Institute, Wollongong, NSW 2522, Australia, [4]Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, Clayton, Vic 3800, Australia,

Corresponding author:

Ronald Sluyter, School of Biological Sciences, University of Wollongong, Wollongong, NSW 2522, Australia. Email address: rsluyter@uow.edu.au

Running title:

P2 receptor-induced ectodomain shedding

Abstract

Ectodomain shedding of integral membrane receptors results in the release of soluble molecules and modification of the transmembrane portions to mediate or modulate extracellular and intracellular signalling. Ectodomain shedding is stimulated by a variety of mechanisms including activation of P2 receptors by extracellular nucleotides. This review describes in detail the roles of extracellular nucleotides and P2 receptors in the shedding of various cell surface molecules, including amyloid precursor protein, CD23, CD62L, and members of the epidermal growth factor, immunoglobulin and tumour necrosis factor families. This review discusses the mechanisms involved in P2 receptor-mediated shedding, demonstrating central roles for the P2 receptors, P2X7 and P2Y2, and the sheddases, ADAM10 and ADAM17, in this process in a number of cell types.

Keywords: purinergic signalling, P2X receptors, P2Y receptors, extracellular ATP, extracellular UTP, metalloprotease.

Introduction

Ectodomain shedding is a post-translational modification of many cell surface molecules that results in their release as soluble molecules, which can subsequently induce cellular responses in an autocrine or paracrine manner, and in the modulation of the intracellular signalling properties of the remaining transmembrane portions [1]. Ectodomain shedding is exhibited by a diverse range of molecules including cell adhesion molecules, and cytokine and epidermal growth factors, and can be stimulated by a variety of mechanisms [1] including the activation of P2 receptors by extracellular nucleotides (Figure 1).

P2 receptors are classified as either P2X or P2Y receptors [2]. P2X receptors are trimeric ligand-gated cation channels that are activated by adenosine 5'-triphosphate (ATP) to mediate the rapid flux of Na^+, K^+ and Ca^{2+}, and in some instances organic ions [3]. In humans and rodents, seven P2X receptor subunits exist (P2X1-P2X7), which combine to form homomeric or heteromeric receptors [4]. P2Y receptors are G protein-coupled receptors and modulate various signalling events including adenylyl cyclase, phospholipase C and ion channel activation [5]. Eight P2Y receptors have been identified in humans and rodents (P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11-P2Y14) [5]. P2Y receptors are predominately activated by ATP or adenosine 5'-diphosphate (ADP), but some are preferentially activated by other nucleotides, such as P2Y2 by uridine 5'-triphosphate (UTP) [5]. In addition, synthetic analogues of ATP and other nucleotides, including 2',3'-O-(4-benzoylbenzoyl) ATP (BzATP) and adenosine 5'-(γ-thio)triphosphate (ATPγS), can activate a number of P2X and P2Y receptors [6]. P2X and P2Y activation stimulates various downstream signalling pathways that affect many cellular processes. Although these pathways remain to be fully elucidated, it is widely accepted that purinergic signalling is involved in physiological and pathophysiological responses including neurotransmission, coagulation, inflammation, tissue regeneration, and cell proliferation, differentiation and death [7].

As discussed below, P2X7 and P2Y2 are the main P2 receptors implicated in nucleotide-induced ectodomain shedding. P2X7 is present on immune, bone, neural, epithelial and other cell types [8]. P2X7 is activated by BzATP and ATP, and to a lesser extent ATPγS [9]. Nicotinamide adenine dinucleotide (NAD) can also activate murine P2X7 following ADP-ribosylation by the ADP-ribosyltransferase ARTC2.2 [10]. P2X7 can be inhibited by broad-spectrum antagonists, including pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), Reactive Blue 2 and suramin, and by more specific antagonists, including Brilliant Blue G (BBG), KN-62, oxidised ATP and A 438079 [9]. P2X7 activity varies between human individuals and mouse strains due to single nucleotide polymorphisms, resulting in loss or gain of function variants, which can be used to establish the role of this receptor in nucleotide-induced responses [11]. P2Y2 is present on neural, bone, epithelial, endothelial, immune and renal cell types [12]. P2Y2 is activated by UTP and ATP, and to a lesser extent ATPγS [13]. Furthermore, there are synthetic P2Y2 agonists including PSB1114, MRS2698 and INS37217 [13], and synthetic P2Y2/P2Y4 agonists such as Diquafosol (INS365), which is used clinically to treat dry eye [6]. P2Y2 can be inhibited by broad-spectrum antagonists including Reactive Blue 2 and suramin [13], and by specific antagonists, such as AR-C 118925XX [6]. This review aims to describe in detail the roles of extracellular nucleotides and P2 receptors in the shedding of various cell surface molecules and the mechanisms involved in this process.

Amyloid precursor protein

The amyloid precursor protein is a single transmembrane receptor with an extracellular N-terminus and intracellular C-terminus (type I integral membrane protein), which can be processed to amyloid-β peptide (Aβ) by either the amyloidogenic or anti-amyloidogenic pathways [14] (Figure 2). Amyloid-β peptide (Aβ) accumulation is a major pathological hallmark of Alzheimer's disease

[14]. In the amyloidogenic pathway, the ectodomain of amyloid precursor protein is cleaved by β-secretase to yield soluble amyloid precursor protein β (sAPPβ) and the remaining transmembrane protein, β-carboxyl-terminal fragment (β-CTF), which is subsequently cleaved by γ-secretase to release Aβ [14]. In the anti-amyloidogenic pathway, the ectodomain of β-amyloid precursor protein is cleaved by the α-secretase activity to yield soluble amyloid precursor protein α (sAPPα) and the remaining transmembrane protein, α-carboxyl-terminal fragment (α-CTF), which is subsequently cleaved by γ-secretase to generate a truncated, non-amyloidogenic Aβ fragment, p3 [14]. Several metalloproteases including ADAM9, ADAM10, ADAM17 and ADAM19 can function as α-secretases [14], while both P2Y2 and P2X7 activation are involved in the release of sAPPα from cells [15, 16].

P2Y2 was first implicated in the shedding of sAPPα using P2Y2-transfected human 1321N1 astrocytoma cells. UTP-induced rapid shedding of sAPPα from 1321N1 cells in a concentration-dependent manner [17]. This process was dependent on extracellular Ca^{2+} and partly on extracellular signal-regulated protein kinase (ERK) phosphorylation, but independent of protein kinase C (PKC), Src and epidermal growth factor receptor (EGFR) activation [17]. Moreover, the metalloprotease inhibitors, phenanthroline and TAPI-2, and the proprotein convertase inhibitor, decanoyl-RVKR-CMK ketone (see Table 1 for sheddase inhibitors used in purinergic signalling studies), impaired UTP-induced sAPPα shedding [17]. Furthermore, short interfering RNA (siRNA) silencing of ADAM10 and ADAM17 reduced UTP-induced sAPPα shedding, and simultaneous silencing of both ADAMs near-completely suppressed this shedding [17]. This indicated that both ADAM10 and ADAM17 are involved in P2Y2-induced sAPPα shedding. Similarly, UTP induced sAPPα shedding via ADAM10 and ADAM17 in rat primary cortical neurons treated with interleukin (IL)-1β (to up-regulate P2Y2) or following transfection with P2Y2 [18]. This study also demonstrated a role for phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)

in this pathway. UTP, and to a lesser extent ATP and BzATP, can also induce slow (48 hours) sAPPα shedding from primary rat cortical astrocytes [19]. Use of P2 antagonists supported a role for both P2Y2 and P2Y4, but not P2Y6, in this process [19]. Both ATP- and UTP-induced sAPPα shedding from primary rat cortical astrocytes was dependent on ERK and p38 mitogen-activated protein kinase (MAPK) activity [19].

