Constitutive lysosome exocytosis releases ATP and engages P2Y receptors in human monocytes

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Summary

Elucidating mechanisms by which Ca^{2+} signals are generated by monocytes is important for understanding monocyte function in health and disease. We have investigated mechanisms underlying Ca^{2+} signals generated following disruption of lysosomes by exposure to the cathepsin C substrate glycyl-L-phenylalanine-β-napthylamide (GPN). Exposure to 0.2 mM GPN resulted in robust increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the absence of extracellular Ca^{2+}. The response was antagonised by thapsigargin and evoked capacitative Ca^{2+} entry. Dantrolene-sensitive Ca^{2+} responses were observed at higher concentrations of GPN (0.4 mM) but not at 0.2 mM. Strikingly, GPN-evoked Ca^{2+} responses and β-hexosaminidase secretion were inhibited by the ATPase/ADPase apyrase. Simultaneous measurement of $[Ca^{2+}]_i$ and extracellular ATP revealed a concomitant secretion of ATP during GPN-evoked Ca^{2+} signalling. Furthermore, the ability of GPN to raise $[Ca^{2+}]_i$ was inhibited by P2Y receptor antagonists or by inhibiting vesicular exocytosis with *N*-ethylmaleimide (NEM). NEM treatment was associated with an inability of GPN to trigger ATP secretion, a drop in baseline $[Ca^{2+}]_i$ and reduction in extracellular ATP concentration. Antagonism of purinergic signalling also caused a reduction in baseline $[Ca^{2+}]_i$. In summary, these data suggest that P2Y receptor activation contributes significantly to GPN-evoked Ca^{2+} signalling, and that constitutive secretion of lysosomal ATP is a major determinant of Ca^{2+} homeostasis in monocytes. Lysosomal Ca^{2+} stores can communicate with ER Ca^{2+} stores either directly through activation of ryanodine receptors, or indirectly through release of ATP and engagement of P2Y receptors.

Key words: ATP, Monocyte, Purinergic signalling, Calcium, Exocytosis

Introduction

Monocytes play an important role in immune defence and the development of chronic inflammatory diseases including atherosclerosis. Changes in cytosolic calcium concentration ($[Ca^{2+}]_i$) constitute a critical requirement for monocyte function including response to pro-inflammatory molecules including chemokines (Gouwy et al., 2008), bacterial lipopolysaccharide (McLeish et al., 1989), chemotaxis (Badolato et al., 1995), phagocytosis (Hishikawa et al., 1991) and cytokine production (MacIntyre et al., 1991). Monocyte calcium dynamics are altered in pathological conditions including sepsis (Shin et al., 1997), *Leishmania* infection (Olivier et al., 1992) and foam cell formation in atherosclerosis (Deng et al., 2005). Understanding mechanisms by which calcium signals are generated and shaped is therefore important for understanding how monocytes function in health and disease.

In resting cells $[Ca^{2+}]_i$ is tightly buffered (typically 100 nM), but it can rise dramatically in response to extracellular cues including hormones and stressors (Berridge et al., 2000). When a calcium signal is generated, calcium enters the cytoplasm through plasma membrane entry mechanisms including translocators (i.e. Na^+/Ca^{2+} exchange) and capacitative Ca^{2+}-entry mechanisms, and/or through release of calcium stored within intracellular compartments (Pozzan et al., 1994; Clapham, 2007). The archetypal calcium store in animal cells is the endoplasmic reticulum (ER). Calcium is pumped into the ER by the thapsigargin-sensitive Ca^{2+}-ATPase SERCA and released through activation of two distinct receptor classes located on the store: (i) inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] receptors and (ii) ryanodine receptors (RyR). Ins(1,4,5)P_3 formation and mediated calcium release are linked to the activation of G-protein-coupled receptors (GPCRs; specifically G_q), conveying extracellular signals from stressors and hormones (Berridge et al., 2000). Ryanodine receptors are widely expressed in muscle and non-muscle cell types, including leukocytes (Hosoi et al., 2001), are activated by increased $[Ca2+]_i$ and facilitate calcium-induced calcium release of the ER store. Activation of ER ryanodine receptors can aid amplification of Ca^{2+} signals generated by the ER but also other potential stores.

It has long been recognised that calcium is stored in acidic organelles distinct from the classical pool of ER calcium. Acidic stores include lysosomes (Lloyd-Evans et al., 2008; Churchill et al., 2002), secretory lysosomes (Holmsen and Weiss, 1979), acidocalcisomes (Luo et al., 2004) and vacuoles (Patel and Docampo, 2010). Moreover, recent studies have demonstrated that such pools are also mobilisable (Churchill et al., 2002; Lange et al., 2009). Leukocyte lysosomes play a key role in immunity and disease (Holt et al., 2006) by inactivating pathogenic organisms, processing antigens for presentation (Mullins and Bonifacino, 2001) and secreting pro-inflammatory molecules (Andrei et al., 2004). Lysosomal calcium stores have been identified in diverse cell types including endothelium (Srinivas et al., 2002), epithelium (Haller et al., 1996), macrophage (Christensen et al., 2002), mesenchymal stem cells (Lee et al.,

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2010) and vascular smooth muscle (Zhang et al., 2010), though their existence in monocytes remains elusive. In this study we sought evidence for functional lysosomal calcium stores in human monocytic cells, their interaction with the ER calcium pool, and explore their contribution to calcium homeostasis and monocyte function. We employ the use of glycyl-L-phenylalanine-β-napthylamide (GPN), a cathepsin C substrate, used extensively to selectively disrupt lysosomes in various cell types (Berg et al., 1994; Haller et al., 1996; Srinivas et al., 2002; Dickinson et al., 2010).

Results

Interaction between GPN-sensitive and thapsigargin-sensitive calcium stores in monocytes

