

P2X7 receptor activation regulates rapid unconventional export of transglutaminase-2

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Abstract

Transglutaminases (TG) are externalized from cells via an unknown unconventional secretory pathway. We show for the first time that purinergic signaling regulates active secretion of TG2, an enzyme with a pivotal role in stabilizing extracellular matrices and modulating cell-matrix interactions in tissue repair. Extracellular ATP promotes TG2 secretion by macrophages, and this can be blocked by a purinergic receptor P2X7 (P2X7R)-selective antagonist. Introduction of functional P2X7R into HEK293 cells is sufficient to confer rapid, regulated TG2 export. By employing pharmacological agents, TG2 release could be separated from P2X7R-mediated microvesicle shedding. Neither, Ca^{2+} signaling alone nor membrane depolarization triggered TG2 secretion which occurred only upon receptor membrane pore formation and without pannexin channel involvement. A gain-of-function mutation in P2X7R associated with autoimmune disease caused enhanced TG2 externalization from cells, and this correlated with increased pore activity. These results provide a mechanistic explanation for a link between active TG2 secretion and inflammatory responses, and aberrant enhanced TG2 activity in certain autoimmune conditions.

Introduction

Unconventional export of cytoplasmic proteins, *i.e.* the processes by which proteins that do not follow the classical ER/Golgi secretory pathway are secreted by cells, is being studied extensively as many molecules that fall into this category constitute potent biological signals with key roles in developmental or inflammatory processes. Such proteins lack posttranslational modifications that occur during ER/Golgi protein maturation but may be subject to *N*-terminal processing and acetylation or acylation (Muesch et al., 1990; Stegmayer et al., 2005). Several fundamentally different mechanisms appear to support unconventional protein secretion including self-sustained or transporter-facilitated direct membrane translocation at the plasma membrane, or release in specialized vesicles, the biogenesis of which is distinct from coat protein complex II-coated vesicles (Nickel and Rabouille, 2009; Rabouille et al., 2012). Neither export through the compartment for unconventional protein secretion/multivesicular body pathway nor direct microvesicle (MV) shedding at the plasma membrane requires membrane translocation of the cargo, and release is thought to occur by vesicle lysis in the extracellular environment.

Transglutaminases (TG) are a family of structurally similar enzymes that post-translationally modify proteins through transamidation, deamidation or esterification of glutaminy residues (Aeschlimann and Thomazy, 2000). Several of these enzymes have well established functions in stabilizing extracellular protein assemblies, including TG2 (wound healing), TG4 (semen coagulation) and factor XIII (blood coagulation) (Aeschlimann and Paulsson, 1994; Lorand and Graham, 2003). More recently, TG3 and TG6 have been implicated in extracellular functions (Zone et al., 2011; Thomas et al., 2013). Despite it being 20 years since we first postulated export of TGs through an unconventional secretory pathway (Aeschlimann and Paulsson, 1994), the underlying process remains elusive. This has gained much attention recently as while matrix stabilization by TG2 is required for an effective tissue repair response, aberrant TG2 action has a central role in pathogenesis of inflammatory diseases and autoimmunity, most notably celiac disease (Aeschlimann and Thomazy, 2000; Iismaa et al., 2009). Externalization from cells appears to control TG2 function as Ca^{2+} -binding serves as a molecular “switch” for its activation, facilitating transition into a conformation that enables catalysis (Pinkas et al., 2007). Early studies pointed to passive release of TG2 through cell damage (Upchurch et al., 1987; Siegel et al., 2008). More recently, several alternative mechanisms for constitutive release of TG2 were proposed, including MV shedding (Antonyak et al., 2011; Van den Akker et al., 2011), or perinuclear import into Rab11-positive recycling endosomes (Zemskov et al., 2011). However, the proposed mechanisms implicated different domains of TG2 (Chou et al., 2011; Zemskov et al., 2011). Furthermore, constitutive export is difficult to reconcile with the lack of a correlation between TG2

synthesis level and extracellular activity, and the fact that export appears to be cell type or differentiation stage specific as exemplified in endochondral bone formation (Aeschlimann et al., 1995). Such sudden, context-dependent externalization of TG2 indicated that its export is regulated by an unidentified signaling event.

One emerging pathway for non-classically secreted proteins including interleukin (IL)-1 β involves P2X7R activation, leading to formation of an inflammasome in a NALP3-dependent manner (Dubyak, 2012; Strowig et al., 2012). Inflammasome assembly drives caspase-1 autoprocessing, maturation of IL-1 β by caspase-1 cleavage and ultimately IL-1 β release (Mariathasan et al., 2006). Activated macrophages derived from P2X7R^{-/-} mice are unable to secrete the mature form of IL-1 family cytokines including IL-1 β and IL-18 (Solle et al., 2001; Pelegrin et al., 2008) and hence, these animals show reduced severity in models of acute inflammatory joint or lung disease (Labasi et al., 2002; Lucatelli et al., 2011; Bartlett et al., 2014).

P2X7R is a member of the P2X family of nucleotide gated ion channels that is activated by high concentrations of extracellular ATP. Besides K⁺ efflux that triggers inflammasome assembly, the ion channel also supports Ca²⁺ and Na⁺ influx, leading to membrane depolarization and activation of intracellular signaling cascades (Coddou et al., 2011; Bartlett et al., 2014). The P2X4R crystal structure confirmed that assembly of 3 subunits, each harboring two transmembrane domains, forms the functional P2X receptor (Kawate et al., 2009). The large extracellular domain has ATP and metal ion binding sites that regulate receptor activation state. Channel opening is associated with conformational changes that reposition the transmembrane segments whereby different states of dilation may be adopted (Hattori and Gouaux, 2012; Jiang et al., 2013). The feature that distinguishes P2X7R from the other P2X family members is a long C-terminal tail (Suprenant et al., 1996; Rassendren et al., 1997) which has been implicated in the process of “membrane pore” formation that enables plasma membrane permeability to larger organic cations (Virginio et al., 1999; Browne et al., 2013).

High extracellular ATP is a consequence of cell damage, and enforced by ATP release from activated innate immune cells. This acts as a danger signal amplification system that spreads the alarm within the local milieu. However, ATP is not only released upon tissue/cell injury or stress, but can also be secreted through membrane channels or secretory vesicles (Garcia and Knight, 2010; Sorge et al., 2012; Burnstock, 2015). Given that TG2 is abundantly secreted in the context of inflammation but that extracellular TG2 also has formative roles in tissue development and homeostasis, we hypothesized that its export may be associated with P2X7R signaling. Here we

show for the first time that rapid TG2 export is regulated by P2X7R-mediated membrane pore formation.

Results

Macrophages secrete TG2 in a P2X7R-dependent manner

The THP-1 monocyte/macrophage cell model was chosen to investigate TG2 export as these cells have been reported to be competent in P2X7R-mediated IL-1 β secretion (Mackenzie et al., 2001). We confirmed initially that activation of inflammasome formation by priming cells with lipopolysaccharide (LPS) for Toll-like receptor (TLR) signaling and subsequent stimulation with ATP induces IL-1 β secretion into the cell supernatant as determined by capture ELISA (Fig. S1A). TG2 is expressed in differentiated macrophages but not in monocyte precursors (Mehta and Lopez-Berestein, 1986). Therefore, THP-1 cells were treated with the phorbol ester TPA to induce differentiation (Fig. S1B), and TG2 upregulation was confirmed by Western blotting of cell lysates (Fig. 1A). We then used the ATP analogue BzATP for P2X7R activation as it shows a high degree of selectivity for P2X7R and does not activate P2Y family ATP-sensing receptors (Coddou et al., 2011). Differentiated cells were BzATP stimulated for 10min, and culture supernatants were collected at the end of agonist treatment (pulse) and after a further 30min in the absence of agonist (chase) to capture immediate and potentially delayed TG2 secretion. BzATP stimulation induced a substantial increase in TG2 secretion as determined by Western blotting of cell-free supernatant (Fig. 1B). TG2 export was blocked by P2X7R antagonist A740003 (Fig. 1B) that inhibits IL-1 β secretion in differentiated monocytes (Honore et al., 2006). To substantiate this finding, we analysed TG2 secretion in response to P2X7R activation in primary human M1 macrophages. BzATP triggered rapid TG2 secretion, contributing to soluble (Fig. 1C) and cell surface associated enzyme (Fig. S1C) whereby the soluble enzyme was undergoing processing generating a ~66kDa species. Processing did not involve inflammasome-associated caspase-1 nor cell surface MT1-MMP cleavage (Belkin et al., 2001) as it occurred in the presence of *N*-acetyl-YVAD-chloromethyl ketone (Ac-YVAD-CMK) or EDTA, respectively (Fig. 1D & S1C). Collectively, these data show that P2X7R regulates not only IL-1 β but also TG2 secretion in macrophages.

Expression of P2X7R confers agonist-inducible, rapid TG2 secretion to HEK293 cells

To investigate whether P2X7R alone was sufficient or other inflammasome pathway components are required for TG2 export, we established HEK293 cells stably expressing wildtype or tagged human P2X7R. This cell model was selected as it lacks endogenous expression of P2X receptor family members (Mackenzie et al., 2005) and secretes mature IL-1 β in response to agonist when co-transfected with P2X7R and pro-caspase-1 (Gudipaty et al., 2003). P2X7R expression was confirmed by Western blotting of cell extracts, whereby for tagged P2X7R a single band reactive with antibodies to P2X7R and the V5-tag was detected (Fig. S2A). Immunocytochemistry

confirmed membrane localization of the receptor in P2X7R cells and its absence in parental cells (Fig. S2B). In order to assess P2X7R functionality, changes in intracellular free Ca^{2+} concentration in response to BzATP were investigated using Fluo-4-AM. Only P2X7R cells but not parental cells responded to this agonist (Fig. S2C). A dose-response analysis for BzATP stimulation of P2X7R cells using Ca^{2+} signaling as a readout derived an apparent K_D of $\sim 75\mu\text{M}$ (Fig. S2D). This is in line with literature data ranging from 40-100 μM depending on extracellular Ca^{2+} concentration (Rassendren et al., 1997). Therefore, stimulation with 100 μM BzATP produced a P2X7R specific and in terms of ligand occupancy, relevant response for further investigation of downstream events.