P2X7 activation can also induce the rapid shedding of sAPPα from neural cell types. Both ATP and BzATP induced sAPPα shedding from human APP-transfected murine Neuro2a neuroblastoma cells, processes impaired by the P2X7 antagonists, BBG and A 438079, as well as by siRNA silencing of P2X7 [20]. Similarly, BzATP also induced sAPPα shedding from human SK-N-BE neuroblastoma cells, a process impaired by the P2X7 antagonists, oxidised ATP and A 438079 [20]. Furthermore, BzATP induced sAPPα shedding from primary astrocytes and neural progenitor cells from wild-type, but not Pfizer P2X7 knockout, mice [20]. The broad-spectrum metalloprotease inhibitors, TAPI-2 and GM 6001, inhibited BzATP-induced sAPPα shedding from Neuro2a cells, but siRNA silencing of ADAM 9, ADAM10 and ADAM17 alone or in combination did not inhibit P2X7-induced shedding of sAPPα from these cells, suggesting the involvement of an alternate α-secretase in P2X7-induced shedding [20]. Additional pharmacological and siRNA evidence from this study [20] and a subsequent study [21] indicated that P2X7-induced shedding of sAPPα from neural cells depends on Rho and MAPK kinase modulation of ERK1/2 and c-Jun N-terminal kinase (JNK) upstream of the intracellular signalling complex, ezrin radixin and moesin, and downstream PI3K.

In contrast to the above studies, BzATP decreased α-secretase activity (as assessed by expression of α-CTF) in murine Neuro2a neuroblastoma cells, while BBG or A 438079 increased basal α-secretase activity in these cells [22, 23], as well as in P2X7-transfected human embryonic kidney (HEK) 293 cells [22]. This increased activity in the presence of P2X7 inhibition was mediated through inhibition of glycogen synthase kinase-3 (GSK-3) [22]. *In vivo* blockade of P2X7

by BBG in a murine model of early onset familial Alzheimer's disease decreased the number of hippocampal plaques, which correlated with decreased GSK-3 activity but increased α-secretase activity [22]. Differences between these and the above studies on P2X7 and amyloid precursor protein processing may possibly be explained by use of different BzATP concentrations, with lower concentrations (<100 μM) decreasing α-secretase activity through P2X7 activation and higher concentrations (>100 μM) increasing α-secretase activity through P2Y2 activation [22, 23].

ARTC2.2

ARTC2.2 is a glycosylphosphatidylinositol-anchored enzyme expressed on the cell surface of murine T cells, which can catalyse the ADP-ribosylation of P2X7 resulting in its activation [10]. NAD induced the rapid shedding of ARTC2.2 from murine YAC-1 lymphoma cells, a process abrogated by either a blocking antibody or blocking nanobody [24]. Moreover, NAD induced ARTC2.2 shedding from splenic $CD4^+$ and $CD8^+$ T cells of wild-type, but not Pfizer P2X7 knockout, mice [24]. The metalloprotease inhibitor, BB-2516 (marimastat), and the ADAM10/ADAM17 inhibitor, GW 280264X, but not the ADAM10 inhibitor, GI 254023X, blocked this process in YAC-1 cells and T cells [24], indicating ADAM17 is the major sheddase involved in P2X7-induced ARTC2.2 shedding. Notably, shed ARTC2.2 retains enzymatic activity, but with a substrate specificity favouring secreted proteins over membrane proteins [24]. To the best of our knowledge this remains the only example of nucleotide-induced shedding of an ectodomain from a cell surface enzyme.

CD14 (lipopolysaccharide receptor)

CD14 is a glycosylphosphatidylinositol-anchored receptor (type V integral membrane protein) expressed on the surface of monocytes and macrophages, and to a lesser extent endothelial and epithelial cells [25]. CD14 functions as a receptor for the lipopolysaccharide (LPS)/LPS binding complex [25]. ATP induced the rapid shedding of CD14 from resting, M1 and M2 murine macrophages, a process impaired by A 438079, or in macrophages from Pfizer P2X7 knockout mice [26]. GM 6001 also impaired ATP-induced CD14 shedding from murine macrophages [26], but the specific metalloprotease mediating this effect is yet to be identified. P2X7-induced CD14 shedding may also occur in humans, as ATP incubation caused a loss in $CD14^{+}$, but not $CD33^{+}$, monocytes, in whole blood [27], however evidence for P2X7 in ATP-induced CD14 shedding in this setting remains to be determined.

CD21 (C3d receptor)

CD21 (C3d receptor) and CD35 (C3d/C4b receptor) comprise the two main complement receptors [28]. In humans different genes code these receptors, whilst in mice they are alternative splice products of the same gene [28]. CD21 is a type I integral membrane glycoprotein predominantly expressed on follicular dendritic cells and B cells [28]. BzATP induced rapid CD21 shedding from human B cells, which could be inhibited by the P2X7 antagonists, KN-62 and oxidised ATP [29] confirming a role for P2X7 in this process. The mechanism by which this process occurs remains unknown. CD21 shedding is a redox-regulated process involving oxidation of a tyrosine kinase pathway and reduction of metalloproteases [30]. Thus, the possibility remains that ATP-induced reactive oxygen species (ROS) formation may be involved in P2X7-induced CD21 shedding.

CD23 (low affinity IgE receptor)

CD23 is a single transmembrane receptor with an extracellular C-terminus and intracellular N-terminus (type II integral membrane protein), and is predominately present on B cells, and some dendritic and epithelial cells [31] CD23 is principally involved in the regulation of immunoglobulin (Ig)E, but can also function as a cell adhesion molecule [32]. Following CD23 shedding, the soluble form exerts cytokine-like properties [33]. ATP and BzATP were first shown to induce the rapid shedding of CD23 from human chronic lymphocytic leukaemic (CLL) lymphocytes [34]. KN-62 and oxidised ATP, impaired this nucleotide-induced CD23 shedding [34], confirming a role for P2X7 in this process. Since then, P2X7-induced CD23 shedding has been described for human monocyte-derived dendritic cells [35], human monocyte-derived Langerhans cells [36], human RPMI 8226 multiple myeloma B cells [37], human B cells [38] and murine B cells [38, 39]. Monocyte-derived dendritic and Langerhans cells, from subjects homozygous for the loss-of-function polymorphism E496A, exhibited attenuated ATP-induced CD23 shedding [35, 36] further supporting a role for P2X7 in this process. Finally, BzATP induced CD23 shedding from CD23-transfected Chinese hamster ovary (CHO) cells [40]. However a direct role for P2X7 in this process was not established in this study, especially given that these cells express both endogenous P2X7 [41] and P2Y receptors [42].