The lysosomotropic agent glycyl-L-phenylalanine-β-napthylamide (GPN) is suggested to raise cytoplasmic $[Ca^{2+}]_i$ by stimulating the osmotic lysis of lysosomes and subsequent release of their Ca^{2+} content. In THP-1 monocytes, application of GPN evoked a transient elevation in $[Ca^{2+}]_i$ which decayed to baseline over several minutes in the absence of extracellular calcium (Fig. 1A). GPN treatment resulted in a loss of Lysotracker staining in pre-stained cells, suggesting GPN is capable of disrupting lysosomes in THP-1 monocytes as observed in other cell types (Fig. 1B,D). Application of the ER calcium store inhibitor thapsigargin also evoked an increase in $[Ca^{2+}]_i$ which was more sustained and approximately 3-fold greater in magnitude to the GPN-evoked response (Fig. 1C,D). Despite the larger magnitude of change in cytosolic calcium evoked by thapsigargin the initial rate of change was greater with GPN, 0.5±0.2 and 1.8±0.3 Ca^{2+} F(%) (n=4-15; P<0.05) for thapsigargin and GPN, respectively. Thapsigargin was also capable of discharging Lysotracker staining (Fig. 1B). GPN was unable to increase calcium following thapsigargin treatment (Fig. 1C,D), suggesting an interaction between ER and lysosomal compartments in monocytes. The interaction could be explained if a direct interaction existed whereby GPN required ER calcium stores to raise $[Ca^{2+}]_i$. To test this, we explored the ability of GPN to evoke a 'calcium add-back' response, characteristic of ER calcium store depletion and capacitive calcium entry at the plasma membrane, observed in other leukocytes. 0.2 mM GPN raised $[Ca^{2+}]_i$ in the absence of extracellular calcium, addition of 1.5 mM $CaCl_2$ induced a large Ca^{2+} influx (Fig. 1E) which was not observed in the absence of GPN (data not shown). This data suggests GPN does cause release of calcium stored within the ER. One possible explanation for this observation is that the osmotic lysis and subsequent release of lysosomal calcium induced by GPN, could trigger release of ER calcium via a calcium-induced calcium release mechanism. We employed the ryanodine receptor antagonist dantrolene to inhibit calcium-induced calcium release. At concentrations of GPN used throughout this study and by others to disrupt lysosomes i.e. 0.2 mM, dantrolene had no effect on the ability of GPN to raise $[Ca^{2+}]_i$. However, increasing GPN to 0.4 mM evoked a more substantial increase in $[Ca^{2+}]_i$ (Fig. 1F,G) which was inhibited by dantrolene (Fig. 1F), suggesting the increase in $[Ca^{2+}]_i$ evoked by 0.4 mM but not 0.2 mM GPN can trigger ryanodine receptor activation and release of ER calcium. Moreover, this data suggests 0.2 mM GPN releases ER calcium via a mechanism other than calcium-induced calcium release. This data suggests that depletion of the ER can disrupt lysosomes and therefore can explain why GPN-evoked increase in $[Ca^{2+}]_i$ is abolished with thapsigargin pre-treatment (Fig. 1C).

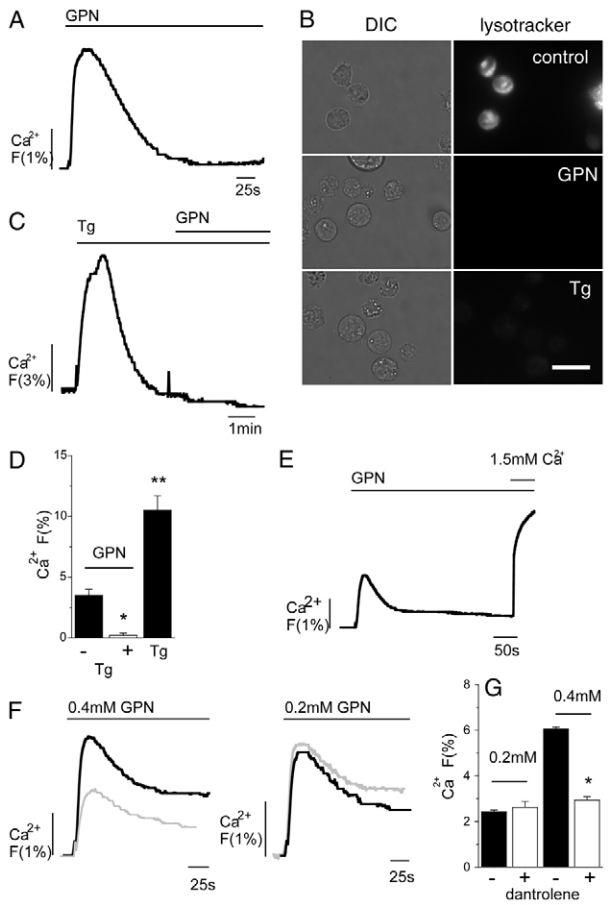

Fig. 1. Interaction between lysosomal and ER calcium stores in human monocytes. (**A**) The lysosomotropic agent GPN transiently mobilises $[Ca^{2+}]_i$ in the absence of external Ca^{2+}, measured in Fluo-4 loaded THP-1 cells (n=15). (**B**) Lysotracker staining (100 nM, 20 mins) of lysosomes can be discharged following exposure to GPN or thapsigargin (Tg; 5 μM; n=4). Scale bar, 20 μm. (**C**) Thapsigargin-evoked $[Ca^{2+}]_i$ mobilisation abolishes subsequent Ca^{2+} response to GPN (n=5). (**D**) Comparison of peak Ca^{2+} responses to GPN in the presence and absence of thapsigargin (5 μM), or to thapsigargin alone (n=5). (**E**) GPN evokes a 'calcium add-back response' following addition of 1.5 mM external $CaCl_2$ after GPN (n=4). (**F**) A higher concentration of GPN (0.4 vs 0.2 mM) evokes an enhanced peak Ca^{2+} response through engagement of ryanodine receptors. Response to 0.2 and 0.4 mM GPN are shown in control cells (black line) and in cells treated with dantrolene (20 μM, 30 mins; grey line). (**G**) The effect of the different concentrations of dantrolene on GPN-evoked Ca^{2+} peak calcium response (n=5; *P<0.01). All experiments are performed in the absence of external Ca^{2+} unless otherwise stated. GPN was used at 0.2 mM unless stated otherwise.

GPN and thapsigargin triggers lysosomes exocytosis in monocytes

As exocytosis of lysosomes has been shown to be stimulated by an increase in cytoplasmic $[Ca^{2+}]_i$ in other leukocytes (Colvin et al., 2010; Chen et al., 2010), we hypothesised that an interaction between the ER and lysosomes could exist in monocytes whereby ER derived calcium promoted exocytosis of lysosomes. Moreover, this could account for the loss of ability

of GPN to raise $[Ca^{2+}]_i$ following thapsigargin treatment (Fig. 1C), as intact lysosomes have already been discharged. To examine lysosome exocytosis, we assayed the extracellular release of the lysosomal marker enzyme β-hexosaminidase. Application of thapsigargin evoked a time-dependent increase in extracellular β-hexosaminidase (Fig. 2A), demonstrating that the release of ER calcium can promote lysosome secretion. Furthermore, GPN also promoted substantial β-hexosaminidase release (Fig. 2B). The rate of GPN triggered β-hexosaminidase secretion was much faster than that of thapsigargin (Fig. 2A,B), which may relate to the quicker calcium rise evoked by GPN with respect to thapsigargin (Fig. 1A,C). Interestingly, GPN-induced β-hexosaminidase secretion was abolished by pre-incubation with NEM (Fig. 2C), suggesting that the NEM-sensitive factor (NSF) and vesicle exocytosis is required for GPN and thapsigargin to trigger β-hexosaminidase secretion. Microscopic examination of monocytes revealed dramatic ruffling of the plasma membrane upon addition of GPN (Fig. 2D), characteristic of exocytosis described in other cell types (Benninger et al., 2009). We next tested the dependency of GPN-evoked calcium response on NEM. The increase in $[Ca^{2+}]_i$ in response to thapsigargin was unaffected by NEM treatment (data not shown), though strikingly the Ca^{2+} response to GPN was abolished (Fig. 2E,F). This data demonstrate that the observed GPN-evoked increase in $[Ca^{2+}]_i$ and β-hexosaminidase secretion are both dependent upon lysosome exocytosis. The calcium response evoked by 0.4 mM GPN was less sensitive to NEM (Fig. 2F). This partial sensitivity is consistent with a calcium rise independent of exocytosis and with the dantrolene sensitivity of the response to 0.4 mM GPN (Fig. 1G). Next we sought to test the dependency of GPN triggered β-hexosaminidase secretion on changes in calcium by buffering cytosolic calcium with BAPTA-AM (50 μM, 30 mins). Incubation of cells with BAPTA-AM abolished the ability of GPN to trigger β-hexosaminidase secretion (Fig. 2G).