We then investigated whether P2X7R activation induces TG2 secretion. TG2 transfected P2X7R cells were treated with agonist for 5, 10 or 30min, followed by a 30min chase period after agonist wash out. Supernatants of both fractions were analysed for TG2 by Western blotting. Within 10min of BzATP application, pulse fractions revealed substantial TG2 secretion in agonist treated but not vehicle treated cells (Fig. 2A). No TG2 export was seen after 5min indicating that kinetics were considerably slower than Ca^{2+} signaling. Interestingly, elevated TG2 levels in the chase fraction were observed in cells that were exposed to BzATP for 5min (Fig. 2A) or even 1min (not shown), indicating that P2X7R activation and not subsequent events occurring upon prolonged agonist exposure triggers TG2 export. As TG2 levels in the chase fraction were agonist exposure time independent (Fig. 2A) it appears that once initiated, the TG2 export mechanism is active over an extended time period and leads to gradual extracellular TG2 accumulation at a constant rate. Note, the amount of secreted TG2 is small compared to the total and hence, export does not deplete cellular TG2 over time period investigated (Fig. 2D, cell lysate). To further demonstrate that this cell response required P2X7R activity, we employed the competitive P2X7R inhibitor A740003. At 5 μM , it completely blocks a rise in $[\text{Ca}^{2+}]_i$ in response to BzATP (Fig. 2B, top panel), and this is reversible upon inhibitor wash out (Fig. 2B, bottom panel). BzATP stimulation of cells in the presence of this inhibitor was unable to trigger TG2 secretion (Fig. 2C), demonstrating that active secretion of TG2 is a P2X7R regulated process.

P2X7R-mediated TG2 export is not linked to loss of cell membrane integrity or apoptosis

Shedding of membrane-bound particles containing TG2 together with the lipid raft protein flotillin-2 was reported (Antonyak et al., 2011). Hence, we investigated whether P2X7R-mediated TG2 secretion correlated with flotillin-2 release. Western blot analysis of cell lysates confirmed that flotillin-2 and TG2 were expressed at comparable levels in P2X7R and parental cells (Fig. 2D, cell lysate). Only P2X7R cells responded to BzATP stimulation with release of flotillin-2 into the cell supernatant, indicating P2X7R-dependent vesicle shedding (Fig. 2D, medium). Similar to TG2,

flotillin-2 was present in the pulse fraction and accumulated in the chase fraction, potentially indicating co-release. To further analyse secreted material and exclude protein release through passive cell lysis we investigated externalization of the cytosolic proteins I κ B α , β -tubulin, as well as of HMGB-1 which is secreted non-classically via the exosome pathway (Lu et al., 2012). We were unable to detect any of these proteins in the cell supernatant after P2X7R activation (Fig. 2E & not shown). However, given that prolonged stimulation of P2X7R can lead to cell death (Mackenzie et al., 2005), and this critically affects the conclusions, we designed experiments to more selectively investigate loss of membrane integrity and apoptosis, respectively. Firstly, release of cytosolic lactate dehydrogenase (LDH) was quantified after stimulation of either P2X7R or parental cells with BzATP for 10min. No P2X7R induced release of LDH was seen (Fig. S3A). Secondly, BzATP treated P2X7R cells were chased for various times up to 22h and assessed for caspase-3 activation by Western blotting. Activated caspase-3 could not be detected at any time (Fig. S3B) whereas within 6h of TNF α stimulation caspase-3 cleavage was evident as reported (Arlt et al., 2003). These data show that TG2 externalization is not related to cell damage or death but is a selective process, possibly linked to P2X7R-dependent membrane changes. This is consistent with activation of P2X7R triggering rapid alterations in membrane topology without causing cell death that completely reverse as $[Ca^{2+}]_i$ falls unless receptor stimulation is sustained for long time periods (Mackenzie et al., 2005).

TG2 localizes to membrane subdomains upon cell stimulation with P2X7R agonist

In P2X7R expressing cells, the prolonged increase in $[Ca^{2+}]_i$ upon BzATP application was followed within 30s by extensive cell blebbing as visualized by real-time microscopy (Fig. 3A, arrows). The term ‘blebbing’ is used here to describe formation of plasma membrane projections due to Rho-dependent actin reorganization that follow P2X7R activation (MacKenzie et al., 2001; Pfeiffer et al., 2004). This response is P2X specific. Stimulation of the parental cells that express P2Y receptors with ATP induced smaller, transient oscillations in $[Ca^{2+}]_i$ but no apparent morphological changes (Fig. 3A). This led us to speculate that TG2 externalization may be linked to membrane bleb formation, and we used GFP-tagged TG2 to monitor its redistribution in live cells. We confirmed that P2X7R activation triggered externalization of tagged TG2 similar to wild-type TG2 (Fig. 3B). Analysis by confocal microscopy revealed a clear ubiquitous cytoplasmic distribution for TG2-GFP (Fig. 3C). Upon P2X7R activation, TG2-GFP was rapidly translocating into membrane blebs, and freely relocalized to sites where new membrane protrusions formed (Fig. 3C, arrow). However, despite abundant bleb formation, careful reconstruction from image sequences revealed that these large membrane protrusions remained continuous with the plasma membrane and were eventually retracted by cells. We obtained similar results for N- and C-terminally tagged TG2

indicating that the position of the tag did not substantially alter protein localization. Although unable to directly visualize TG2 release, a noticeable reduction in fluorescence upon P2X7R activation indicated that the intracellular pool of TG2 was rapidly diminishing consistent with its relocation into the medium (Fig. 3B).

P2X7R agonist-induced TG2 secretion is independent of MV shedding

As small vesicles may be released by cells that are beyond the resolution of conventional confocal microscopy, we used light scattering combined with particle tracking to further analyse cell free supernatants for nanoparticles. A robust increase in particle shedding by P2X7R cells upon BzATP treatment was observed during stimulation and in the subsequent chase period (Fig. 4A). Most of the secreted particles had diameters of 81-262 nm (Fig. 4B) in line with more variably sized MV, rather than exosomes that originate from multivesicular bodies, are size-constrained and typically <90nm (Cocucci et al., 2009). TG2 expression modestly increased the proportion of larger particles (Fig. 4B) but did not significantly alter total particle release (Fig. 4A). To understand if TG2 localizes in MV, freshly harvested conditioned medium was subjected to differential centrifugation and resulting pellets and supernatant were analysed by Western blotting (Fig. 4C). TG2 mainly localized to the 100,000xg supernatant fraction containing soluble proteins (S5), with some TG2 found in very large aggregates or associated with organelles (P2) but not in the MV fraction (P4). To substantiate this, MV were separated using a sucrose density gradient (Fig. 4D). Again, TG2 was predominantly in the soluble protein fraction. These data suggest that while P2X7R activation induces abundant MV release by cells, secreted TG2 is not apparently MV-associated but present in free form.

Extracellular Ca²⁺ regulates TG2 externalization but its secretion is independent of catalytic enzyme functions

TG2 secretion was effectively stimulated by P2X7R activation in media that contain 0.9mM Ca²⁺, which is similar to the free ionized extracellular Ca²⁺ concentration estimated at 1.1-1.3mM (Riccardi and Kemp, 2012), but surprisingly not in media containing high Ca²⁺ (Fig. 1D & 5A). BzATP treatment of cells in the absence of Ca²⁺ lead to enhanced TG2 secretion during stimulation only (Fig. 5A), indicating that TG2 export was faster but not sustained. In contrast, flotillin-2 release occurring at 0.9mM Ca²⁺ was greatly reduced when cells were stimulated with agonist at either 0 or 2.2mM Ca²⁺ (Fig. 5A). This shows that TG2 and flotillin-2 secretion is differentially affected by [Ca²⁺]_{ex} and hence, that the underlying mechanisms are distinct. As MV shedding is a Ca²⁺-dependent process, TG2 release in Ca²⁺-free medium supports a vesicle-independent mode of release in line with previous data (Fig. 4).

To exclude Ca^{2+} -dependent loss of externalized TG2 due to cell surface retention through interaction with substrates or autocatalytic crosslinking, we compared secretion of wild-type TG2 with crosslinking incompetent TG2 $\text{C}^{277\text{S}}$ (Stephens et al., 2004). BzATP stimulation of cells induced export of both TG2 and TG2 $\text{C}^{277\text{S}}$ at 0.9mM Ca^{2+} but not at 2.2mM (Fig. 5B). This indicates that the lack of TG2 secretion at high $[\text{Ca}^{2+}]_{\text{ex}}$ is not due to TG2 activity but may reflect differences in P2X7R activation state. This is further supported by high $[\text{Ca}^{2+}]_{\text{ex}}$ affecting flotillin-2 release as well (Fig 5A). Besides cation transport, activation of P2X7R can lead to “membrane pore” formation which manifests as apparent permeability of the plasma membrane to cationic molecules such as YO-PRO1 (Virginio et al., 1999; Pelegrín, 2011; Browne et al., 2013). Measurement of YO-PRO1 uptake confirmed that P2X7R cells but not parental cells form membrane pores upon BzATP treatment (Fig. 5C), and that the dye uptake rate is inversely correlated to $[\text{Ca}^{2+}]_{\text{ex}}$ (Fig. 5D). Ca^{2+} -nucleotide interactions could potentially limit the effective agonist concentration. However, the observed BzATP dose response is not consistent with this explanation (Fig. S3C). Therefore, Ca^{2+} likely acts in our experiments as an allosteric regulator of P2X7R, either directly or indirectly inhibiting receptor activation as previously suggested (Yan et al., 2011). Taken together, this suggests that high $[\text{Ca}^{2+}]_{\text{ex}}$ is an important negative regulator of TG2 secretion, whereby Ca^{2+} ions appear to regulate P2X7R activation rather than influencing TG2 activity during export.

TG2 export is linked to P2X7R-mediated membrane pore formation

To assess the contribution of the initial ion flux on TG2 secretion, calmidazolium was employed. It is an inhibitor with broad selectivity for voltage gated fast acting Na^+/K^+ and L-type Ca^{2+} -channels that also inhibits the initial ATP-evoked ion flux through P2X7R without affecting the downstream membrane pore formation (Virginio et al., 1997). Calmidazolium has an extracellular mode of action on P2X7R. BzATP induced TG2 export in P2X7R cells was unaffected by the presence of calmidazolium but flotillin-2 secretion was blocked (Fig. 6A). The inhibitor had no effect on pore formation activity of P2X7R (Fig. 6B) but substantially reduced the rise in $[\text{Ca}^{2+}]_i$ mediated by P2X7R activation (Fig. 6C). This indicates that TG2 secretion is linked to P2X7R-dependent pore formation but not the initial ion flux and associated membrane depolarization.

Given that TG2 secretion was induced by P2X7R activation in Ca^{2+} -free medium but the kinetics of export were altered (Fig. 5A), we investigated whether Ca^{2+} release from intracellular stores plays a role. P2X7R cells were pre-loaded with Ca^{2+} chelator BAPTA-AM to buffer free cytosolic Ca^{2+} prior to BzATP stimulation in Ca^{2+} -free medium. This reduced TG2 release to near baseline levels (Fig. S3D), confirming that Ca^{2+} signaling has a role in TG2 export as previously

suggested (Zemskov et al., 2011). Conversely, cyclopiazonic acid (CPA) was applied to inhibit the SERCA Ca^{2+} transporter to trigger a rise in $[\text{Ca}^{2+}]_i$ in the absence of P2X7R activation. CPA addition alone was unable to induce TG2 secretion (Fig. S3D), despite inducing peak $[\text{Ca}^{2+}]_i$ of the same magnitude as P2X7R activation when used at 20 μM (Fig. S3E). This indicates that a rise in $[\text{Ca}^{2+}]_i$ by itself is not sufficient to induce TG2 export.