P2X7-induced CD23 shedding is primarily mediated by ADAM10. Comparisons of phorbol ester- and P2X7-induced shedding of CD23 and CD62L from CLL cells revealed that this process was mediated by different metalloproteases [34]. First, either phorbol ester treatment or P2X7 activation induced CD62L shedding, but only P2X7 activation induced CD23 shedding. Second, the hydroxamic acid-based protease inhibitor of Zn^{2+}-dependent metalloprotease, Ro 31-9790, inhibited P2X7-induced CD23 shedding more potently than P2X7-induced CD62L shedding. A role for ADAM10 in ATP- or BzATP-induced CD23 shedding was first shown in human histiocytic lymphoma U937 cells using an inhibitory prodomain construct of ADAM10, A10-(23-213) [43], and subsequently in CD23-transfected CHO cells and murine B cells using GI 254023X [39, 40].

However, a direct role for P2X7 in CD23 shedding was not established in any of these studies. Subsequently, the broad-spectrum metalloprotease inhibitors, BB-94 (batimastat) and GM 6001, were shown to impair P2X7-induced CD23 shedding from RPMI 8226 cells [37] supporting a role for a metalloprotease in this process. Finally, using GI 254023X, direct evidence for ADAM10 in mediating P2X7-induced CD23 shedding was established in RPMI 8226 cells [44], and in human and murine B cells [38].

P2X7-induced CD23 shedding appears to occur independently of ion channel activity. Studies of P2X7-induced CD23 shedding in RPMI 8226 cells indicated that neither K^+ efflux, Na^+ influx, Ca^{2+} influx nor increases in intracellular Ca^{2+} were essential for this process [45]. In fact, extracellular Ca^{2+} partly impaired P2X7-induced CD23 shedding from CLL cells [34]. Similarly, Mn^{2+} and Mg^{2+} partly impaired P2X7-induced CD23 shedding from these cells [34]. However the mechanism by which these divalent cations impair P2X7-induced CD23 shedding remains unknown. Finally, attempts using small molecule inhibitors in RPMI 8226 cells have failed to establish a role of many enzymes, commonly downstream of P2X7 activation, in P2X7-induced CD23 shedding including PKC, JNK, Rho kinase, PI3K, GSK-3, MAPK, acid sphingomyelinase and phospholipases [45].

CD44 (phagocyte glycoprotein-1)

CD44 (phagocyte glycoprotein-1) is a type I integral membrane glycoprotein expressed on leukocytes, fibroblasts, and epithelial and endothelial cells [46]. CD44 plays roles in cell adhesion, lymphocyte signalling, inflammation, angiogenesis and tumour metastasis [46]. ATP, but not ADP, induced the rapid shedding of CD44 from murine P388D1 lymphoid tumour cells, which was impaired by KN-62 and short hairpin RNA silencing of P2X7 [47], indicating a role for P2X7 in the process. In contrast, ATP was unable to alter the expression of cell surface CD44 on CLL cells [34].

Thus, P2X7-induced CD44 shedding may be cell or species specific. Nevertheless, the mechanism involved in P2X7-induced CD44 shedding from P388D1 cells remains undefined. ADAM10 [48], ADAM17 [49], MMP (matrix metalloproteinase) 9 [50] and MMP14 [51] are involved in constitutive CD44 shedding, and thus one or more of these sheddases may play a role in P2X7-induced CD44 shedding. Notably, the glycosaminoglycan chains of soluble CD44 have been reported to associate with P2X7 to function as a positive allosteric modulator of P2X7 activation [52]. Thus, it was proposed that P2X7 activation results in CD44 shedding, with the resulting soluble CD44 forming part of a regulatory positive feedback loop facilitating ATP-induced cell signalling via P2X7 [52, 53]. Whether other glycosaminoglycan chain-containing ectodomains shed following P2X7 activation can also modulate P2X7 remains unknown.

CD62L (L-selectin)

CD62L (L-selectin) is a type I integral membrane glycoprotein present predominately on leukocytes [54]. CD62L is involved in constitutive trafficking of lymphocytes through lymphoid organs, and rolling of leukocytes on inflamed vascular endothelium [54]. Seminal studies by Wiley and colleagues demonstrated that P2X7 activation could induce the rapid shedding of CD62L, revealing for the first time the potential importance of extracellular nucleotides and P2 receptors in ectodomain shedding. ATP was first shown to induce rapid CD62L shedding from the surface of CLL cells [55]. Adenosine, ADP and UTP did not cause CD62L shedding, while oxidised ATP inhibited both ATP- and BzATP-induced CD62L shedding from these cells [55]. Furthermore, KN-62 inhibited ATP-induced CD62L shedding from CLL lymphocytes [34, 56], while BzATP-induced CD62L shedding was impaired in CLL cells expressing non-functional P2X7 [57]. Collectively, confirming a role for P2X7 in this process.

Following the studies above, it was shown that P2X7 activation can induce CD62L shedding from human B cells and T cells [29], and subsets of these cells from humans or mice including $CD27^-$ and $CD27^+$ B cells [29], and $CD4^+$ and $CD8^+$ T cells [29, 58-60]. In murine T cells, NAD was also able to induce P2X7-induced CD62L shedding [61, 62]. ATP-induced CD62L shedding was impaired in lymphocytes, as well as monocytes, from subjects coding the P2X7 E496A loss-of-function polymorphism [60, 63] and from Pfizer P2X7 knockout mice [64]. In contrast, BzATP-induced CD62L shedding was found to be more rapid in T cells from GlaxoSmithKline P2X7 knockout mice [65]; presumably due to the presence of the highly functional escape variant P2X7k in T cells from these mice [66, 67].

A physiological role for P2X7-induced CD62L shedding was implicated in T cell transendothelial migration to the heart in a murine model of Duchenne muscular dystrophy [68]. This study showed that treatment of *mdx/mdx* mice with the P2X7 antagonist, BBG, allowed the transendothelial migration of T cells to the heart by allowing sustained CD62L expression [68]. This suggested that impaired P2X7-induced CD62L shedding might contribute to the pathology of Duchenne muscular dystrophy. In contrast, oxidised ATP did not affect the loss of CD62L during CLL lymphocyte transmigration [69], suggesting P2X7 may not play a role in this process. This difference may be due to different experimental conditions including cell type and species.

ADAM17 is thought to be the principal sheddase involved in P2X7-induced CD62L shedding. A metalloproteases was first implicated in this process when Ro 31-9790 was shown to inhibit P2X7-induced CD62L shedding in CLL cells [34]. Subsequently, a role for ADAM17 in this process was shown in murine B cells and T cells [39, 40]. In the absence of ADAM17, ADAM10 is able to mediated BzATP-induced CD62L shedding [39, 40]. However BzATP-stimulated CD62L shedding mediated by ADAM17 was significantly more rapid than shedding mediated by ADAM10 [39], corroborating a role for ADAM17 as the principal sheddase. Such studies highlight the

complexity in ectodomain shedding, often revealing dominant but redundant roles for many sheddases.