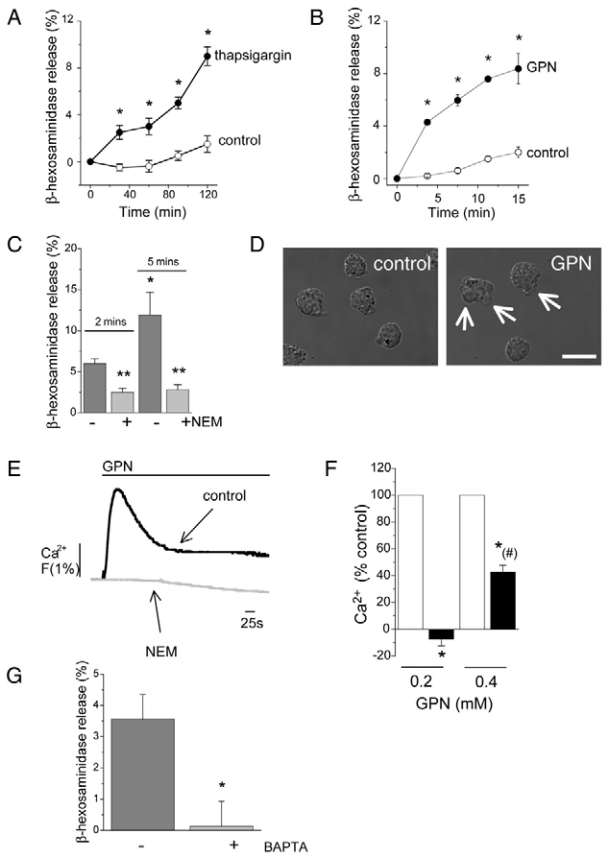

Fig. 2. GPN-evoked Ca^{2+} responses are dependent upon lysosome exocytosis. Thapsigargin (5 μM; **A**) and GPN (0.2 mM, **B**) stimulate release of lysosomal enzyme β-hexosaminidase in THP-1 monocytes (n=5; *P<0.01). Control responses are with DMSO vehicle. (**C**) Inhibition of vesicular exocytosis by *N*-ethylmaleimide (1 mM, 15 mins; NEM) inhibits time-dependent GPN-evoked β-hexosaminidase release (n=6; *P<0.05; **P<0.01). (**D**) GPN (0.2 mM, 5 mins) stimulates membrane protrusions and reorganisation (arrows). Scale bar: 20 μm. (**E**,**F**) NEM pre-treatment (1 mM, 15 mins) abolishes 0.2 mM GPN-evoked Ca^{2+} responses (n=5; *P<0.01 vs control) but not response to 0.4 mM GPN (n=3; #P<0.01 vs 0.2 mM GPN with NEM). White bars, control; black bars, NEM. (**G**) β-hexosaminidase release 2 mins after GPN (0.2 mM) application with and without cytosolic Ca^{2+} buffering with BAPTA-AM (50 mM, 30 mins; n=4; *P<0.01).

GPN-evoked Ca^{2+} response is dependent upon secreted ATP

As exocytosis of lysosomes is associated with release of ATP in other cell types including neuronal astrocytes (Zhang et al., 2007), we sought to determine whether the Ca^{2+} response to GPN was dependent upon extracellular ATP. In paired experiments were $[Ca^{2+}]_i$ and the extracellular ATP concentration ($[ATP]_{ext}$) were sampled simultaneously, it is evident that a release of ATP is associated with the GPN-evoked Ca^{2+} response (Fig. 3A). At rest, a basal $[ATP]_{ext}$ of approximately 35 nM was detectable, which rose to about 100 nM at the peak of the GPN-evoked Ca^{2+} response (Fig. 3B). The GPN-evoked increase in $[ATP]_{ext}$ was abolished following treatment with BAPTA-AM or apyrase (Fig. 3B). BAPTA-AM treatment also reduced $[ATP]_{ext}$ in the absence of GPN (Fig. 3B). As observed for GPN-evoked β-hexosaminidase secretion and $[Ca^{2+}]_i$ increase, ATP secretion was also inhibited by NEM (Fig. 3C). NEM had no direct effect itself on the luciferase-luciferin assay or β-hexosaminidase secretion assays used in this study (supplementary material Fig. S1). In further experiments we tested whether scavenging the GPN-evoked released ATP impacted on the Ca^{2+} response. Surprisingly treatment with apyrase inhibited the GPN-evoked Ca^{2+} response by over 80% (Fig. 3D). This effect was not observed following heat-inactivation of apyrase, suggesting ATP/ADP hydrolase activity is required for the inhibitory action of apyrase (Fig. 3D,E). These data suggest that the GPN-evoked release of ATP mediates the GPN-evoked Ca^{2+} increase. MTT cell viability assays where performed to exclude GPN-induced cell lysis as an alternative route of ATP release (Fig. 3F).

GPN requires engagement of P2Y receptors to elevate $[Ca^{2+}]_i$

Previous studies have demonstrated that monocytes abundantly express ATP-activated purinergic receptors (Moore et al., 2001). As the GPN-evoked rise in $[ATP]_{ext}$ is insufficient to activate P2X receptors (North, 2002; Fountain and North, 2006), we sought to test an interaction between GPN and P2Y receptor activation. ATP, ADP, UTP and UDP all stimulated significant increases in $[Ca^{2+}]_i$ (Fig. 4A,B). All responses were inhibited by pre-incubation with thapsigargin or the phospholipase C inhibitor U-73122 (Fig. 4C). These data suggest that P2Y-receptor-mediated responses are completely dependent upon Ins(1,4,5)P_3