TG2 secretion is pannexin independent but enhanced by activating mutations in P2X7R

P2X7R-mediated membrane pore formation has been proposed to relate to P2X7R channel dilation upon saturation of ATP binding sites, possibly combined with acquisition of additional subunits (Browne et al., 2013) or alternatively, by coupling to another channel, *i.e.* pannexin-1 (Pelegriin and Suprenant, 2007; Gulbransen et al., 2012). We evaluated the latter by treating cells with pannexin inhibitors. Neither the peptidic competitor $^{10}\text{Panx}$ (Pelegriin and Suprenant, 2007) nor trovafloxacin (Poon et al., 2014) had any effect on BzATP stimulated YO-PRO1 uptake (Fig. 7A) or TG2 export. We therefore sought to clarify whether the C-terminally truncated P2X7R splice variant B that lacks pore forming ability (Adinolfi et al., 2010) supports TG2 secretion. However, expression of this variant after site-specific stable integration or transient transfection was very low as determined by Western blotting of cell lysates (Fig. 7B), and we were unable to confirm cell surface localization with P2X7R extracellular domain antibodies. Nevertheless, we attempted to confirm agonist-mediated membrane depolarization using the sensitive voltage sensing FRET probes CC2-DMPE and DiSBAC₂ (Wolff et al., 2003). Only wild type P2X7R expressing cells showed membrane channel activity (response ratio for P2X7R: $1.53 \pm 0.04^{\text{BzATP}}$, $2.17 \pm 0.12^{\text{KCl}}$, $1.07 \pm 0.06^{\text{control}}$; for P2X7R variant B: $1.10 \pm 0.03^{\text{BzATP}}$, $1.74 \pm 0.10^{\text{KCl}}$), suggesting altered trafficking and degradation of the truncated receptor variant.

A mutation in mouse P2X7R renders it deficient in pore forming activity (Sorge et al., 2012). As the affected sequence motif in the P2X7R C-terminal domain is conserved in human, we have generated cells expressing human P2X7R with an analogous mutation, P⁴⁵¹L (Fig. 7B,C). However, these cells formed membrane pores in response to BzATP as revealed by YO-PRO1 uptake (Fig. 7D). This led us to investigate the gain of function P2X7R variant, A³⁴⁸T, that confers increased risk for autoimmune disease in man (Stokes et al., 2010) to substantiate a link between pore formation and TG2 secretion. Cells expressing P2X7R A³⁴⁸T (Fig. 7B,C) had a substantially increased propensity to form membrane pores as evidenced by enhanced peak pore activity (Fig. 7D) and by pore formation at very low BzATP concentrations (Fig. 7E). This enhanced pore activity was reflected in a corresponding increase in TG2 export (Fig. 7F,G). Interestingly, we also observed BzATP-induced secretion of thioredoxin-1 (Fig. 7H), an enzyme that can re-activate

oxidatively inactivated TG2. This not only indicates that membrane pore activity controls the rate of TG2 export but that it leads to co-secretion of TG2 with thioredoxin-1 (Fig. 8).

Discussion

We identify P2X7R as the central regulator of the pathway that enables active export of TG2 and its co-activator, thioredoxin-1. The action of both of these enzymes has been linked to specific immune responses (Ismaa et al., 2009; Jaeger et al., 2013), and this may therefore constitute a pathway for export of proteins relevant to innate immunity. Besides having roles in re-instating tissue integrity following injury or associated with infection control, TGs including TG2 have been implicated in immune regulation (Toth et al., 2009; Loof et al., 2011). Here, we show that in monocytes/macrophages, purinergic signaling triggered rapid TG2 export in the absence of TLR engagement, and this response was P2X7R dependent but did not require caspase-1 activity. Likewise, introduction of P2X7R in HEK293 cells devoid of other inflammasome components (Lu et al., 2012) instated agonist-regulated rapid TG2 export. Taken together, the data demonstrate that P2X7R signaling alone is sufficient to trigger TG2 export, and involvement of an inflammasome-independent mechanism of export is further supported by the fact that externalized TG2 was not vesicle-associated or co-secreted with exosome-associated HMGB-1.

A redox sensitive Cys switch promotes oxidative inactivation of TG2 (Stamnaes et al., 2010), a mechanism that is thought to contribute to rapid enzyme inactivation in the extracellular milieu (Jin et al., 2011) and thereby, to prevent aberrant crosslinking that may lead to fibrosis and potentially autoimmunity via neo-epitope formation (Aeschlimann and Thomazy, 2000; Ismaa et al., 2009). However, it has been shown that extracellular matrix-associated TG2 can be reactivated by thioredoxin-1 released from activated monocytes in inflammation (Jin et al., 2011). Cell surface associated thioredoxin-1 plays a key role in innate immunity, particularly in mucosal epithelia where it activates β -defensin-1 (Jaeger et al., 2013). Interestingly, thioredoxin-1 is also an unconventionally secreted protein (Rubartelli et al., 1992), and our results show that it is in fact co-secreted with TG2. We speculate that thioredoxin-1 may not primarily act on pre-existing extracellular TG2 but may have a role as a chaperone during active TG2 export, enabling conversion of TG2 into its active conformation. Such a mechanism could explain why in celiac disease active TG2 accumulates in the intestinal mucosa (Korponay-Szabo et al., 2004).

Purinergic signaling fulfills the pre-requisites for a unifying pathway regulating TG export

As TG2, and also other TGs, can be externalized by a range of cells including myeloid, mesenchymal, endothelial and epithelial cells (Aeschlimann and Thomazy, 2000; Nurminskaya and Belkin, 2012), it is implausible that this involves multiple highly divergent mechanisms as proposed. P2X7R is not restricted to the hematopoietic lineage as originally thought but is also

widely distributed among mesenchymal, endothelial and epithelial cells, and in the central and peripheral nervous system (Bartlett et al., 2014). Activation of P2X7R occurs not only in conjunction with injury, cell stress and inflammatory processes but has major independent roles in the musculoskeletal (Garcia and Knight, 2010) and nervous system (Burnstock, 2015), contexts within which TG2-mediated extracellular reactions are also prevalent (Aeschlimann et al., 1995; Ismaa et al., 2009; Thomas et al., 2013).

Unlike previous work, our data implicate a regulated pathway in TG2 export. This mechanism may be activated to a varying extent under different conditions. A key finding here is that Ca^{2+} levels present in many media formulations impair TG2 release. Our data with catalytically inactive TG2 C²⁷⁷S show that this is not related to regulation of TG2 by Ca^{2+} but due to suppressed P2X7R functionality in line with evidence suggesting that divalent cations including Ca^{2+} allosterically inhibit P2X7R (Yan et al., 2011). Therefore, varying extracellular Ca^{2+} or ATP concentrations may explain some contradictory findings in the literature. It is worth noting that modest shear stress during medium exchange or passaging may trigger cellular ATP release (Rumney et al., 2012) and trigger P2X7R-mediated TG2 release at low (0-1mM) but not high ($\geq 2\text{mM}$) $[\text{Ca}^{2+}]_{\text{ex}}$. Hence, endogenous P2X7R activation may explain apparently “constitutive” TG2 secretion.

Crucially, in our HEK293 model TG2 is not retained at the cell surface or internalized, unless an appropriate cell surface receptor is introduced (Fig. S4). Hence, TG2 export can be directly assessed by quantification in the cell supernatant. Thus, our system is overcoming intrinsic difficulties that hampered progress in the analysis of TG2 export previously, including quantification of cell surface associated TG2 without disrupting cell integrity or endocytic TG2 uptake and retrograde transport. This together with modulation of the different P2X7R activities with small molecules or by mutagenesis provided strong evidence for a direct link between P2X7R signaling and TG2 export. Our data is not contradictory to passive TG2 release as a consequence of a substantial insult including mechanical damage (Upchurch et al., 1987) or TLR-engagement (Siegel et al., 2008), or to vesicle-associated TG2 release under circumstances such as serum starvation-associated cell stress (Antonyak et al., 2011). Rather, it suggests that purinergic signaling links controlled TG2 export to specific extracellular functions. Furthermore, given that MV-associated TG2 co-localized with fibronectin (Antonyak et al., 2011) an extracellular enzyme localization is implied. Therefore, it is possible that TG2 preferentially binds to plasma membrane subdomains where specific types of MV form (pericellular matrix reorganization) explaining the apparent association but that this occurs subsequent to membrane translocation.

Different activities of TG2 as well as sequence motifs for interaction with proteins and phospholipids were implicated in the export process (Balklava et al., 2002; Scarpellini et al., 2009; Chou et al., 2011; Zemskov et al., 2011). Our data show that transamidation activity is dispensable for export and that tagging TG2 with GFP does not prevent export, thereby excluding a terminal targeting signal. Blocking TG2 interaction with classically secreted proteins such as fibronectin, syndecans and integrins may alter extracellular localization or endocytic uptake and trafficking of TG2 (Antonyak et al., 2011; Chou et al., 2011; Zemskov et al., 2011) but cannot explain how the implied membrane translocation occurs.

Mechanistically, TG2 export is linked to the secondary permeability pathway

Several lines of evidence show that TG2 export is linked to the “membrane pore” activity associated with P2X7R activation (Fig. 8). Our data show that TG2 export is mechanistically separate from MV shedding. In line with this, P2X7R induces bleb formation and MV shedding through actin reorganization via MAPK p38 and Rho activation whereas YO-PRO1 uptake by cells is insensitive to cytochalasin-D (Pfeiffer et al., 2004). We further show that TG2 export is not induced by a $[Ca^{2+}]_i$ rise alone, nor abrogated by pharmacological suppression of P2X7R ion channel function without affecting membrane pore formation. In contrast, introducing a mutation in P2X7R that enhanced pore activity resulted in accelerated TG2 export.

P2X7R is the only P2X receptor where membrane pore formation is consistently observed and this activity is therefore a defining feature of it. While mechanistically not fully understood, it requires the extended unique C-terminal intracellular tail (Smart et al., 2003; Sun et al., 2013). Recent data suggest that large cations can pass through the P2X7R channel itself, and that blocking the channel prevents dye uptake by cells (Browne et al., 2013). However, a larger channel diameter than expected from available structural data (Hattori and Gouaux, 2012) would be required to adequately explain permeation of some molecules, and a more substantial conformational change than predicted from existing structural data is indeed supported by a recent study (Allsopp and Evans, 2015). Interestingly, P2X7R also couples to effectors implicated in dye permeability and a sustained $[Ca^{2+}]_i$ elevation by itself was shown to trigger membrane pore opening (Bartlett et al., 2014). In our HEK293 model, calmidazolium attenuated Ca^{2+} influx while membrane pore activity was unaffected suggesting that distinct permeation pathways are involved. Pannexin-1 is not involved as shown with inhibitors, consistent with data of others (Sun et al., 2013). Physiologically, the secondary permeability pathway may have a role in release of secondary messengers, *e.g.* glutamate release in P2X7R expressing HEK293 cells was reported (Cervetto et al., 2013). Given

the delay between P2X7R-dependent Ca^{2+} signaling and detection of changes in extracellular TG2, we cannot exclude a role of a secondary messenger system. However, it is conceivable that this pathway constitutes a pore through which proteins can be trafficked via co-translocational unfolding (Rodriguez-Larrea and Bayley, 2014).