Other studies have identified other molecules important in P2X7-induced CD62L shedding. An inhibitor of phosphatidylserine exposure, 4,4'-diisothiocyanatosilbene-2,2'-disulphonic acid, prevented P2X7-induced CD62L shedding from murine effector/memory $CD4^+$ T cells [70], indicating a role for rapid, non-apoptotic phosphatidylserine exposure in this process although the precise mechanism involved remains undefined. P2X7-induced CD62L shedding does not involve conventional PKC isoforms [55], PI3K, ERK or the NADPH oxidase [71]. In contrast, P2X7-induced loss of CD62L from human $CD4^+$ T cells was enhanced by diphenyleneiodonium, rotenone and antimycin A, which uncouple complexes of the mitochondrial respiratory chain to cause ROS formation [71]. Moreover, rottlerin, an inhibitor of novel PKC isoforms that can also promote ROS formation, potentiated P2X7-induced CD62L loss from human $CD4^+$ T cells [71]. Although this study did not directly study ADAM17, ROS are able to activate ADAM17 via oxidation of cysteine motifs, which are critical for CD62L cleavage [72]. Therefore, ROS may play a role in P2X7-induced activation of ADAM17 and subsequent shedding of CD62L.

CD206 (macrophage mannose receptor 1)

CD206 (macrophage mannose receptor 1) is a type I integral membrane glycoprotein mainly expressed on the surface of macrophages and dendritic cells, where it functions as an antigen receptor promoting phagocytosis of pathogens [73]. As for CD14, ATP induced the rapid shedding of CD206 from resting, M1 and M2 murine macrophages, a process impaired by A 438079 and GM 6001, as well as in macrophages from Pfizer P2X7 knockout mice [26]. The specific metalloprotease mediating P2X7-induced CD206 shedding is yet to be determined.

CD324 (E-cadherin)

CD324 (E-cadherin) is a type I integral membrane glycoprotein that mediates cell-to-cell adhesion in epithelial tissues, while loss of this molecule promotes cell migration including epithelial tumour metastasis [74]. In contrast to most studies that use exogenous ATP to induce ectodomain shedding, one study showed that melittin, the major component of bee venom, induced the rapid shedding of CD324 from human HaCaT keratinocytes via a purinergic pathway [75]. Although evidence for the specific P2 receptor involved in this process is limited, melittin caused ATP release from HaCaT keratinocytes, and the ATP-degrading enzyme, apyrase, and broad-spectrum P2 antagonists, PPADS, suramin and Evans Blue, impaired ERK1/2 phosphorylation, which was associated with melittin-induced CD324 shedding. Furthermore, P2X7-transfected, but not mock transfected, HEK 293 cells showed increased melittin-induced phosphorylation of ERK, which could also be abrogated by apyrase [75]. Collectively, this suggested a role for an ATP-P2X7 axis in melittin-induced CD324 shedding. Finally, BB-2516, GI 254023X and GW 280264X inhibited melittin-induced CD324 shedding [75], thus, supporting a role for ADAM10 in this pathway.

CXCL16 (CXC-chemokine ligand 16)

CXCL16 (CXC-chemokine ligand 16) is a type I integral membrane glycoprotein, which predominantly functions as an adhesion receptor for cells expressing the CXC-chemokine receptor CXCR6, but can also serve as a scavenger receptor capable of binding cells displaying exposed phosphatidylserine or oxidised low-density lipoprotein [76]. Following shedding, soluble CXCL16 can induce lymphocyte chemotaxis, promote angiogenesis and prevent excitotoxicity of neurons [76]. P2X7 activation can induce CXCL16 shedding and this process is mediated by ADAM10. ATP and BzATP, but neither ADP nor UTP, induced the rapid shedding of CXCL16 from RPMI

8226 cells, a process impaired by the P2X7 antagonists, KN-62 and AZ 10606120 [44]. Moreover, BB-94 and GM 6001, as well as GI 254023X, impaired ATP-induced CXCL16 shedding [44].

Epidermal growth factor members

Activation of EGFR involves the binding of members of the epidermal growth factor (EGF) family including amphiregulin, betacellulin EGF, heparin-binding EGF-like growth factor (HB-EGF) and transforming growth factor-α (TGF-α) [77]. EGF family members are type I integral membrane proteins and ectodomain shedding of these proteins generates soluble ligands for EGFR [77]. EGF members are involved in the modulation of cell proliferation, apoptosis and migration, and play important roles in processes such as bone formation, wound healing and tumourigenesis [77].

Amphiregulin

ATP and ATPγS induced slow (24 hours) shedding of amphiregulin from LPS-stimulated human monocyte-derived and murine bone marrow-derived dendritic cells [78]. UTP also induced amphiregulin shedding from LPS-stimulated murine bone marrow-derived, but not from human monocyte-derived, dendritic cells [78]. Moreover, suramin inhibited ATPγS-induced amphiregulin shedding from human monocyte-derived dendritic cells [78], but the specific P2 receptors involved were not identified. Notably, amphiregulin released from murine bone marrow-derived dendritic cells stimulated tumour growth *in vivo* [78] indicating that nucleotides can confer tumourigenic properties to dendritic cells by inducing amphiregulin release. This contrasts the anti-tumourigenic properties of dendritic cells following P2X7 activation and subsequent NLRP3 inflammasome stimulation to promote anti-tumour immunity [79].

Betacellulin

BzATP induced the rapid shedding of betacellulin from betacellulin-transfected CHO cells, and from P2X7 and betacellulin co-transfected murine embryonic fibroblasts (mEFs) [39, 40]. BzATP-induced betacellulin shedding was impaired by BB-2516 and GI 254023X, as well as by transfection of dominant-negative ADAM10 plasmid DNA [40]. GI 254023X impaired BzATP-induced betacellulin shedding in ADAM17 knockout mEFs (which expressed ADAM10). Conversely, BzATP did not induce betacellulin shedding in ADAM10 knockout mEFs (which expressed ADAM17) [40]. Collectively, this indicated that P2X7 stimulates ADAM10 to induce betacellulin shedding.

Heparin-binding epidermal growth factor-like growth factor (HB-EGF)

The first indication of nucleotide-induced HB-EGF release was indirectly shown using a neutralising antibody against HB-EGF, which inhibited ATP-induced mitogenic effects in guinea pig Muller glial cells [80]. This study postulated that ATP-induced P2Y activation lead to the release of HB-EGF from cells, which mediated EGFR activation [80]. Later, direct evidence of nucleotide-induced HB-EGF shedding was shown using ATPγS in HB-EGF-transfected SV-40 immortalised human corneal epithelial cells [81, 82]. Wounding, which increased ATP in the culture medium, as well as ADP, also induced rapid HB-EGF shedding from these cells [81, 82]. Reactive Blue 2 inhibited ATPγS-induced HB-EGF shedding [81] indicating a role for a P2 receptor in this process. GM 6001, GW280264X [81] and GI 254023X [82] inhibited HB-EGF shedding from these cells. Furthermore, the mitogen-activated protein kinase kinase (MEK) and ERK1/2 inhibitors, PD98059 and U0126 respectively, impaired ATPγS- and wound-induced HB-EGF shedding [81, 82]. Overall, these studies suggest that ADAM10, ADAM17, MEK and ERK1/2 are involved in nucleotide-induced HB-EGF shedding from corneal epithelial cells, and that this process may be relevant during wound healing. However since Reactive Blue 2 and ATPγS can

inhibit and activate a range of P2X and P2Y receptors, respectively [6] further studies are required to determine which P2 receptor(s) is (are) involved in this process.