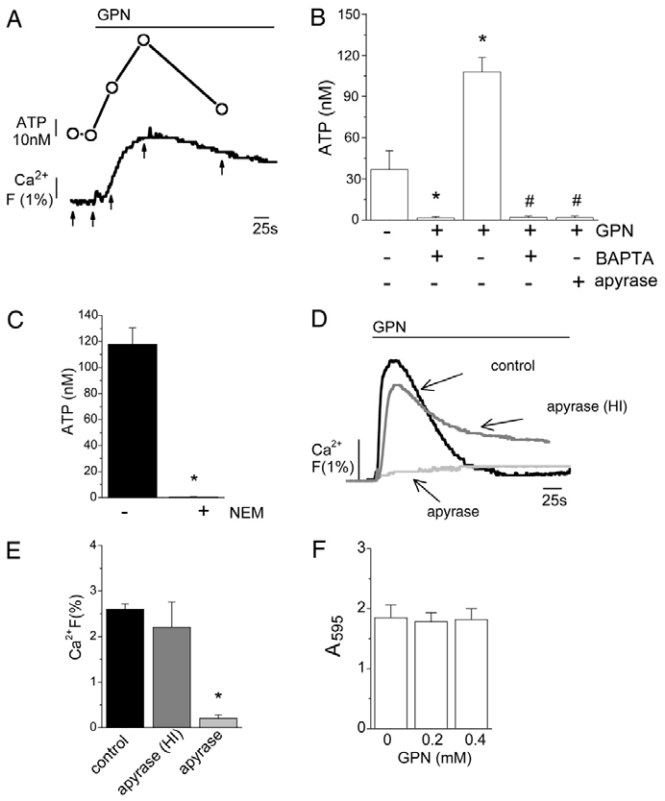

Fig. 3. GPN-evoked Ca^{2+} responses couple to and are dependent upon vesicular secreted ATP. (**A**) Simultaneous measurement of GPN-evoked Ca^{2+} response and extracellular ATP concentration. Arrow denotes sampling points for ATP measurement. (**B**) Summarised extracellular ATP concentration in unstimulated and GPN-stimulated THP-1 monocytes. Addition of BAPTA-AM (50 μM, 30 mins) or apyrase (4 IU/ml) abolishes GPN-evoked external ATP increase (n=4-6; *P<0.01 test vs control; #P<0.01 test vs GPN alone). (**C**) Inhibition of vesicular exocytosis by NEM (1 mM, 15 mins) abolishes GPN-evoked ATP secretion (n=5; *P<0.01). (**D**) Scavenging extracellular ATP with apyrase (4 IU/ml) attenuates GPN-evoked Ca^{2+} responses. Heat-inactivated (HI) apyrase is ineffective (n=5). (**E**) Average peak Ca^{2+} responses evoked by GPN with and without ATP scavenging (n=5; *P<0.01). (**F**) MTT cell viability assay. MTT absorbance at 595 nm of control monocytes or monocytes exposed to 0.2 or 0.4 mM GPN for 30 mins (n=3; P>0.05).

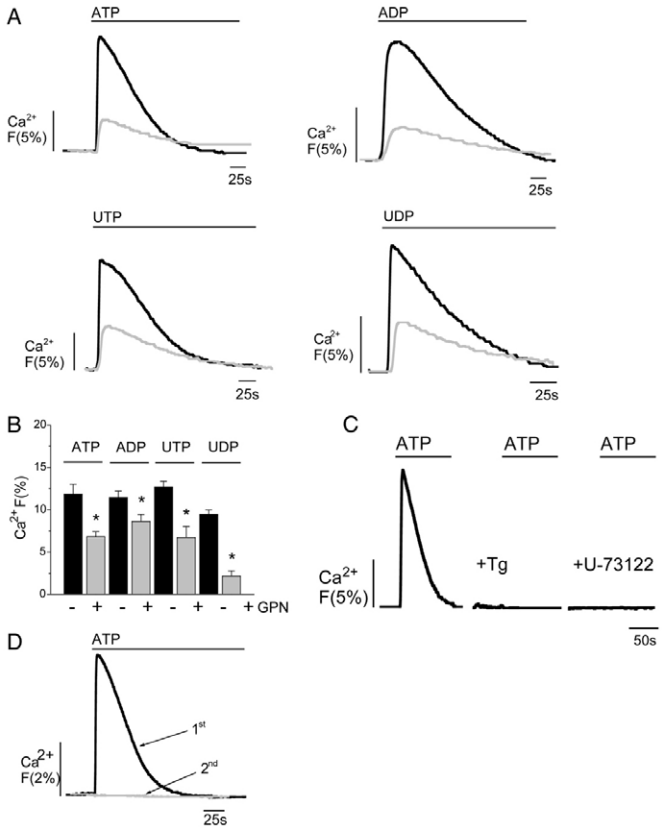

Fig. 4. P2Y-receptor-mediated Ca^{2+} responses are attenuated by GPN treatment. (**A**) Ca^{2+} responses evoked by 30 μM nucleotides (ATP, ADP, UTP or UDP) with (grey line) or without (black line) GPN pre-incubation (0.2 mM, 15 mins). (**B**) Average peak Ca^{2+} response (n=6; *P<0.05). (**C**) Inhibition of ATP-evoked Ca^{2+} response by pre-incubation with thapsigargin (5 μM, 15 mins) and or U-73122 (10 μM, 15 mins) demonstrates dependency of ATP response on phospholipase C activity and ER calcium mobilisation (n=4). (**D**) Desensitisation of ATP-evoked Ca^{2+} responses by repeated ATP application. Response to 30 μM ATP with 10 min intervals between applications. All experiments are performed in the absence of external Ca^{2+}.

generation and release of ER calcium. Despite this, GPN pre-treatment was able to inhibit nucleotide-evoked Ca^{2+} responses in THP-1 monocytes (Fig. 4A,B). This is unlikely to represent a dependency upon release of stored lysosomal calcium as the response is entirely dependent upon ER calcium release (Fig. 4C). Rather this is likely to be due to GPN-evoked ATP release (Fig. 3A) causing a desensitisation of subsequent nucleotide responses. Indeed, we observed that the response to GPN requires engagement of P2Y receptors, as antagonising P2Y receptors with suramin or MRS-2578 significantly impairs GPN-evoked $[Ca^{2+}]_i$ (Fig. 5), and Ca^{2+} responses to exogenous ATP display marked desensitisation (Fig. 4D). Consistent with results given in Fig. 1G and Fig. 2F, the calcium response to 0.4 mM GPN is less sensitive to P2 receptor antagonists (Fig. 5C). This further demonstrates that the additional calcium responses evoked by 0.4 mM GPN is independent upon exocytosis and P2Y receptor engagement but dependent upon direct release of ER calcium via ryanodine receptors.