TG2 activation is biological context-dependent

Given its requirement for high extracellular ATP concentration, P2X7R will primarily be activated after injury, in the context of inflammation, or in the tumor microenvironment. Enhanced TG2 expression by resident fibroblasts and infiltrating myeloid cells is an integral part of the tissue repair response and leads to accumulation of extracellular TG2. TG2 secretion is thought to bring about its activation via Ca^{2+} -induced conformational changes (Pinkas et al., 2007). However, it is possible that high extracellular nucleotide concentrations at sites of injury or inflammation not only activate P2X7R itself but also control TG2 activation as purine nucleotides are allosteric inhibitors, although the apparent binding affinity for ATP is low ($\sim 1\text{mM}$) compared to GTP ($\sim 3\mu\text{M}$) (Han et al., 2010; Thomas et al., 2013). Furthermore, a proposed heparan sulfate binding site is unique to the GTP-induced conformation (Lortat-Jacob et al., 2012) and such an interaction may stabilize this conformation and prevent Ca^{2+} binding. Therefore, it is worth noting that signaling functions for extracellular nucleotide bound enzyme have been postulated (Johnson and Terkeltaub, 2005; Toth et al., 2009).

Implications for TG2-mediated disease processes

P2X7R is highly polymorphic, and it has become increasingly clear that some amino acid substitutions predispose to disease (Bartlett et al., 2014). We have shown here that a polymorphism in the second transmembrane domain that is associated with autoimmune disease (Stokes et al., 2010) facilitates membrane pore formation leading to enhanced TG2 secretion. This opens the possibility that the threshold for activation of TG2 export differs between individuals depending on their *P2RX7* genotype, and this may constitute a risk factor for diseases where TG2 mediated reactions cause pathology. This extends to animal models of disease. Notably, in contrast to mouse strain 129, the C57BL/6 background widely used in genetic studies carries P2X7R P⁴⁵¹L which lacks the capacity to form membrane pores (Sorge et al., 2012). Different mouse lines may therefore differ with regards to the capacity for active TG2 export.

In conclusion, we have demonstrated that TG2 export is regulated by purinergic signaling, and that P2X7R plays a central role in this process. Our findings provide an explanation for the link between high levels of extracellular TG2 activity and inflammatory responses, and thereby identify

a new avenue to limit TG2 activity therapeutically in conditions where enzyme function directly drives pathogenic processes including fibrotic disease and gluten related disorders.

Materials and methods

Cell culture

THP-1 monocytic leukemia cells were grown in suspension in RPMI1640 medium containing 10% heat inactivated FBS, streptomycin, and penicillin. Mononuclear cells were isolated from heparinised human blood on Ficoll-Plaque PREMIUM (GE Healthcare), washed in PBS, and cultured for 7 days as THP-1 cells but with addition of 20ng/ml human GM-CSF (Preprotech) to derive M1 macrophages (with informed consent of donors and approval of the Research Ethics Committees: REC10/MRE09/28). HEK293 flp-in cells (Invitrogen) were cultured in DMEM containing 10% FBS, above antibiotics and 100µg/ml zeocin (Invitrogen). Experiments were conducted without antibiotics.

Generation of stably transfected cell lines

P2X7R was amplified by PCR from image clone (ID:4298811) using primers specified in Table S1 to generate wild-type, truncated and V5-tagged coding sequences, which were cloned into pcDNA5/V5-His/FRT vector (Invitrogen). Constructs for P2X7R mutants were generated by site directed mutagenesis using oligonucleotides given in Table S1. The coding sequence of all constructs was verified by sequencing. Cell lines were generated by co-transfection of P2X7R and recombinase (pOG44, Invitrogen) expression vectors into HEK293 flp-in cells using FuGENE 6 (Promega), followed by selection of stable transfectants with hygromycin B (100µg/ml).

THP-1 cell differentiation and activation

Cells were differentiated with 0.5µg/ml TPA, and for IL-1β upregulation, treated with 100ng/ml LPS for 24h. For activation, cells (1×10^6 /well) were suspended in PSS (10mM HEPES/NaOH, pH 7.4, 147mM NaCl, 2mM KCl, 1mM MgCl₂, indicated CaCl₂ concentration, and 12mM glucose) and ATP stimulated. Medium was carefully collected and rendered cell-free by centrifugation (1,500xg, 10min). Cells were extracted on ice in 20mM HEPES/NaOH, pH 7.4, 150mM NaCl, 1mM EGTA, 1% Triton-X100, 0.25% deoxycholate, 10% glycerol, 1mM PMSF and 1mM *N*-ethylmaleimide, and the extract cleared by centrifugation (15,000xg, 10min, 4°C). IL-1β concentration in conditioned media (100µl) was determined by capture ELISA (Ready-SET-Go Set, eBioscience).

Immunocytochemistry

Cells grown on poly-L-lysine coated coverslips were fixed with 2% paraformaldehyde/PBS for 10min, and permeabilized in 0.1% Triton-X100/PBS. After blocking of non-specific binding with 1% BSA/PBS, P2X7R was detected with 2 μ g/ml anti-P2X7R antibodies (sc-25698, Santa-Cruz) and Alexa Fluor 488-conjugated secondary antibodies. Coverslips were mounted using Vectashield containing DAPI.

[Ca²⁺]_i measurements in individual cells

Fluo-4-AM (Invitrogen) Ca²⁺ indicator was prepared in DMSO containing 20% Pluronic F-127. Cells (7x10⁴/well) in poly-L-lysine coated glass bottom dishes (50mm; MatTek) were loaded for 20min with 3 μ M Fluo-4-AM in OptiMEM (Invitrogen). Medium was replaced with fresh OptiMEM, and cells were monitored by confocal microscopy during ATP or BzATP (Sigma) stimulation at 37°C/5% CO₂. Real-time videos were acquired (2.62s/frame, 63x objective) using sequential scanning. For experiments with P2X7R antagonist, cells were Fluo-4-AM loaded in OptiMEM containing 5 μ M A740003 (Tocris) prior to stimulation with agonist in A740003-containing OptiMEM. Images were analyzed using the LAS-AF software (Leica).

Analysis of TG2 externalization

Differentiated THP-1 cells (1x10⁶/well) and primary macrophages (1x10⁵/well, 24-well plates) were stimulated with P2X7R agonists in OptiMEM. HEK293 P2X7R or parental cells (1.5x10⁵/well, 24-well plates) were transfected with 0.5 μ g expression construct for wild-type TG2 or TG2 C²⁷⁷S (Stephens et al., 2004), or GFP-tagged TG2 (Table S2) using FuGENE-6. After 48h, cells were washed with pre-warmed/gassed OptiMEM, and stimulated with P2X7R agonist or CPA (Merck-Millipore) in OptiMEM (250 μ l/well). For inhibitor studies, cells were treated with 5 μ M A740003, 1 μ M calmidazolium chloride (Merck-Millipore), 10 μ M BAPTA-AM (Merck-Millipore) or vehicle for 10min, and then BzATP stimulated in presence of respective inhibitors as indicated. Caspase-1 inhibitor Ac-YVAD-CMK was prepared fresh in OptiMEM and diluted to 100 μ M final concentration in experiments. Cell supernatant (pulse fraction) was collected, and cells washed with and subsequently incubated in pre-warmed/gassed OptiMEM without agonist for 30min (chase fraction). Conditioned media from four wells (six wells for macrophages) were combined, and rendered cell-free by centrifugation (1,500xg, 10min) for analysis. Cell surface protein labeling with Sulfo-NHS-SS-biotin and purification was carried out with the Pierce cell surface protein isolation kit.

Immunoblotting

Lyophilized (500 μ l) or ethanol precipitated (1.3ml, macrophages) conditioned media were reconstituted in 1/10th or 1/50th of original volume of 12.5mM Tris/HCl, pH 6.8, 4M urea, 2% SDS, 20mM EDTA, 2% β -mercaptoethanol, and 15% glycerol. Protein concentrations of extracts were determined with Bicinchoninic Acid Protein Assay. 20 μ l reconstituted media or 10 μ g cell extract together with Amersham LMW-SDS markers were separated on 4-20% SDS-PAGE Tris/glycine gels (Invitrogen) under reducing conditions, and transferred onto nitrocellulose membranes. For thioredoxin-1 detection, ethanol precipitated (1:9,v/v) proteins (600 μ l medium) were resuspended as above, and separated in 16% SDS-PAGE Tricine gels (Invitrogen) calibrated with Broad Range marker (11-190kD; NEB). Antibody labeling was performed as described (Aeschlimann et al., 1993) using monoclonal CUB7402 to TG2 (0.2 μ g/ml), TUB2.1 to β -tubulin (2.6 μ g/ml), to flotillin-2 (0.5 μ g/ml; 610383, BD-Biosciences), to HMGB-1 (0.73 μ g/ml; ab184203, Abcam), to V5-tag (20ng/ml) or polyclonal anti-I κ B α (1 μ g/ml; sc-371, Santa-Cruz), anti-caspase-3 (40ng/ml; 9662, Cell-Signaling), anti-P2X7R C-terminus (1 μ g/ml) or anti-P2X7R extracellular domain (1.7 μ g/ml; APR-008, Alomone Labs) antibodies. Anti-thioredoxin-1 antibodies (1:200; FL105, Santa-Cruz) were used with 5% casein as blocking agent. Bound antibodies were detected with HRP-conjugated secondary antibodies and Amersham ECLTM Plus/Prime. TG2 band intensity was quantified by densitometry using Image Lab 5.1 software (Bio-Rad).

Analysis of cell damage and apoptosis

To assess cell integrity, LDH release was measured using CytoTox-ONETM HMI Assay (Promega). Cells (1.2x10⁵/well, 24-well plate) were BzATP-treated in 300 μ l OptiMEM for 10min (n=4), and cell-free conditioned media (100 μ l) analyzed for LDH. For estimation of total LDH, a replicate well set was subjected to cell lysis.

To assess whether treatment induced cell death, P2X7R cells were BzATP stimulated and chased as described, and where indicated subsequently cultured in serum containing DMEM for up to 22h. Cell extracts and particulate material recovered from conditioned media were analysed for caspase-3 by immunoblotting.