Transforming growth factor-α (TGF-α)

Several studies have shown that nucleotides induce TGF-α shedding from cells. BzATP induced the rapid shedding of TGF-α from TGF-α-transfected CHO cells [40], and from P2X7 and TGF-α co-transfected mEFs [39, 40]. TGF-α shedding from CHO cells was impaired by BB-2516, but not GI 254023X [40], excluding a role for ADAM10 in the process. P2X7 was thought to be involved in this process, but direct evidence for this receptor is required, especially since P2Y receptors have also been implicated in TGF-α shedding as discussed below.

Both ATP and UTP can induce rapid TGF-α shedding from TGF-α-transfected CHO cells, which express P2Y2 mRNA [42]. The broad-spectrum metalloprotease inhibitor, TAPI-2, inhibited ATP-induced TGF-α shedding from CHO cells, but ATP did not induce TGF-α shedding from CHO cells deficient in ADAM17 [42]. Moreover, ATP, ATPγS and UTP induced TGF-α shedding from EC-4 murine fibroblasts (which express ADAM17), but not from EC-2 murine fibroblasts (which are ADAM17 deficient) [42]. Collectively, this data indicates that P2Y2 activation stimulates ADAM17 to induce the rapid shedding of TGF-α, but an additional role for P2X7 in this process cannot be excluded. Further data from this study, using the Ca^{2+} chelators, 1,2-bis(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid (BAPTA-AM) and ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), indicates that this process is regulated by both intracellular and extracellular Ca^{2+} [42]. Furthermore, this process involved mitochondrial, but not NADPH oxidase, ROS formation, as the ROS scavenger N-acetylcysteine, and the mitochondrial complex inhibitors, rotenone and myxothiazol, but not the NADPH oxidase inhibitor, apocynin, impaired ATP-induced TGF-α shedding [42].

ATP also induced TGF-α shedding from human bronchial epithelial 1 cells over 2 hours [83], while melittin (which causes ATP release) induced rapid TGF-α shedding from HaCaT keratinocytes [75]. Although a role for P2 receptor activation in this process was only assessed in the former study and then only using the broad-spectrum P2 antagonist, suramin, this further supports a role for P2 receptor activation in TGF-α shedding. Notably both studies identified ADAM17 as the main sheddase involved in this process [75, 83]. Furthermore, through use of siRNA knockdown and chemical inhibitors, ATP-induced ADAM17 stimulation in human bronchial epithelial 1 cells was mediated by a pathway involving activation of the NADPH oxidase homolog dual oxidase 1 and ERK1/2 [83].

Collectively, the above studies suggest a role for both P2X7 and P2Y2 receptor activation in nucleotide-induced TGF-α shedding, and that this process is primarily mediated by ADAM17. However, further evidence is required to establish the specific roles for each receptor in this process.

Immunoglobulin superfamily members

Extracellular nucleotides induce the shedding of several molecules of the Ig superfamily including CD54, CD56, CD126, T cell immunoglobulin and mucin domain (TIM)-2, and possibly major histocompatibility complex (MHC) class I molecules.

CD54 (intercellular adhesion molecule-1, ICAM-1)

CD54 (intercellular adhesion molecule-1, ICAM-1) is a type I integral membrane glycoprotein important in cell adhesion mediating immune cell trafficking, antigen presentation and cell signalling [84]. BzATP stimulated the rapid shedding of CD54 from P2X7 and CD54 co-transfected mEFs, and from CHO cells [40], but a direct role for P2X7 in this process in CHO cells was not

directly established. GI 254023X inhibited BzATP-induced CD54 shedding in ADAM17 knockout mEF cells (which expressed ADAM10), but not in ADAM10 knockout mEF cells (which expressed ADAM17) [40]. Further, BzATP failed to induce CD54 shedding in ADAM10/ADAM17 knockout mEF cells [40], but this process could be reversed following transfection with ADAM17 [39]. BzATP-induced CD54 shedding from CHO cells was impaired by BB-2516 but not by GI 254023X [40]. Collectively, this suggested that ADAM17 is the main sheddase involved in BzATP-induced CD54 shedding when both ADAM10 and ADAM17 are present.

CD56 (neural cell adhesion molecule, NCAM)

CD56 (neural cell adhesion molecule, NCAM) is an integral membrane glycoprotein and comprises three major forms, with molecular weights of 120, 140 and 180 kDa [85]. These molecules are involved in cell adhesion, migration, and survival, as well as axon guidance and synaptic targeting [85]. ATP induced the shedding of all three forms from embryonic rat hippocampal neurons and from CD56-transfected L929 murine fibroblasts over 1.5 to 6 hours [86]. ATP, at concentrations of 2.5 mM, was required to induce CD56 shedding [86], indicating a possible role for P2X7 in this process. The broad-spectrum metalloprotease inhibitors, BB-3103 and GM 6001, impaired ATP-induced CD56 shedding from CD56-transfected L929 cells [86]. In contrast, ATP-induced CD56 shedding was independent of lysosomal, proteasomal and calpain proteolytic activity, as well as PI3K and PKC activation [86]. Finally, the extracellular ATP binding site of CD56 was not required for ATP-induced CD56 shedding [86], indirectly supporting a role for a P2 receptor in this process.

CD126 (interleukin-6 receptor, IL-6 receptor)

IL-6 is a cytokine involved in homeostasis, as well as immune responses, and is secreted by both immune and non-immune cells [87]. This cytokine induces the proliferation and differentiation of T cells, macrophages and neutrophils, and can induce fever in the presence of tumour necrosis factor

(TNF) and IL-1β [87]. The biological activities of IL-6 are mediated by binding CD126 (IL-6 receptor), a type I integral membrane glycoprotein, which subsequently recruits two integral membrane glycoproteins, CD130 (gp130), to form a signalling complex [87]. Alternatively, CD126 shed from cells can bind IL-6 to form a complex with two membrane CD130 molecules to mediate signalling in cells that do not express CD126 or have shed this receptor [87]. P2X7 can mediate the rapid shedding of CD126. BzATP induced the rapid shedding of CD126 from wild-type murine splenocytes, a process impaired by KN-62 or in splenocytes from Pfizer P2X7 knockout [88]. Notably, CD126 serum concentrations from these knockout mice are reduced compared to wild-type mice, suggesting a role for P2X7 in CD126 shedding *in vivo* [88]. GI 254023X and GW 280264X both inhibited BzATP-induced CD126 shedding in P2X7 and CD126 co-transfected NIH3T3 mouse embryonic fibroblasts and HEK 293 cells, while the ADAM10 prodomain, A10-(23-213), inhibited BzATP-induced CD126 shedding from wild-type murine T cells and T cells with minimal ADAM17 expression [88]. Thus, indicating that ADAM10, but not ADAM17, plays a predominant role in P2X7-induced CD126 shedding.