Constitutive release of ATP and P2Y receptor activation control Ca^{2+} homeostasis in monocytes

Our prior observations demonstrate that apyrase inhibits the GPN-induced increase in $[ATP]_{ext}$ (Fig. 3D). However, we also observe that apyrase treatment significantly reduces baseline $[ATP]_{ext}$ (Fig. 3B). In paired experiments where baseline $[Ca^{2+}]_i$ and $[ATP]_{ext}$ are measured simultaneously, application of NEM reduced both $[Ca^{2+}]_i$ and $[ATP]_{ext}$ (Fig. 6A). These data suggest inhibition of the exocytotic release of ATP is a determinant of baseline $[Ca^{2+}]_i$. To test this further, we examined the effect of apyrase and purinergic receptor antagonists on resting $[Ca^{2+}]_i$. Application of apyrase (Fig. 6C), but not heat-inactivated apyrase (Fig. 6D), caused a reduction in resting $[Ca^{2+}]_i$, as did the broad-spectrum P2 receptor antagonist suramin (Fig. 6E). The selective $P2Y_{11}$ antagonist NF380 also reduced baseline $[Ca^{2+}]_i$ (Fig. 6F), though the selective $P2Y_6$ antagonist MRS2578 caused a small transient increase in calcium which returned to a pre-application baseline (data not shown). Interestingly, the P1 adenosine receptor antagonist CGS15943 elevated resting $[Ca^{2+}]_i$, suggesting tonic P1 receptor activation negatively regulates resting $[Ca^{2+}]_i$ in monocytes (Fig. 6G). In a concluding series of experiments we

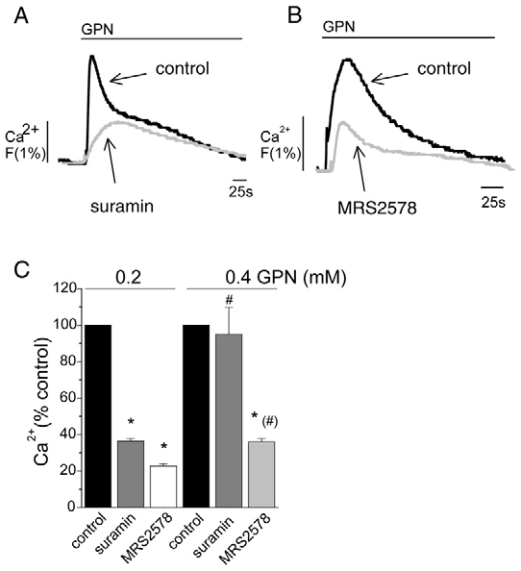

Fig. 5. GPN-evoked Ca^{2+} responses require engagement of purinergic receptors. (**A**,**B**) Effect of P2Y receptor antagonists suramin (100 μM; A) and MRS2578 (1 μM; B) on GPN-evoked Ca^{2+} responses in THP-1 monocytes. (**C**) Sensitivity of calcium responses to P2 receptor antagonists when exposed to 0.2 mM and 0.4 mM GPN (n=3-5; *P<0.05 vs control; #P<0.05 vs 0.2 mM GPN equivalent. Average peak Ca^{2+} responses in control conditions and in the presence of antagonist (n=5; *P<0.05).

sought to examine whether disrupting lysosomes with GPN could impact on monocyte function in vitro. In Transwell migration assays, THP-1 monocytes migrated toward 1 μM ATP (Fig. 7). Migration was inhibited by suramin (Fig. 7), suggesting engagement of P2 receptors is required for ATP-evoked migration. Importantly, GPN abolished migration (Fig. 7), suggesting disruption of lysosomes impairs the ability of monocytes to migrate towards ATP. GPN, apyrase or P2 receptor antagonists had no effect on basal cell migration (Fig. 7C). Monocytes also migrated towards serum yet this was not inhibited by GPN or apyrase (Fig. 7D).

Discussion

GPN-induced Ca^{2+} mobilisation

Exposure of human monocytes to the cathepsin C (dipeptidyl peptidase I) (Muno et al., 1993) substrate GPN at low millimolar concentrations caused robust elevation in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. GPN is a useful pharmacological tool for the selective disruption of lysosomes, capable of distinguishing lysosomes from endosomes and autophagosomes (Berg et al., 1994). The proposed mechanism of action of GPN is osmotic lysis of lysosomes attributed to increased luminal tonicity following GPN hydrolysis by cathepsin C (Srinivas et al., 2002), consequently released stored Ca^{2+} into the cytoplasm. Our data are constituent with the notion that GPN disrupts lysosomes, as Lysotracker staining in monocytes is discharged following only short exposures to GPN (Fig. 1B). However, it is clear from our current study that GPN also disrupts lysosomal Ca^{2+} pools by triggering their exocytosis as GPN-evoked β-hexosaminidase secretion is attenuated following inhibition of vesicular exocytosis by *N*-ethylmaleimide (Fig. 2C). Experiments with BAPTA-AM demonstrate that exocytosis is dependent upon a rise in intracellular calcium (Fig. 2G, Fig. 3B).

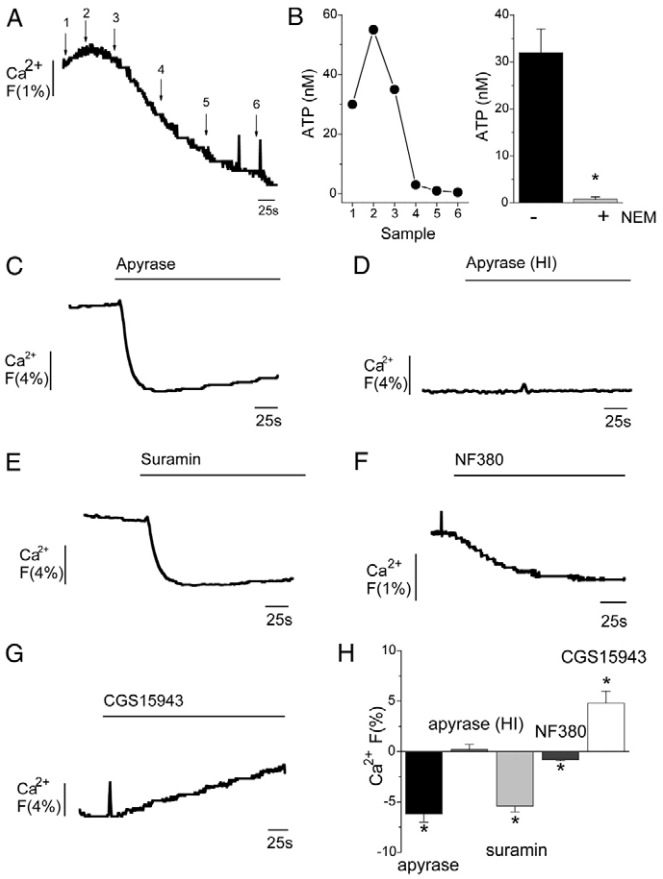

Fig. 6. Constitutive vesicular secretion of ATP controls Ca^{2+} homeostasis in human monocytes. (**A**,**B**) Simultaneous measurement of basal intracellular Ca^{2+} concentration (A) and extracellular ATP concentration (B) following inhibition of vesicular exocytosis by NEM. Arrows denote sampling points for extracellular ATP. Bar chart represents extracellular ATP concentration at sampling points 1 and 6 as given in A (n=5; *P<0.01). (**C-G**) Effect of antagonising purinergic receptors on baseline intracellular Ca^{2+} concentration. (C,D) Effect of scavenging extracellular ATP with apyrase (4 IU/ml; C) or heat-inactivated (HI) apyrase (D). (E-G) Effect of non-selective P2 receptor antagonist suramin (100 μM), selective $P2Y_{11}$ antagonist NF380 or P1 receptor antagonist CGS15943 (1 μM) on basal intracellular Ca^{2+} concentration. (**H**) Average effects of pharmacological interventions on basal intracellular Ca^{2+} concentration (n=5; *P<0.05).