Localization of GFP-tagged TG2 using confocal microscopy

P2X7R cells on poly-L-lysine coated coverslips were transfected with constructs for expression of N- or C-terminally GFP-tagged TG2. After 24h, the coverslip was mounted for microscopy into a customized holder using silicone grease. Cells were kept in OptiMEM at 37°C/5% CO₂, and stimulated with a defined volume of agonist solution to obtain 1mM ATP or 100 μ M BzATP while

monitoring GFP fluorescence and acquiring real-time movies.

Detection and isolation of MV

TG2 or mock transfected P2X7R cells were stimulated with 100 μ M BzATP or vehicle. Freshly collected conditioned media were rendered cell-free by centrifugation (1,500 \times g, 10min) and supernatants analysed for MV by particle tracking using the NanoSight LM12 system with a high sensitivity camera (Webber and Clayton, 2013). Five 60s videos per sample (1-5 \times 10⁸ particles/ml) were recorded at 25.6 frames/s (gain=250), and analysed using the NTA2.3 software. Alternatively, freshly collected conditioned media were subjected to differential centrifugation at 4°C, with 1,500 \times g for 10min, followed by 3,000 \times g for 20min, and then either 10,000 \times g for 30min and 100,000 \times g for 1h or subjected to density gradient centrifugation. Supernatant (1.0 ml) was carefully layered on Tris-buffered sucrose step gradient (0/20/60%) and centrifuged at 100,000 \times g for 90min. Fractions (~1ml) constituting top layer and 20/60% interface (MV fraction) as well as pellet were collected. Proteins were precipitated with 9 volumes of ethanol at 4°C, and analysed using immunoblotting.

P2X7R “membrane pore” activity

Cells in poly-L-lysine coated black optical 96-well plates (Nunc, 165305) were placed in PSS containing 0-2mM Ca²⁺ and 1 μ M YO-PRO1 (Invitrogen). The plate was transferred to a FLUOstar Omega reader (BMG Labtech) equilibrated to 37°C/5% CO₂. BzATP was injected to obtain 0-500 μ M final concentration (n=3) and fluorescence measured (4mm orbital area) every 40s for 30min. Where indicated, cells were preincubated with 100 μ M ¹⁰Panx (Tocris) for 10min or 10-100 μ g/ml trovafloxacin (Sigma) for 30min and stimulated in the presence of inhibitors. After normalization for well-specific fluorescence, average YO-PRO1 fluorescence of unstimulated cells was subtracted from that of agonist stimulated cells to correct for bleaching. Dye uptake rates were derived by linear regression of data from initial 5min.

[Ca²⁺]_i measurements in plate format

Cells (3 \times 10⁴/well) in optical 96-well plates were loaded with Fluo-4-AM, washed, and placed in fresh OptiMEM (90 μ l/well). After measuring baseline fluorescence, different concentrations of BzATP (0-300 μ M) or medium alone were injected (10 μ l/well). Fluorescence changes were measured in well mode over 20s, with 40 0.1s intervals followed by 0.4s intervals. Data from eight wells per condition were averaged. Fluorescence of control was subtracted from data with agonist treatment to correct for bleaching. Data (F =fluorescence, t =time) for the first 10s were fitted using equation 1 to estimate the maximal fluorescence value (F_{max}):

$$F = \frac{1 - e^{-(A - F_{max})kt}}{F_{max} - \frac{1}{A}e^{-(A - F_{max})kt}} + C \quad (1)$$

whereby k is the association constant, A is a function of agonist concentration and C is a constant for baseline correction. The association constant obtained from data fitting was $k=1.8 \times 10^{-6} \text{M}^{-1} \text{s}^{-1}$. F_{max} was then plotted against the agonist concentration to derive a dose-response curve.

Membrane potential analysis

Voltage sensor probes, coumarin-labeled phospholipid CC2-DMPE (FRET donor) and oxonol dye DiSBAC₂(3) (acceptor), were from Invitrogen. Cells (3×10^4 /well) in optical 96-well plates were loaded with $10 \mu\text{M}$ CC2-DMPE in FRET buffer (10mM Hepes/NaOH, pH 7.4, 160mM NaCl, 0.9mM CaCl₂, 1mM MgCl₂, and 10mM glucose) containing $200 \mu\text{g/ml}$ Pluronic F-127 for 30min, washed, and incubated in $100 \mu\text{l}$ $10 \mu\text{M}$ DiSBAC₂(3) in FRET buffer for 20min. 10min after addition of tartrazine (1.2mM), fluorescence measurements ($\lambda_{\text{Ex}}=420\text{-}10\text{nm}$, $\lambda_{\text{Em}}=460\text{-}10\text{nm}/550\text{-}10\text{nm}$) were conducted in well mode at $37^\circ\text{C}/5\% \text{CO}_2$. Gain was adjusted to yield similar baseline readings for each fluorophor at resting potential. Following baseline acquisition, $10 \mu\text{l}$ 0.82M KCl, $100 \mu\text{M}$ BzATP, or buffer control were injected while monitoring fluorescence. Following subtraction of signal without cells, the signal ratio (SR) before and at equilibrium after depolarization was calculated, and the response ratio (RR) derived: $\text{RR} = \text{SR}^{\text{depol}} / \text{SR}^{\text{pol}}$.

Statistics

One-way ANOVA was used and significance between groups determined with Tukey post-test, whereby $p < 0.05$ was considered significant.

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Author contributions

DA conceived the study. MA, RG, SD and VK carried out the experiments. MA and DA analyzed all the experiments. MA and DA wrote the manuscript with input from all authors.

Conflict of interests

Authors declare no conflict of interest.

Abbreviations list

Ac-YVAD-CMK, *N*-acetyl-YVAD-chloromethyl ketone;
BAPTA-AM, (1,2-*bis*(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid) acetoxymethylester;
BzATP, benzoylbenzoyl-ATP;
CPA, cyclopiazonic acid;
Fluo-4-AM, Fluo-4 acetoxymethylester;
HMGB-1, high-mobility group box protein-1;
I κ B α , nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor alpha;
IL, interleukin;
LDH, lactate dehydrogenase;
LPS, lipopolysaccharide;
MV, microvesicles;
NALP, NACHT domain-, leucine-rich repeat-, and PYD-containing protein;
P2X, purinergic receptor (ion channel);
P2X7R, P2 receptor X7;
P2Y, purinergic receptor (G-protein coupled);
PSS, physiological salt solution;
SERCA, Sarco(Endo)plasmic reticulum Ca²⁺ ATPase;
TG, transglutaminase;
TLR, Toll-like receptor;
TPA, 12-*o*-tetradecanoyl-phorbol-13-acetate.

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Figures

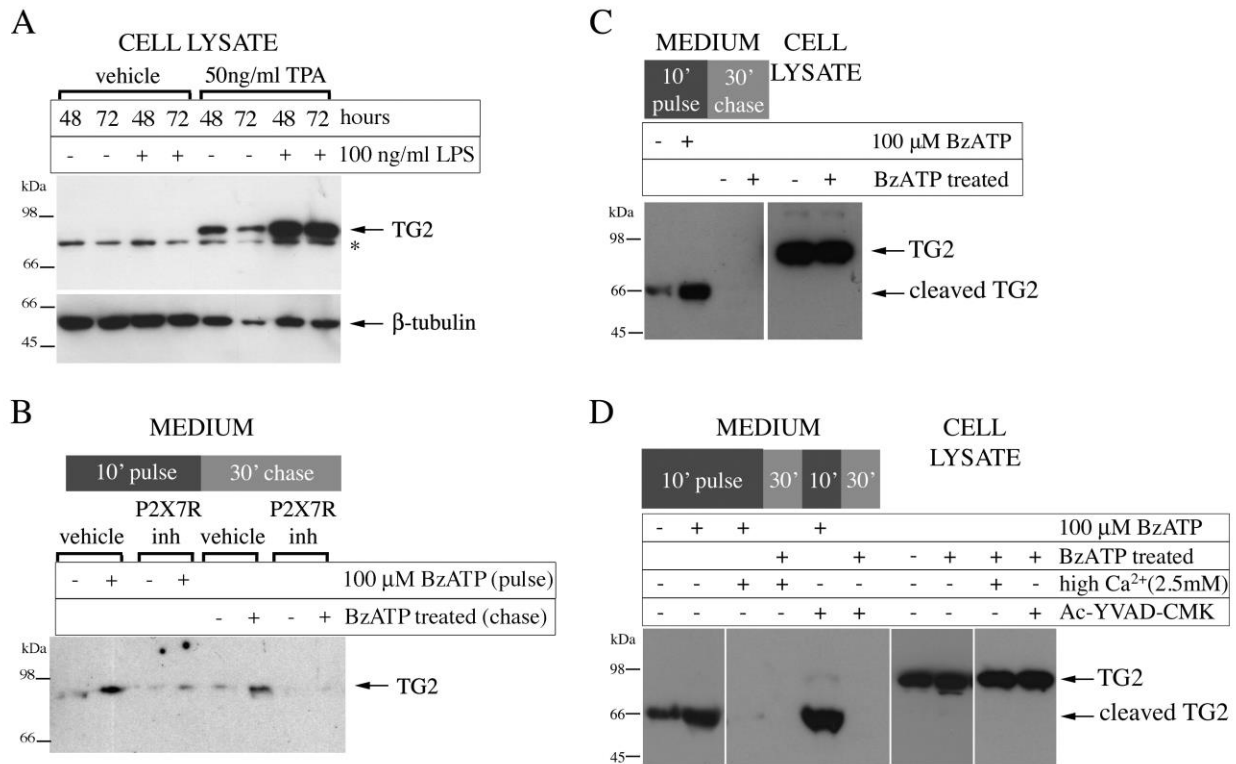


Fig. 1 P2X7R inhibition blocks TG2 secretion by macrophages.

(A) Differentiated monocytes express TG2. THP-1 cells were differentiated for indicated time with TPA and stimulated with LPS as indicated. Cell extracts were analysed by Western blotting for TG2, or β -tubulin as a loading control (*=non-specific reactivity).

(B) TG2 export requires P2X7R activity. Differentiated THP-1 cells were pre-treated with vehicle or 5 μ M P2X7R inhibitor A740003 for 10min, then stimulated as indicated with BzATP for 10min with or without inhibitor (pulse). Cells were chased for 30min in P2X7R agonist/antagonist-free medium. Collected media of pulse and chase (200 μ l) were rendered cell-free by centrifugation, and analyzed for TG2 by Western blotting.

(C,D) P2X7R activation triggers TG2 secretion in peripheral blood mononuclear cell-derived macrophages. Macrophages were BzATP stimulated and chased as in B, and collected media of pulse and chase analyzed for TG2 by Western blotting alongside the cell lysates (C). The presence of 100 μ M Ac-YVAD-CMK did not prevent externalization or cleavage of TG2, indicating a caspase-1 independent process (D).