Major histocompatibility complex (MHC) molecules

MHC class I molecules are cell surface receptors comprised of a single transmembrane protein paired with β_2 microglobulin, and are highly expressed on nearly all cell types [89]. MHC class I molecules present antigenic peptides to $CD8^+$ T cells [89]. BzATP was shown to induce the loss of cell surface MHC class I molecules from murine $CD8^+$ T cells [58], but the identity of the P2 receptor involved, and whether this loss was due to shedding or internalisation was not determined. However, ATP also induced the rapid loss of MHC class I molecules from murine bone marrow-derived macrophages from wild-type, but not Pfizer P2X7 knockout, mice [90]. Furthermore, the P2X7 antagonists, A 438079 and A 740003, impaired ATP-induced MHC class I molecule loss from these cells from wild-type mice [90]. Collectively, confirming a role for P2X7 in this process.

Use of GM 6001, or the Zn^{2+} chelator, N,N,N′,N′-tetra-2-picolylethylenediamine, failed to impair ATP-induced MHC class I molecule loss [90] suggesting that this process was not due to shedding. Moreover, the lysosome cysteine protease inhibitor, E64, or the proteasome inhibitor, MG132, failed to impair ATP-induced MHC class I loss [90] suggesting that this process was not due to a lysosome-mediated pathway. Thus, the authors proposed that ATP-induced MHC class I loss was the result of microvesicle release rather than shedding. Consistent with this, P2X7 activation induced the release of MHC class II molecule-containing microvesicles and exosomes from murine macrophages and dendritic cells [91, 92], but did not induce a loss of MHC class II molecules from the surface of CLL cells [34]. Finally, P2X7 activation inhibited the release of the non-classical MHC molecule, soluble human leukocyte antigen-G, from LPS-activated peripheral blood mononuclear cells [93]. Collectively, these studies demonstrate a complex role for P2X7 activation in the loss of MHC molecules from cells, and it remains to be determined if P2 receptor activation induces ectodomain shedding of MHC molecules.

T cell immunoglobulin and mucin domain-2 (TIM-2)

T cell immunoglobulin and mucin domain (TIM) molecules are type I integral membrane glycoproteins comprising three members in humans (TIM-1, TIM-3 and TIM-4) and eight members (TIM1-8) in mice [94]. TIM-2 is present in mice, but not humans, and functions as a H-ferritin receptor on T helper 2 cells and B cells to modulate immune responses [94]. BzATP induced the rapid shedding of TIM-2 from murine splenic B cells, as well as from P2X7 and TIM-2 co-transfected HEK 293 cells [95]. Both GI 254023X and GW 280264X inhibited BzATP-induced TIM-2 shedding from these cells [95], supporting a role for ADAM10 in this process. However a direct role for P2X7 activation in BzATP-induced TIM-2 shedding from splenic B cells remains to be established.

Tumour necrosis factor-related members

TNF and TNF receptors (TNFRs) belong to TNF and TNFR superfamilies, respectively, and have numerous roles in biology including the immune, nervous and skeletal systems [96]. TNF is a proinflammatory cytokine that plays a critical role in inflammation and immunity by binding to TNFRs [97]. TNF functions as a transmembrane protein (to transmit signals as either a ligand or as a receptor) and as a soluble protein (ligand) following shedding by ADAM17 [97]. The type I integral membrane glycoprotein, CD27, is another member of the TNFR superfamily with important roles in immunity and tolerance [98]. CD27 binds CD70 and functions as both a membrane receptor and soluble molecule with biological activity [98].

CD27

ATP induced a rapid loss of surface CD27 from murine B and T cells [99]. In contrast to most other studies, which utilised enzyme-linked immunosorbent assays or immunoblotting to directly demonstrate P2 receptor-induced ectodomain shedding, surface plasmon resonance was used to show that supernatants from ATP-treated splenocytes displayed significant binding to immobilised anti-CD27 antibody [99] indicating that ATP-induced loss of CD27 is due to shedding. BzATP induced CD27 shedding more potently than ATP, whilst ATP-induced CD27 shedding was impaired by KN-62 [99] indicating a role for P2X7 activation in this process. GM 6001 also impaired P2X7-induced CD27 shedding [99], indicating a role for a metalloprotease in this process, but the identity of which remains unknown. Inhibitors of tyrosine kinases, PI3K, MEK and p38 MAPK did not impair P2X7-induced CD27 shedding [99]. Of note, NAD, released during tissue dissociation, promoted the P2X7-mediated loss of cell surface CD27 from murine splenic T regulatory cells and liver NK T cells, a process that could be impaired with a blocking nanobody

against ARTC2.2 [100, 101]. Thus, P2X7 activation can induce CD27 shedding from various lymphocyte subsets.

CD120a (tumour necrosis factor receptor 1, TNFR1)

The P2Y2 and P2Y4 agonist, UTP, induced the rapid shedding of CD120a (tumour necrosis factor receptor 1, TNFR1) from SV-40 immortalised human corneal epithelial cells, a process that was impaired by the broad-spectrum metalloprotease inhibitor, TAPI-1 [102]. Moreover, eye drops containing the P2Y2/P2Y4 agonist, Diquafosol, increased soluble CD120a in tears of patients with short break-up time dry eye [102]. Collectively, this suggests that P2Y2 or P2Y4 activation can induce CD120a shedding. Of note, P2Y2 is required for sustained TNF-induced Ca^{2+} oscillations in murine lung endothelial cells, a process that accompanies ADAM17-mediated CD120a shedding [103], providing indirect evidence that P2Y2 activation can induce CD120a shedding.

Tumour necrosis factor (TNF)

BzATP induced the rapid shedding of TNF from P2X7 and TNF co-transfected mEFs [40], and from either ADAM17 knockout mEF cells (which expressed ADAM10) or ADAM10 knockout mEF cells (which expressed ADAM17) [40]. Further, GI 254023X impaired BzATP-induced shedding of TNF in ADAM17 knockout mEF cells, but not in ADAM10 knockout mEF cells or normal CHO cells [40]. This indicates that ADAM10 contributes to BzATP-induced TNF shedding only in the absence of ADAM17, and that ADAM17, when present, is the main sheddase involved in this process. The contribution of P2X7 in this process remains to be established.