Ca^{2+}-dependent exocytosis of lysosomes has also been described in fibroblasts and epithelia (Rodríguez et al., 1999; Martinez et al., 2000), with raised $[Ca^{2+}]_i$ promoting exocytosis through activation of various regulated proteins associated with lysosomes (Holt et al., 2006; Higashio et al., 2008). Inhibition of GPN-evoked β-hexosaminidase secretion in monocytes implicates a NEM-sensitive factor (NSF) regulates lysosome exocytosis following raised $[Ca^{2+}]_i$, suggesting regulation by SNAREs (Rao et al., 2004). Exocytosis of monocytic lysosomes can occur following activation by inflammatory stimulants including bacterial lipopolysaccharide and contributes to the processing and secretion of interleukin-1β (IL-1β) (Andrei et al., 2004).

Interaction between lysosomal and ER Ca^{2+} stores in monocytes

The data presented here suggests that communication between lysosome and ER Ca^{2+} pools is bidirectional and both direct,

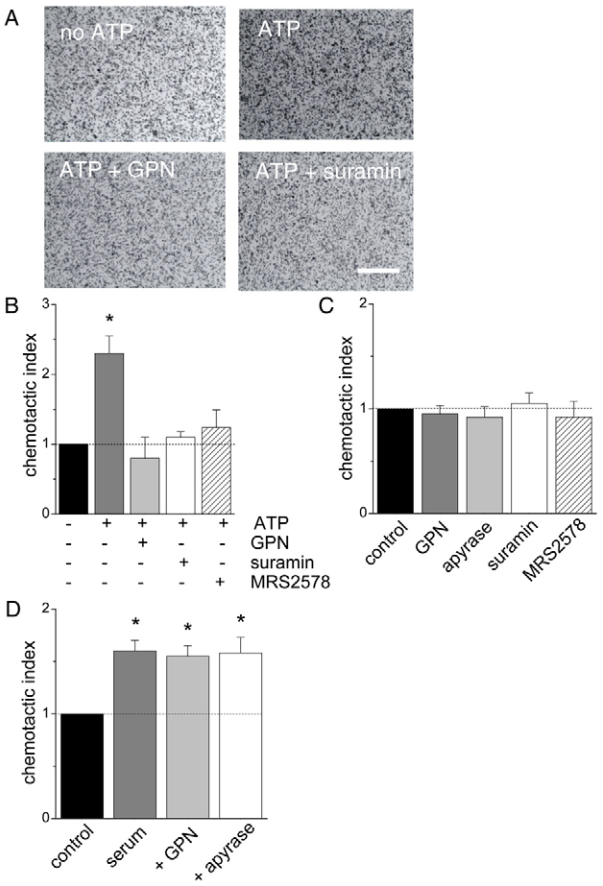

Fig. 7. GPN inhibits chemotactic migration of monocytes toward ATP. (**A**) Transwell migration assays using 1 μM ATP as the chemoattractant in the lower chamber. Representative Transwell membranes showing THP-1 monocytes stained with Crystal Violet following migration (1 hour) with ATP, in the absence of ATP, and with ATP in the presence of GPN (0.2 mM) or suramin (100 μM). Scale bar: 200 μm. (**B**) Average chemotactic index of monocytes for ATP in different experimental conditions (n=5; *P<0.05). (**C**,**D**) Effect of GPN, apyrase and P2 receptor antagonists on basal cell migration (C) and with the addition of 1% serum (D) (n=3 or 4; P>0.05).

through activation of ER ryanodine receptors, and indirect through release of ATP and activation of cell surface PLC-coupled purinergic receptors. Exposure to thapsigargin was sufficient to discharge Lysotracker staining, stimulate β-hexosaminidase secretion and ablate GPN-evoked Ca^{2+} responses. This data suggests ER calcium release is capable of promoting lysosome exocytosis. This can explain why GPN fails to elicit a Ca^{2+} response following exposure to thapsigargin. Studies in epithelial cells demonstrate that lysosomal Ca^{2+} pools can be mobilised by Ins(I,4,5)P_3 (Haller et al., 1996); however, similar studies in endothelium reveal lysosomal Ca^{2+} is not linked to thapsigargin-sensitive stores (Srinivas et al., 2002). It is evident that release of ER Ca^{2+} can be triggered directly by GPN-evoked release of lysosomal Ca^{2+} through engagement of ER ryanodine receptors, as the enhanced Ca^{2+} response to higher concentrations (0.4 mM) of GPN (presumably working by disrupting greater numbers of lysosomes) is antagonised by dantrolene (Fig. 1F). These findings are consistent with the identification of functional ryanodine receptors in monocytes by others (Bracci et al., 2007; Cardaba and Mueller, 2009; Guidarelli et al., 2009). The Ca^{2+} influx observed following GPN-evoked Ca^{2+} responses after Ca^{2+} add-back is reminiscent of capacitative calcium entry (CCE) observed following depletion of ER. Our data is consistent with activation of CCE pathways in corneal endothelium following disruption of lysosomal Ca^{2+} pools (Srinivas et al., 2002).

ATP secretion couples lysosomal and ER Ca^{2+} stores via P2Y receptor activation