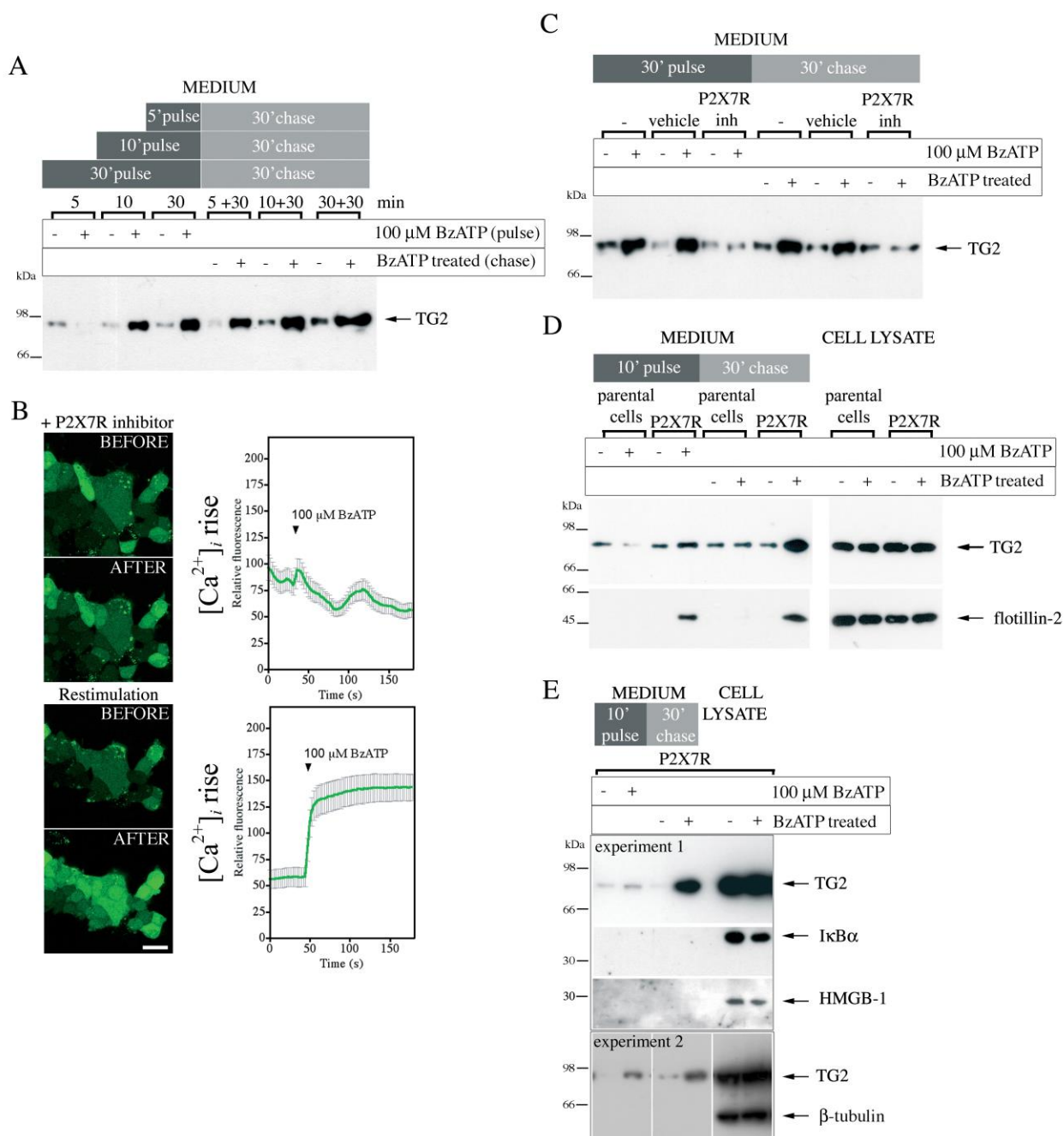


Fig. 2 P2X7R activation mediates TG2 externalization.

(A) Analysis of TG2 secretion in HEK293 P2X7R cells. TG2 transfected cells were stimulated with BzATP or vehicle for indicated time (pulse), then incubated for 30min in agonist-free medium (chase). TG2 secretion into cell-free supernatants was assessed by Western blotting.

(B) Inhibitor A740003 reversibly blocks P2X7R activation. P2X7R cells were incubated with Fluo-4-AM and 5 μ M P2X7R inhibitor for 20min prior to BzATP stimulation in the presence of inhibitor

(top), washed with inhibitor-free medium for 5min, and then re-stimulated with BzATP (bottom). Fluorescence ($\lambda_{\text{Ex}}=488\text{nm}$, $\lambda_{\text{Em}}=500\text{-}535\text{nm}$) change in individual cells was monitored by confocal microscopy (mean \pm s.e.m., n=30) (right). Optical sections of the same field before and 180s after BzATP addition are shown (left). Bar=25 μm .

(C) P2X7R inhibitor blocks TG2 secretion. TG2 transfected P2X7R cells were pre-treated with P2X7R inhibitor or vehicle for 10 min before BzATP stimulation as indicated. TG2 release into media was assessed as in *A*.

(D,E) Cells release membrane-bound particles upon P2X7R activation. TG2 transfected P2X7R or parental cells were BzATP stimulated for 10min, and chased in agonist-free medium. Conditioned media and cell lysate were analysed by Western blotting for TG2 and the MV marker flotillin-2 (D) or as a control, β -tubulin, I κ B α and HMGB-1 (E).

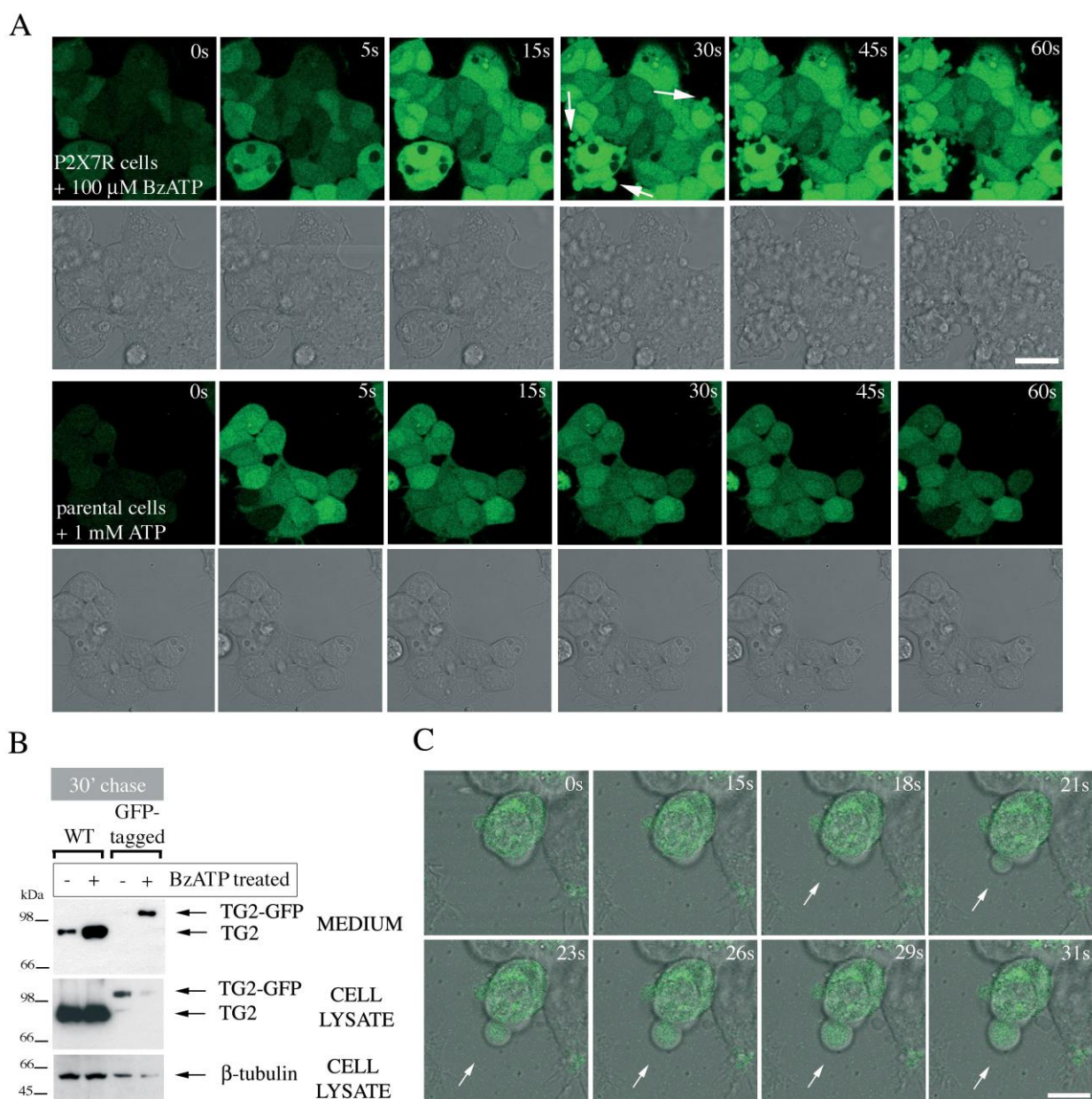


Fig. 3 Membrane blebs induced by P2X7R activation contain TG2.

(A) P2X7R signaling induces rapid membrane blebbing. Fluo-4-AM loaded P2X7R cells were stimulated with BzATP while acquiring fluorescence and phase contrast images by real-time microscopy to visualize morphological changes and Ca^{2+} signaling simultaneously (top). Membrane blebs are indicated by arrows. ATP stimulation of parental cells induces oscillating Ca^{2+} signals but no overt morphological changes (bottom). Bar=25 μm .

(B,C) TG2 redistributes into membrane blebs. To confirm export of tagged TG2, TG2 (WT) or TG2-GFP expressing P2X7R cells were stimulated with 100 μM BzATP for 10min, chased for

30min in agonist-free medium, followed by analysis of conditioned media and cell extracts for TG2 by Western blotting (B). To localize GFP-tagged TG2 during BzATP stimulation, real-time confocal microscopy was employed. Genesis of a membrane bleb is depicted (arrow), with an optical section of GFP fluorescence overlaid onto phase contrast images to correlate morphological changes with changes in TG2 distribution (C). Bar=25 μ m.