Summary

In conclusion, it is evident that extracellular nucleotides through the activation of P2 receptors can mediate the ectodomain shedding of numerous cell surface molecules. Both P2X7 and P2Y2 appear to be the major P2 receptors involved in this process, with well-established roles for various cell surface molecules including amyloid precursor protein, CD23, CD27 and CD62L, although a role for P2X7 in amyloid precursor protein shedding remains to be clearly defined. Importantly, a number of studies have shown that P2 receptor-mediated ectodomain shedding for some molecules, such as ARTC2.2, CD62L and CD126, can occur *in vivo*, but such evidence for most molecules is still required. In addition, the biological significance of P2 receptor-mediated ectodomain shedding remains to be determined in nearly all cases. In most instances it remains unknown if the soluble molecules or the remaining transmembrane regions, following P2 receptor-mediated ectodomain shedding, have any biological properties, although this is often inferred from ectodomain shedding studies initiated by pathways other than purinergic signalling. Further, a large body of evidence supporting a role for P2 receptor-induced ectodomain shedding is based on immortalised cell lines and in many cases transfection of P2 receptors into such cell lines. Thus, further work is required to study this process in primary cells expressing endogenous P2 receptors, especially in human cell types. In this regard, the identity of the P2 receptors involved in nucleotide-induced ectodomain shedding remains to be determined in many cases. Finally, although the major sheddases, most notably ADAM10 and ADAM17, have been identified in many examples of P2 receptor-induced ectodomain shedding, it remains unclear in most instances how such metalloproteases are activated downstream of P2 receptor activation.

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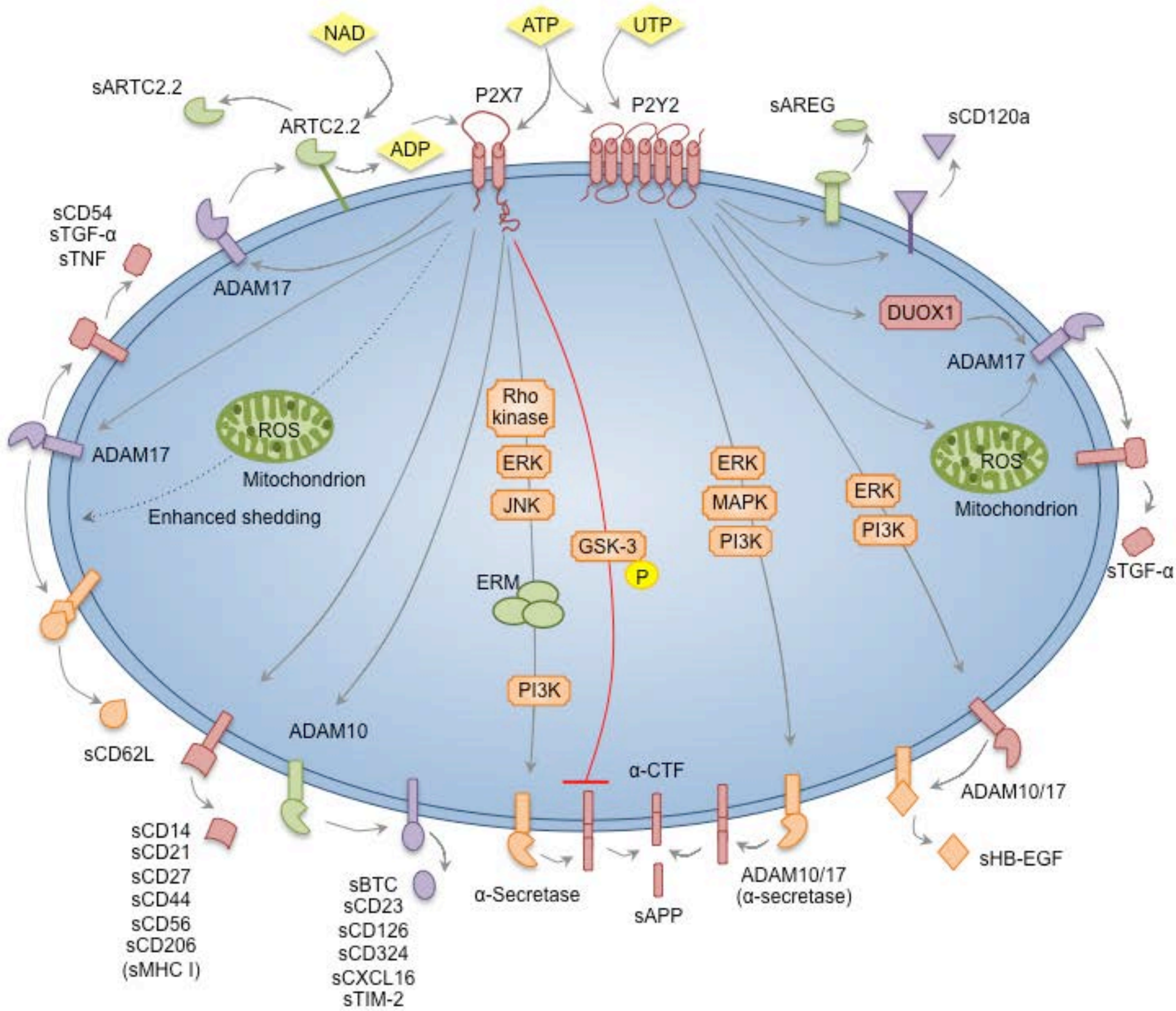
Figure legends

Figure 1 Nucleotide-induced ectodomain shedding. ATP activates P2X7 receptors. NAD can also activate P2X7 via the ADP-ribosyltransferase, ARTC2.2. P2X7 activation can stimulate ADAM10 to induce the shedding of BTC, CD23, CD126, CD324, CXCL16 and TIM-2. P2X7 activation can stimulate ADAM17 to induce the shedding of ART2.2C, CD54, CD62L, TGF-α and TNF. P2X7-induced shedding of CD62L can be enhanced by mitochondrial ROS formation. P2X7 activation can stimulate a α-secretase to induce APP shedding via an intracellular signalling cascade involving Rho-kinase, ERK, JNK, ERM and PI3K. An opposing role for P2X7 in APP processing is also suggested, whereby APP processing is inhibited by GSK-3 downstream of P2X7 activation. Finally, P2X7 activation can induce the shedding of CD14, CD21, CD27, CD44, CD56, CD206, and possibly MHC class I molecules, but the sheddases involved remain unknown. ATP and UTP can stimulate P2Y2 receptors. P2Y2 activation can stimulate ADAM17, via DUOX1 and mitochondrial ROS generating pathways, to induce the shedding of TGF-α. P2Y2 activation can stimulate ADAM10 and ADAM17 to induce the HB-EGF shedding via an intracellular signalling cascade involving ERK and PI3K. P2Y2 activation can also stimulate ADAM10 and ADAM17, which serve as an α-secretase, to induce APP shedding via an intracellular signalling cascade involving ERK, MAPK and PI3K. Finally, P2Y2 activation (or possibly activation of other P2Y receptors) can induce the shedding of AREG and CD120a, but the sheddases involved remain

unknown. *Abbreviations:* ADAM, a disintegrin and metalloprotease; ADP, adenosine 5'-diphosphate; APP, amyloid precursor protein; AREG, amphiregulin; ATP, adenosine 5'-triphosphate; BTC, betacellulin; CD21, complement receptor 2; CD23, IgE receptor; CD27, tumor necrosis factor receptor; CD44, hyaluronic acid receptor; CD54, intercellular adhesion molecule-1; CD56, neural cell adhesion molecule; CD62L, L-selectin; CD120a, tumor necrosis factor receptor 1, CD126, interleukin-6 receptor; CD324, E-cadherin; CTF, carboxyl-terminal fragment; DUOX1, NADPH oxidase homolog dual oxidase 1; ERK, extracellular signal-regulated kinase; ERM, ezrin radixin moesin; GSK, glycogen synthase kinase; HB-EGF, heparin-binding-epidermal growth factor; JNK, c-Jun N-terminal kinase; MHC, major histocompatibility complex; NAD, nicotinamide adenine nucleotide; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; ROS, reactive oxygen species; TIM, T cell immunoglobulin and mucin domain; TGF, transforming growth factor; TNF, tumor necrosis factor; UTP, uridine 5'-triphosphate.