Scavenging extracellular ATP/ADP using apyrase severely attenuates Ca^{2+} responses evoked by GPN (Fig. 3). Heat-inactivated apyrase had no effect suggesting the ATP diphosphohydrolase is necessary requirement for inhibition. An apyrase-resistant component to the GPN-evoked Ca^{2+} response is apparent (Fig. 3D), which may represent raised $[Ca^{2+}]_i$ due to lytic activity of GPN on lysosomes. In experiments where extracellular ATP and $[Ca^{2+}]_i$ are simultaneously measured, it is clear that the GPN-evoked Ca^{2+} response is associated with ATP secretion (Fig. 3). As for GPN-evoked β-hexosaminidase secretion, the GPN-evoked Ca^{2+} response is also ablated following inhibition of vesicular exocytosis by *N*-ethylmaleimide. This data strongly suggest the source of extracellular ATP is exocytosis of lysosomes. Astrocytes also respond to elevated $[Ca^{2+}]_i$ by vesicular release of ATP (Zhang et al., 2004a; Zhang et al., 2004b). Some elegant studies have demonstrated that the vesicles responsible for exocytotic release of ATP are lysosomes (Zhang et al., 2007). Granular release of ATP/ADP is important for platelet function (Daniel et al., 1998). The peak extracellular ATP concentration following GPN exposure was approximately 100 nM, which is sufficient to activate P2Y receptors but not P2X receptors (North, 2002; von Kügelgen, 2006). In addition, apyrase attenuates GPN-evoked Ca^{2+} responses in the absence of extracellular Ca^{2+} (Fig. 3D), suggesting a dependency on release of intracellular calcium rather than calcium influx. P2Y receptors differ in their activation profile by adenine (ATP, ADP) and uracil nucleotides (UTP, UDP) (von Kügelgen, 2006). We observed Ca^{2+} responses to micromolar ATP, ADP, UTP and UDP in THP-1 monocytic cells (Fig. 4). Human primary monocytes and monocytic cells express various P2X and P2Y receptor subtypes, of which $P2Y_2$, $P2Y_6$ and $P2Y_{11}$ are expressed by unstimulated THP-1 cells (Moore et al., 2001; Li and Fountain, 2012). $P2Y_2$, $P2Y_6$ and $P2Y_{11}$ are all PLC-coupled receptors and hence have the capacity to mobilise ER Ca^{2+} through Ins(I,4,5)P_3 synthesis when activated. Antagonism by suramin is not sufficient to discriminate between $P2Y_2$, $P2Y_6$ or $P2Y_{11}$ (von Kügelgen, 2006), though antagonism by MRS2578 demonstrates activation of $P2Y_6$ is concomitant with the GPN-evoked Ca^{2+} response. Though UDP is the preferred agonist at $P2Y_6$ receptors, ADP also activates the receptor (Robaye et al., 1997). The effect of apyrase, a specific adenosine diphosphatase, on the GPN-evoked Ca^{2+} response implicates extracellular ATP or ADP as mediators, though co-secretion of uridine nucleotides cannot be excluded.

Effect of GPN on P2Y-receptor-mediated Ca^{2+} responses

Pre-treatment with GPN attenuated P2Y-receptor-mediated Ca^{2+} responses in THP-1 monocytes (Fig. 4). This may suggest that P2Y receptors couple to lysosomal Ca^{2+} stores and that depleting the store with GPN subsequently decreases the magnitude of P2Y-receptor-mediated responses. However, we suggest that the effect of GPN on P2Y-receptor-mediated Ca^{2+} responses is due to desensitisation of P2Y receptors following GPN-evoked ATP

release. This notion is supported by several observations. Firstly, ATP-evoked Ca^{2+} responses are completely inhibited by thapsigargin and U-73122 (Fig. 4C). The effect of thapsigargin could possibly be explained by disruption of lysosomal Ca^{2+} stores by lysosome exocytosis (Fig. 2A); however, inhibition by U-73122 suggests a requirement for phospholipase C activity and therefore Ins(I,4,5)P_3-dependent release of ER Ca^{2+} is a more plausible explanation. Secondly, ATP-evoked Ca^{2+} responses are highly sensitive to desensitisation by repeated agonist application (Fig. 4D). P2Y receptor activation could also explain why GPN triggers CCE (Fig. 1E). Our data also raises the possibility that agonists which release ER Ca^{2+} could also induce lysosomal ATP secretion. Indeed, the ATP release is associated with reception of chemoattractant stimulates such as *N*-formyl-methionyl-leucly-phenylalanine in neutrophils (Chen et al., 2006) though the signalling coupling to ATP secretion is not clear. We suggest the chemotactic response to ATP is impaired due to GPN-evoked ATP secretion and P2Y receptor desensitisation (Fig. 4).

Constitutive vesicular ATP release contributes to resting $[Ca^{2+}]_i$

Application of apyrase (but not heat-inactivated apyrase) or suramin caused a robust decrease in baseline $[Ca^{2+}]_i$ which slowly recovered over several minutes post exposure (Fig. 6). In luminometer experiments, the amount of extracellular ATP measured in resting cells was around 30 nM (Fig. 6). Basal secretion of ATP has been observed in other cell types including renal epithelium (Hovater et al., 2008), osteoblasts (Genetos et al., 2005), pituitary cells (Li et al., 2011) and astrocytes (Coco et al., 2003). The basal level of ATP secreted by THP-1 cells breaches the activation threshold for many P2Y receptors (Parr et al., 1994; von Kügelgen et al., 2006). Though the level of pericellular ATP is likely to be much higher, the absence of extracellular calcium in these experiments precludes any involvement of P2X receptors. Of the G_q-coupled P2Y receptors activated by ATP or ADP, $P2Y_2$, $P2Y_6$ and $P2Y_{11}$ are expressed by THP-1 monocytes (Moore et al., 2001). $P2Y_2$ and $P2Y_{11}$ are both antagonised by suramin (Communi et al., 1999; von Kügelgen, 2005) and $P2Y_{11}$ selectively antagonised by NF340 (Meis et al., 2010). $P2Y_2$ and $P2Y_{11}$ mediated response are completely blocked at 100 µM suramin (Communi et al., 1999; von Kügelgen, 2005) as used in Fig. 6, and therefore the smaller effect of NF380 on baseline $[Ca^{2+}]_i$ suggests a major contribution of $P2Y_2$, for which no selective antagonists are available. The effect of suramin, apyrase and NF380 are therefore best explained if constitutive activation of P2Y receptors contributes to resting $[Ca^{2+}]_i$ in these experiments, though the failure of MRS2578 to reduce baseline calcium suggest $P2Y_6$ is not constitutively active. Conversely, antagonism of P1 adenosine receptors with CGS15943 raises baseline $[Ca^{2+}]_i$. These data suggest tonic P1 receptor activation negatively regulates resting $[Ca^{2+}]_i$, though the precise mechanism requires further investigation. The basal concentration of extracellular ATP is achieved through vesicular release of ATP, as *N*-ethylmaleimide caused a concomitant reduction in extracellular ATP and $[Ca^{2+}]_i$. The likely source of vesicular ATP is lysosomes as *N*-ethylmaleimide suppresses β-hexosaminidase secretion (Fig. 2). Furthermore, it is evident that monocytes caused a time-dependent conditioning of buffer with β-hexosaminidase in the absence of stimulant (Fig. 2), strongly suggesting that lysosomes undergo constitutive exocytosis. Experiments with BAPTA-AM demonstrate that buffering cytosolic calcium reduces the amount of constitutively secreted ATP (Fig. 3B), suggesting that constitutive as well as stimulated lysosome exocytosis is calcium dependent in monocytes.