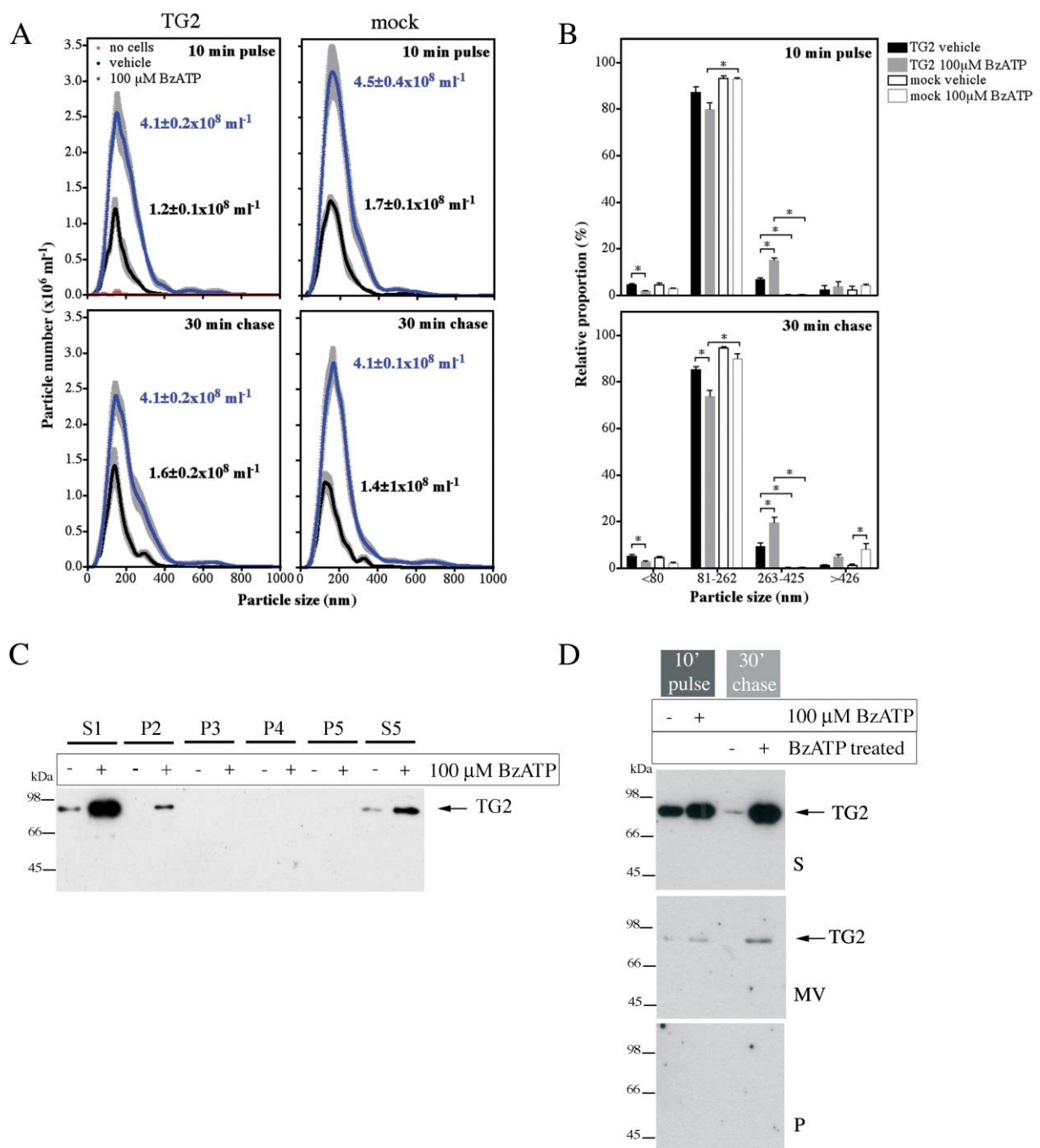


Fig. 4 P2X7R-mediated TG2 export is not due to MV release.

(A,B) Analysis of vesicle release by nanoparticle tracking. TG2 or mock transfected P2X7R cells were BzATP stimulated for 10min, chased for 30min in agonist-free medium, and conditioned media analysed for nanoparticles using light scattering in combination with particle tracking (Nanosight). Particle distribution and total particle concentration is shown (mean±s.e.m.; n=5) (A). Particles were broadly assigned to 4 fractions based on volume: representing exosomes (~60nm;

≤ 80 nm diameter), MV (~ 145 nm; 81-262nm), larger vesicles (~ 335 nm; 263-425nm) and aggregates/membrane blebs (≥ 426 nm) (B).

(C,D) Analysis of isolated MV for TG2. Cell-free media (S1) of BzATP or control treated cells were subjected to differential centrifugation (P=pellet; S=supernatant): in *C*, 3000xg twice (P2, P3), 10,000xg (P4), and 100,000xg (P5, S5) and in *D*, 3,000xg followed by separation of MV on a sucrose cushion, and fractions analysed for TG2 by Western blotting.

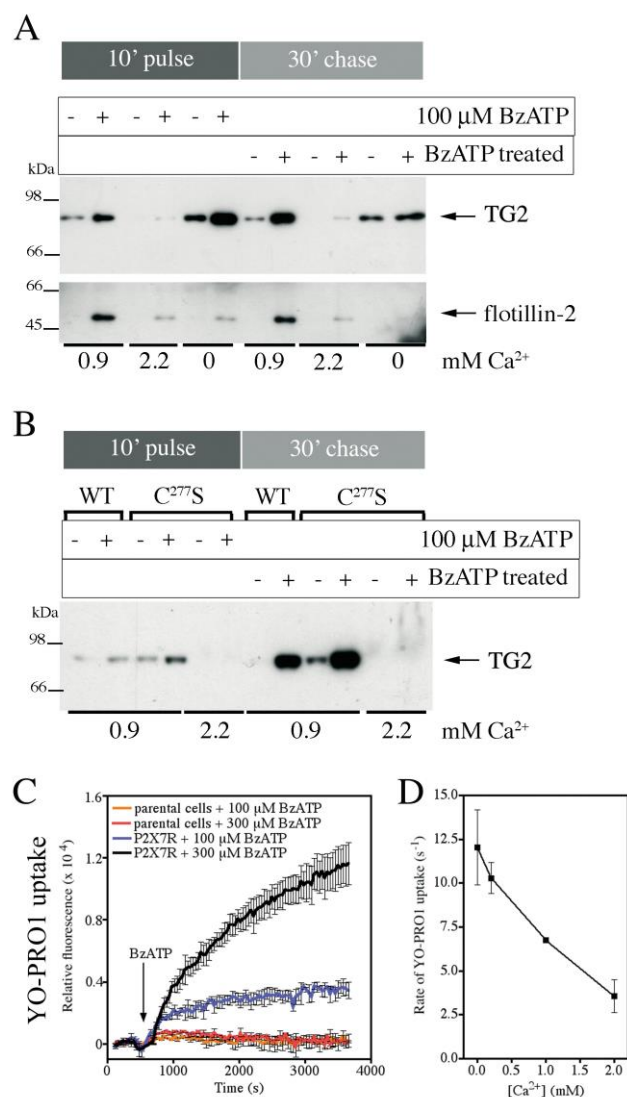


Fig. 5 Extracellular Ca^{2+} regulates TG2 secretion.

(A) P2X7R-mediated TG2 export at different $[\text{Ca}^{2+}]_{\text{ex}}$. P2X7R cells expressing TG2 were BzATP stimulated for 10min in medium containing 0.9 or 2.2mM Ca^{2+} or in Ca^{2+} -free medium, and chased for 30min in respective media without BzATP. Conditioned media were analyzed by Western blotting for TG2 and flotillin-2.

(B) TG2 catalytic activity is not required for P2X7R-mediated export. P2X7R cells expressing TG2 or TG2 $\text{C}^{277\text{S}}$ mutant were BzATP stimulated in medium containing 0.9 or 2.2mM Ca^{2+} and TG2 export was assessed as above.

(C,D) $[\text{Ca}^{2+}]_{\text{ex}}$ regulates P2X7R activity. P2X7R or parental cells were stimulated with BzATP as indicated in PSS containing YO-PRO1 and different concentrations of Ca^{2+} . To determine YO-

PRO1 uptake by cells after BzATP application, changes in well-specific fluorescence ($\lambda_{\text{EX}}=480\text{-}10\text{nm}$, $\lambda_{\text{EM}}=520\text{-}10\text{nm}$) were monitored over time. A representative experiment of dye uptake in Ca^{2+} -free PSS is shown as mean \pm s.e.m. of 2 wells (C). In *D*, the initial rates of YO-PRO1 uptake at different $[\text{Ca}^{2+}]_{\text{ex}}$ in response to $300\mu\text{M}$ BzATP are given (mean \pm s.e.m.; $n=2$).

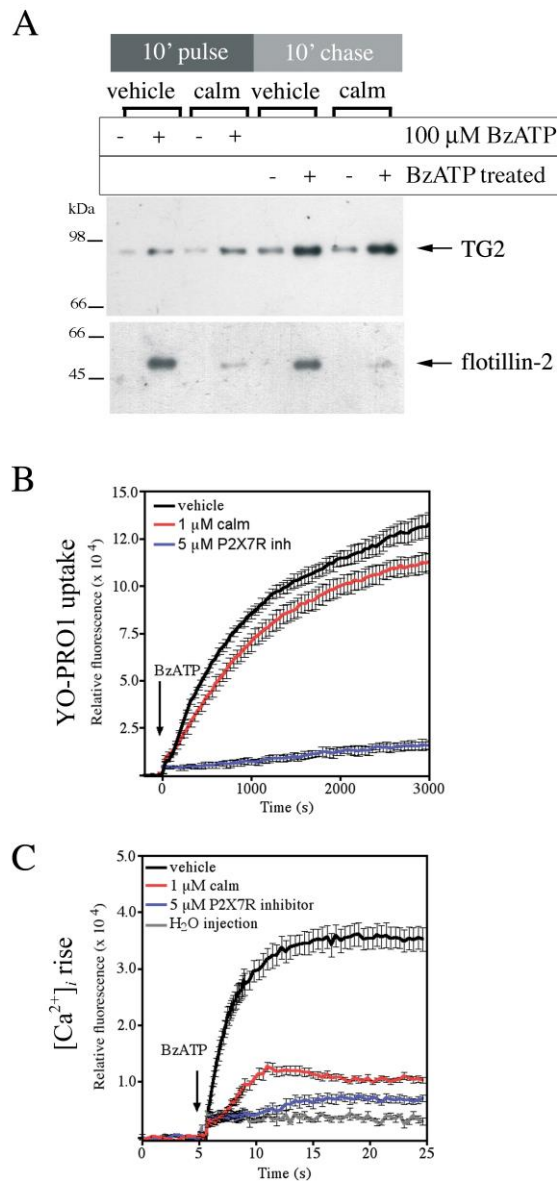


Fig. 6 TG2 export is independent of K^+ efflux and membrane depolarization.

(A) Calmidazolium (calm) blocks flotillin-2 but not TG2 release. TG2 transfected P2X7R cells were pre-treated for 10min and then BzATP stimulated in medium containing 1 μ M calm or vehicle. Cells were chased in agonist-free medium, and conditioned media analyzed by Western blotting for TG2 and flotillin-2.

(B) Calm does not affect P2X7R-dependent “membrane pore” formation. P2X7R cells were pre-treated with calm, P2X7R inhibitor A740003 or vehicle for 10min prior to stimulation with 100 μ M BzATP in the presence of respective inhibitors or carrier in PSS containing YO-PRO1 and 0.9mM

Ca²⁺. Dye uptake was monitored over time. Results are shown as mean±s.e.m. of 2 wells, and is representative of 3 independent experiments.