Figure 2 APP processing pathways. The amyloidogenic pathway (left) is (1) initiated with the cleavage of APP by β-secretase (2) to generate sAPPβ and β-CTF. (3) β-CTF is subsequently cleaved by γ-secretase (4) to generate Aβ. The anti-amyloidogenic pathway (right) is (1) initiated with the cleavage of APP by α-secretase (2) to generate sAPPα and α-CTF. (3) α-CTF is subsequently cleaved by γ-secretase (4) to generate truncated Aβ peptide termed p3. *Abbreviations:* APP, amyloid precursor protein; sAPPβ, soluble amyloid precursor protein β; β-CTF, β-carboxyl-terminal fragment; sAPPα, soluble amyloid precursor protein α; α-CTF, α-carboxyl-terminal fragment. (After [14].)


NAD
ATP
UTP
sARTC2.2
ARTC2.2
ADP
P2X7
P2Y2
sAREG
sCD120a
sCD54
sTGF-α
sTNF
ADAM17
DUOX1
ADAM17
ADAM17
ROS
Mitochondrion
Enhanced shedding
Rho kinase
ERK
JNK
GSK-3
P
ERM
PI3K
ERK
MAPK
PI3K
ERK
PI3K
ROS
Mitochondrion
sTGF-α
sCD62L
ADAM10
α-CTF
ADAM10/17
sCD14
sCD21
sCD27
sCD44
sCD56
sCD206
(sMHC I)
sBTC
sCD23
sCD126
sCD324
sCXCL16
sTIM-2
α-Secretase
sAPP
ADAM10/17
(α-secretase)
sHB-EGF

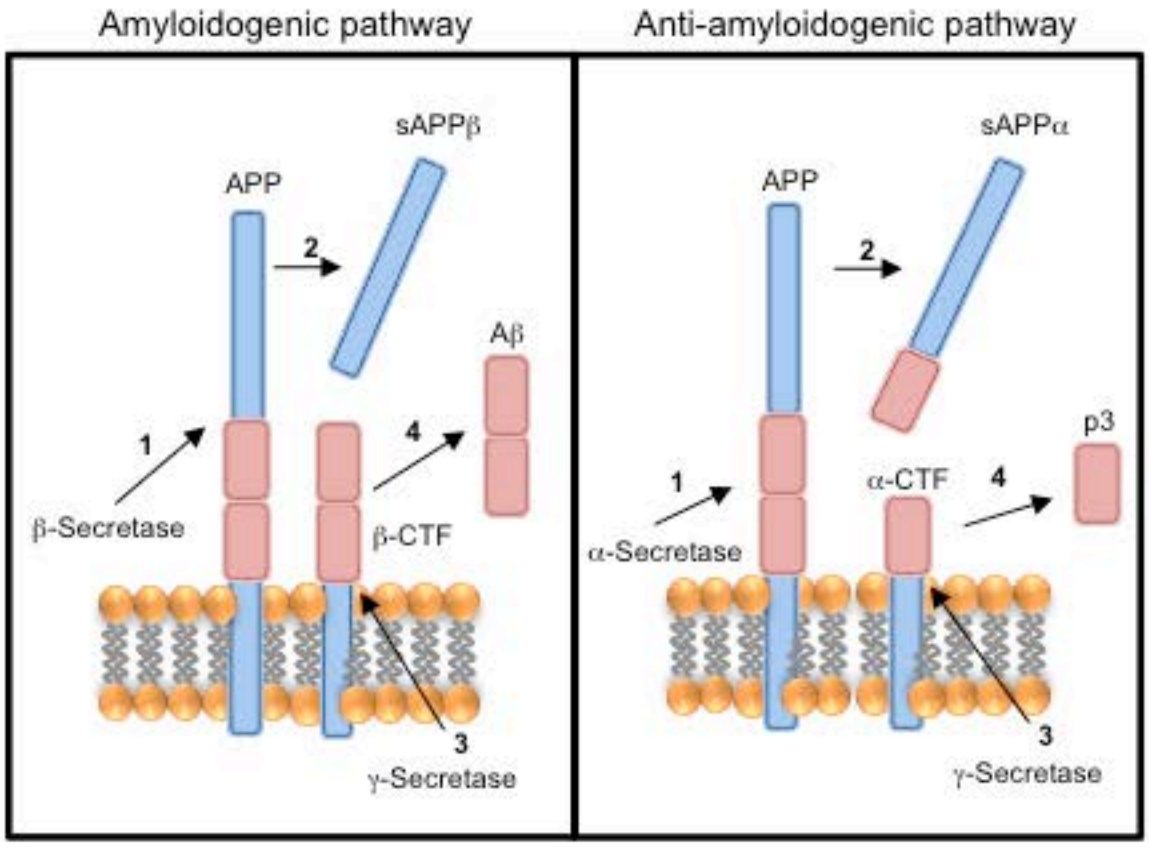

Amyloidogenic pathway
Anti-amyloidogenic pathway
sAPPβ
APP
2
Aβ
1
4
β-Secretase
β-CTF
3
γ-Secretase
sAPPα
APP
2
p3
1
α-CTF
4
α-Secretase
3
γ-Secretase

Table 1 Sheddase inhibitors used in studies of nucleotide- and P2 receptor-induced ectodomain shedding

Inhibitor	Target	Reference
A10-(23-213)[a]	ADAM[b]10	[43]
BB-2516 (marimastat)	Metalloproteases (broad-spectrum)	[104]
BB-3103	Metalloproteases (broad-spectrum)	[105]
BB-94 (batimastat)	Metalloproteases (broad-spectrum)	[106]
Decanoyl-RVKR-CMK	Convertase (non-selective)	[107]
GI 254023X	ADAM10	[108]
GM 6001	Metalloproteases (broad-spectrum)	[109]
GW 280264X	ADAM10 and ADAM17	[108]
Phenanthroline	Metalloproteases (broad-spectrum)	[110]
Ro 31-9790	Metalloproteases (broad-spectrum)	[111]
TAPI-1	Metalloproteases (broad-spectrum)	[112]
TAPI-2	Metalloproteases (broad-spectrum)	[113]

[a]Prodomain construct, [b]ADAM, a disintegrin and metalloprotease