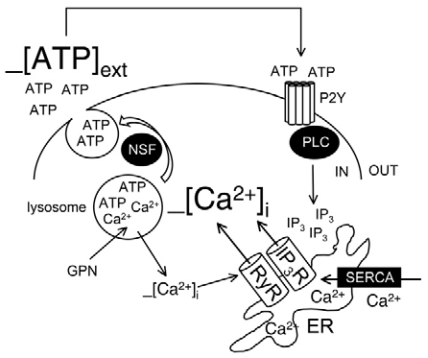

Fig. 8. Bidirectional communication between lysosomal and ER Ca^{2+} in monocytes. Lysosome to ER: release of lysosomal Ca^{2+} by the cathepsin C substrate GPN stimulates lysosome exocytosis by a NEM-sensitive-factor (NSF)-dependent pathway causing extracellular release of ATP. ATP activates cell surface phospholipase C (PLC)-coupled P2Y receptors to generate Ins(1,4,5)P_3 (IP_3), activate Ins(1,4,5)P_3 receptor (IP_3R) and release ER Ca^{2+}. Release of lysosomal Ca^{2+} can also directly release ER Ca^{2+} through activation of ryanodine receptors (RyR). ER to lysosome: release of ER Ca^{2+} stimulates exocytosis of lysosomal Ca^{2+} stores.

Implications for GPN as a pharmacological tool

Investigation into the function and regulation of acidic Ca^{2+} stores has increased greatly in the past 10 years with many landmark discoveries (Calcraft et al., 2009; Patel and Docampo, 2010). In many such studies GPN is a central pharmacological tool used to destroy lysosomes and their subsequent contribution to cellular Ca^{2+} signals. The data presented here suggests that secreted ATP may be a common feature when cells are exposed to GPN and that consequent second messenger signalling through P2Y receptor activation may have to be taken into consideration. However, it remains to be demonstrated whether GPN-evoked ATP secretion is observed outside cells of hematopoietic lineage that possess Ca^{2+}-sensitive secretory lysosomes (Holt et al., 2006). Nucleotide-evoked Ca^{2+} responses appear unaffected in corneal endothelium following exposure to GPN (Srinivas et al., 2002).

Conclusions

The data presented here suggests that constitutive vesicular secretion of ATP regulates Ca^{2+} homeostasis in THP-1 human monocytic cells via tonic activation of P2Y receptors. ATP secretion and subsequent P2Y receptor activation contributes significantly to Ca^{2+} signals generated by lysosome disruption with the cathepsin C substrate GPN. Bidirectional communication exists between lysosomal and ER Ca^{2+} stores via several routes (i) ER to lysosome: release of ER Ca^{2+} triggers lysosome exocytosis; (ii) lysosome to ER: activation of ryanodine receptors; and (iii) lysosome to ER: release of ATP and mobilisation of ER calcium through activation of PLC-coupled P2Y receptors (Fig. 8).

Materials and Methods

Intracellular calcium measurements

THP-1 monocytes were cultured in RPMI 1640 medium with L-glutamine and 10% (v/v) serum at 37°C and 5% CO_2. Cultures contained 50 IU/ml penicillin and 50 µg/ml streptomycin. Cultures were maintained between 1×10^5 and 8×10^5 cells/ml. For intracellular calcium measurements, 1×10^6 cells/ml were loaded with Fluo-4 in SBS

buffer containing (mM): NaCl, 130; KCl, 5; $MgCl_2$, 1.2; $CaCl_2$, 1.5; D-glucose, 8; HEPES, 10; pH 7.4) plus 0.01% (w/v) pluronic acid and 2 μM Fluo-4-AM. Cells were loaded for 1 hour at 37℃. Cells were resuspended at 1×10^6 cells/ml in SBS buffer supplemented with 1 mM EGTA and without $CaCl_2$. Fluo-4 fluorescence (494 nm excitation; 516 nm emission) was sampled at 1 s intervals using a Hitachi F-2000 fluorescence spectrophotometer. Cells in a quartz cuvette were continuously agitated by means of a magnetic stirrer. Maximum fluorescence (F_{max}) signals were generated by addition of 40 μM digitonin in the presence of 1.5 mM $CaCl_2$. Calcium responses to drugs were expressed at a percentage of F_{max}.

β-hexosaminidase secretion assay

THP-1 monocytes were resuspended at 2×10^6 cells/ml in SBS buffer (no calcium+1 mM EGTA) in 24-well plates. Cells were equilibrated in SBS 3 hours prior to experimentation. β-hexosaminidase activity in 200 μl supernatant samples was measured by incubation with 200 μl of 1 mM *p*-nitrophenyl *N*-acetyl-*β*-*D*-glucosaminide in 0.1 M citrate buffer (0.05 M citric acid, 0.05 M sodium citrate, pH 4.5) for 1 hour at 37℃. Reactions were quenched by addition of 400 μl 0.1 M sodium carbonate buffer. Absorbance was read at 405 nm. Absorbance values were background subtracted using zero time-point values and normalised to maximal signals generated following cell lysis with 1% (v/v) Triton X-100.

ATP secretion assay

Sample ATP was assayed by means of an ATP bioluminescence assay kit (CLSII kit, Roche). Extracellular ATP was simultaneously measured with intracellular calcium by sampling supernatants from Fluo-4-loaded cells undergoing intracellular calcium measurements as described above. 150 μl samples were withdrawn and cells sedimented at 4℃. Clarified samples were mixed 1:4 with luciferase reagent before luminescence measurements were made using a Modulus Luminometer (Turner BioSystems) and 7 s integration time.

Transwell migration assay

Migration assays were performed in 24-well plates using polyethylene terephthalate (PET) membrane Transwell inserts with 3 μm pores (BD Biosciences). 1×10^6 monocytes in RMPI without serum were added to upper chambers and 1 μM ATP chemoattractant to lower chambers. GPN or suramin was added to upper wells where appropriate. Cell migration was allowed to progress for 1 hour at 37℃ with 5% CO_2 in a humidified incubator. Inserts were fixed with −20℃ methanol, upper chambers were swabbed and cells stained with 0.5% (w/v) Crystal Violet. Migrated cells were scored using an Olympus CKX41 inverted microscope equipped with a Leica digital camera. Basal migration was assessed in the absence of chemoattractant in the lower chamber.

Microscopy

Differential interference contrast and fluorescent images were obtained using an Olympus IX71 inverted microscope equipped with a Hamatasu digital camera. For Lysotracker staining, monocytes were incubated with 100 nM Lysotracker DND-99 in Ca^{2+}-free SBS buffer for 20 mins prior to exposure to drugs. Drugs were delivered by bath perfusion.

Cell viability assays

THP-1 monocytes (1×10^6) were exposed to GPN at 0.2 and 0.3 mM for 30 mins at 37℃. Cell viability for assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cells were incubated with 12.5 mg/ml MTT for 1 hour at 37℃ followed by solubilisation in DMSO and measurement of absorbance at 595 nm.

Chemicals and reagents

All chemicals were purchased from Sigma with the exception of GPN (Insight Biotechnology Ltd), Fluo-4-AM and Lysotracker (Invitrogen), Suramin, MRS2578 and CGS15943 (Tocris). THP-1 cells where procured from ATCC.

Temperature conditions

All experiments were performed at room temperature (22℃) unless otherwise stated.

Statistical analysis

Data is expressed as means ± s.e.m. Hypothesis testing was by means of paired Student *t*-tests.

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