(C) Calm ameliorates large rise in [Ca²⁺]_i. Fluo-4-AM loaded P2X7R cells were pre-treated with calm, P2X7R inhibitor or vehicle for 20min prior to stimulation with 100μM BzATP in the presence of inhibitors or carrier. Fluorescence change ($\lambda_{Ex}=485-12\text{nm}$, $\lambda_{Em}=520-10\text{nm}$) relative to control in response to agonist treatment was monitored (mean±s.e.m. of 8 replicate wells).

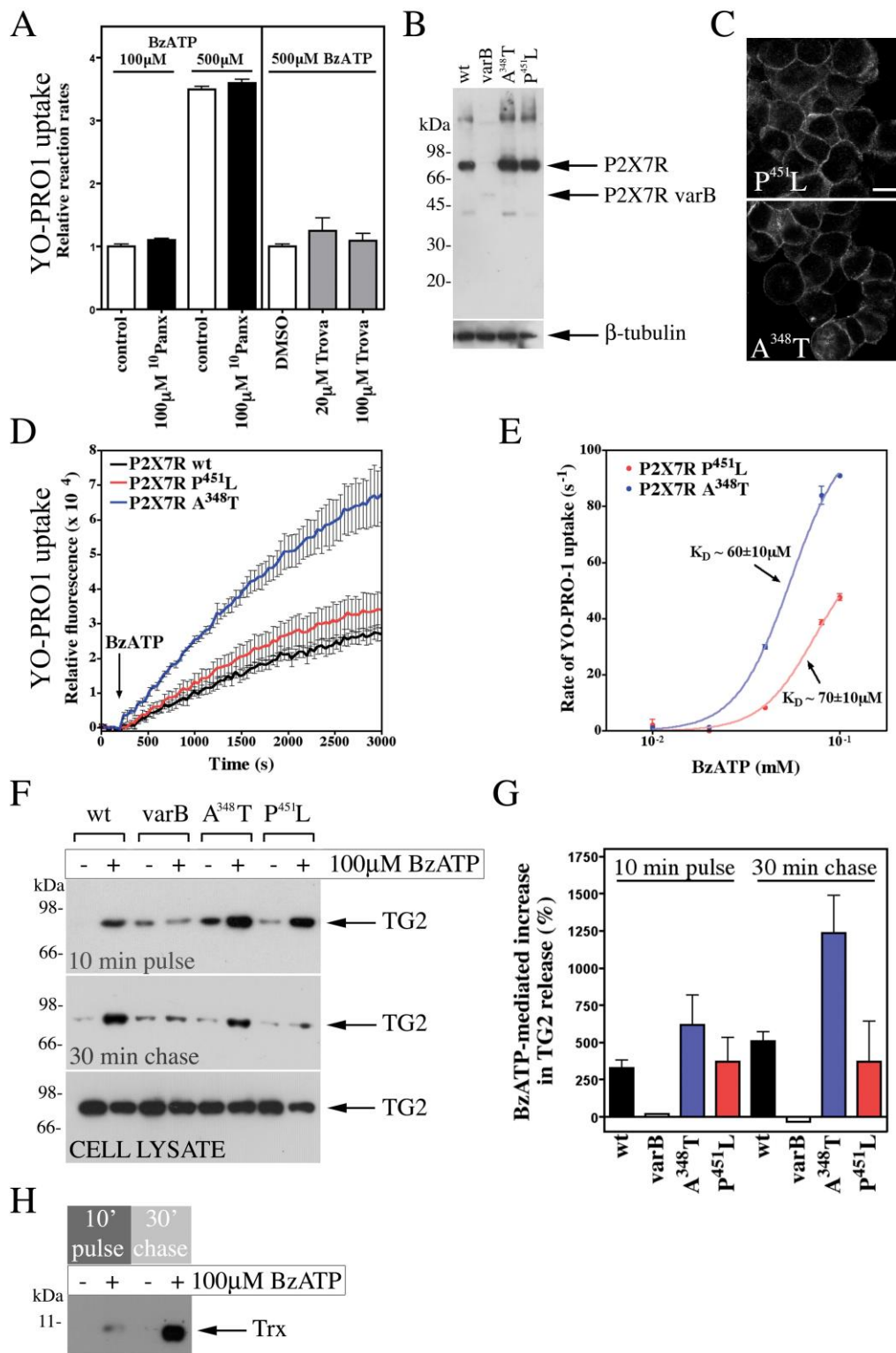


Fig. 7 P2X7R-mediated membrane pore formation is required for TG2 externalization.

(A) P2X7R-mediated pore formation is pannexin independent. P2X7R cells were pre-treated with 10 Panx or trovafloxacin (Trova) as indicated, and then BzATP stimulated in PSS with respective

inhibitors, YO-PRO1 and 0.9mM Ca²⁺. Results are given as initial rates of dye uptake relative to control. Pannexin inhibitors did not affect dye uptake, neither at limiting nor saturating agonist concentration.

(B,C) Characterization of expression of mutant P2X7Rs. Extracts of cells stably expressing wildtype (wt), A³⁴⁸T or P⁴⁵¹L P2X7R, or P2X7R variant B (varB) were analysed by Western blotting with antibodies to P2X7R extracellular domain, and to β -tubulin as a loading control (B). Membrane localization of receptor was confirmed by immunocytochemistry (C; compare to Fig. S2B). Images reflect an optical section acquired by confocal microscopy. Bar=12.5 μ m.

(D,E) Pore formation is enhanced in cells expressing P2X7R A³⁴⁸T. YO-PRO1 uptake following stimulation of cells with 100 μ M BzATP is shown as mean fluorescence \pm s.e.m. (D). Comparison of initial rate of YO-PRO1 uptake for P2X7R A³⁴⁸T and P⁴⁵¹L expressing cells highlights increased pore activity for P2X7R A³⁴⁸T but unchanged ligand regulation (E).

(F-H) TG2 export correlates with receptor pore activity. TG2 transfected cells expressing P2X7R variants were BzATP stimulated for 10 min, and chased in agonist-free medium. Conditioned media were analyzed by Western blotting for TG2 (F), and results (n=3) quantified by densitometry (G). Note, cell lysates confirm comparable TG2 expression levels in different cell lines (F). For thioredoxin-1 (Trx) detection, media (P2X7R cells) were analysed by Western blotting after separation in 16% SDS-PAGE Tricine gels (H).

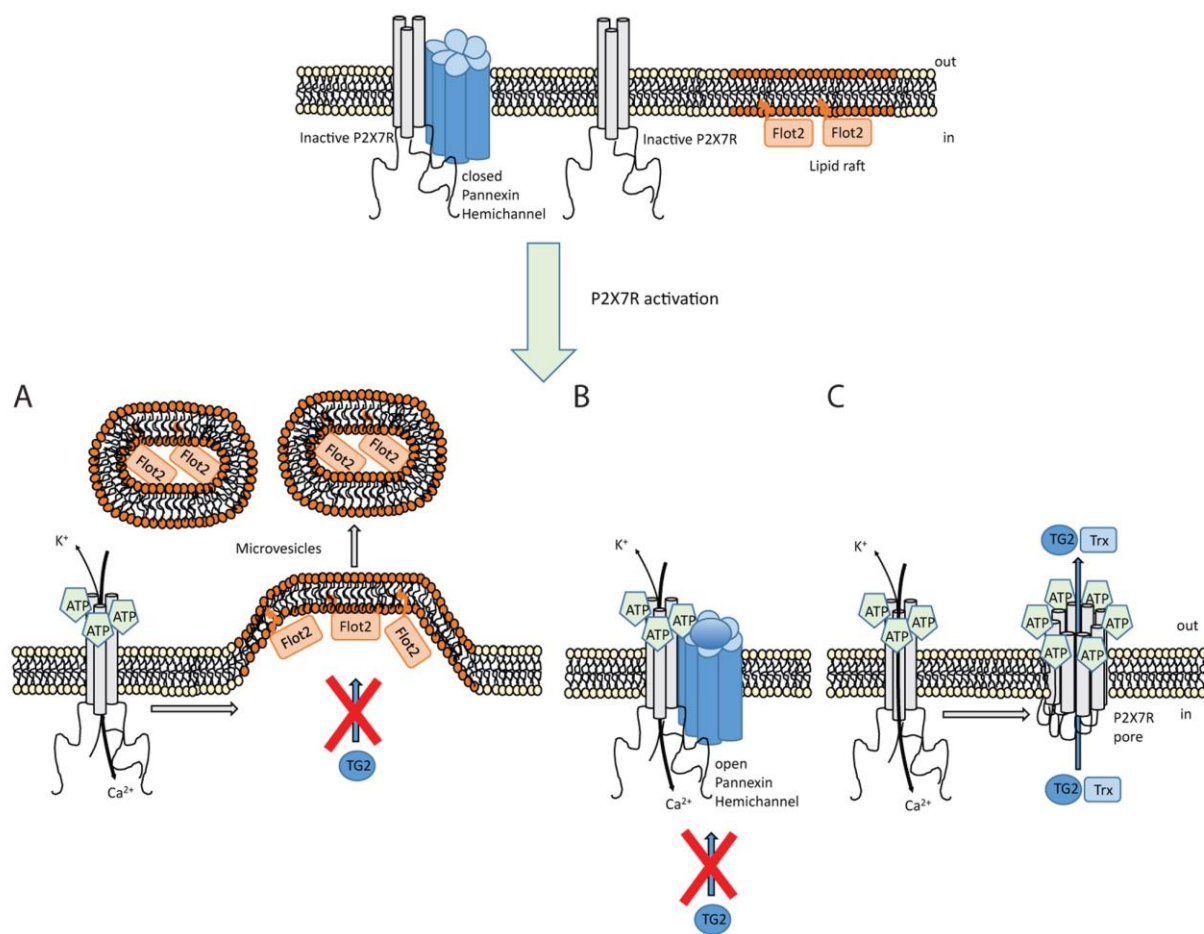


Fig. 8 Mechanism controlling TG2 export.

Schematic showing different events occurring upon P2X7R activation by ATP. (A) Ion channel activity triggers intracellular signaling that results in actin reorganization and MV shedding. However, these MV do not contain TG2. (B) Coupling between P2X7R and pannexin-1 triggers hemichannel pore opening. TG2 secretion was unaffected by blocking pannexin-1 channels. (C) P2X7R itself can form a membrane pore through conformational changes and possibly receptor oligomerization in a process that involves the extended intracellular C-terminal sequence. TG2 secretion is associated with this membrane pore activity but independent of ion channel function, and occurs in conjunction with thioredoxin-1 externalization. As thioredoxin can reactivate TG2 functionally blocked in an oxidized state, this may ensure that externalized TG2 has transamidation activity.