Lifecycle of Weibel-Palade bodies

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Summary
Weibel-Palade bodies (WPBs) are rod or cigar-shaped secretory organelles that are formed by the vascular endothelium. They contain a diverse set of proteins that either function in haemostasis, inflammation, or angiogenesis. Biogenesis of the WPB occurs at the Golgi apparatus in a process that is dependent on the main component of the WPB, the haemostatic protein von Willebrand Factor (VWF). During this process the organelle is directed towards the regulated secretion pathway by recruiting the machinery that responds to exocytosis stimulating agonists. Upon maturation in the periphery of the cell the WPB recruits Rab27A which regulates WPB secretion. To date several signaling pathways have been found to stimulate WPB release. These signaling pathways can trigger several secretion modes including single WPB release and multigranular exocytosis. In this review we will give an overview of the WPB lifecycle from biogenesis to secretion and we will discuss several deficiencies that affect the WPB lifecycle.

Zusammenfassung
Weibel-Palade-Körperchen (WPK) sind längsovale sekretorische Organellen, die vom Gefäßendothel gebildet werden. Sie enthalten diverse Proteine, die an der Blutstillung, Entzündung bzw. Angiogenese beteiligt sind. Die Biogenese der WPK erfolgt im Golgi-Apparat. Der Prozess hängt vom Hämostase-Protein von-Willebrand-Faktor (VWF) ab, der den Hauptbestandteil der WPK bildet. Während dieses Prozesses werden die Organellen in Richtung des regulierten Sekretionspfades geleitet, indem der Mechanismus aktiviert wird, der durch Stimulation von Agonisten auf die Exozytose reagiert. Bei Reifung in der Zellperipherie rekrutiert das WPK Rab27A, das die WPK-Sekretion reguliert. Bisher sind mehrere Signalwege bekannt, die die WPK-Freisetzung stimulieren. Die Signalwege können verschiedene Sekretionsmodi auslösen, wie beispielsweise die einzelne WPK-Freisetzung und die multigranuläre Exozytose. Dieser Artikel enthält eine Übersicht über den WPK-Lebenszyklus von der Biogenese bis zur Sekretion. Wir besprechen außerdem mehrere Mängel, die den WPK-Lebenszyklus betreffen.

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Weibel-Palade bodies (WPBs) are lysosome-related storage organelles of vascular endothelial cells which are secreted upon physiological stimuli, such as injury or inflammation (1, 2). They were discovered in 1964 by Ewald Weibel and George Palade and are characterised by their typical elongated shape that can measure 0.1–0.2 µm in width and up to 5 µm in length (1, 3).

In the lumen of the WPB fine tubules are present which are densely packed and are formed by the haemostatic glycoprotein von Willebrand Factor (VWF) (3–5). Exocytosis of VWF from WPBs is one of the crucial steps in haemostasis and facilitates rapid control of bleeding by facilitating platelet adhesion and aggregation at sites of vascular damage (2, 6).

> Defects in WPB formation and secretion are therefore prone to result in prolonged bleeding.

Especially mutations in VWF were shown to influence WPB formation as VWF itself functions as the major driving force in WPB biogenesis (7). In addition to VWF, many other proteins are stored in WPBs such as P-selectin, osteoprotegerin, angiopoietin-2, insulin-like growth factor-binding protein 7 (IGFBP-7), and in specialised subsets of endothelial cells also Factor VIII is found (8–14).

> This diverse set of proteins suggests that WPB exocytosis may also contribute to processes other than haemostasis, including inflammation and angiogenesis.

In this review, we will give and overview on the recent findings on WPB formation and secretion and we will discuss several deficiencies that influence the lifecycle of WPBs.

VWF multimerisation and tubule formation

The lifecycle of the WPB starts at the Golgi apparatus by tubule formation of multimeric VWF, the prerequisite component of the WPB (4, 15, 16). Correct processing of VWF is fundamental for WPB formation and is largely determined by the conserved structural domains of VWF. VWF is synthesised in the endoplasmic reticulum (ER) as monomers which consist of a signal peptide, a propeptide (741 aa, 100 kDa) and mature VWF (2050 aa, 260 kDa) (17).

The first steps towards VWF storage in WPBs start at the ER. After cleavage of the signal peptide, monomeric VWF subunits dimerise in a tail-to-tail fashion at the C-terminal cystein knot (CK) domains (17). At the Golgi apparatus, the VWF dimers subsequently multimerise by head-to-head disulfide bond formation. For this multimerisation step, the propeptide of VWF is crucial (18). It is cleaved from the mature VWF by furin and acts as a scavenger to facilitate disulfide bond formation in the low pH of the trans Golgi network (TGN) (19–21). Usually, a low pH is not very favourable for disulfide bond formation but it seems to be required for VWF multimerisation. Modulating the pH using monensin, ammonium chloride or chloroquine was shown to reduce multimerisation in a dose-dependent manner (17).

VWF multimers can leave the TGN using two distinct routes: They can be secreted constitutively or are stored in WPBs. The main difference between the two pathways seems to be found in the multimeric composition of the VWF multimers (22, 23). The highest molecular weight multimers are preferentially stored in WPBs as they are much more potent in binding platelets (24) which is beneficial when VWF is released in response to vascular injury.

To store VWF multimers in WPBs a drastic rearrangement has to occur. Guided by the propeptide – which stays non-covalently attached to the mature VWF subunit – the VWF multimers change conformation. The propeptide and part of the mature VWF (D'D3 assembly) rearrange into a right-handed helical core structure which has a 25 nm outer-diameter and 12 nm inner-diameter (5, 25). The rest of the protein arranges itself around this helical structure by folding into a so-called dimeric bouquet (26). *In vitro* it was shown by Huang et al. (5) that the formation of the helical core depends on an acidic and calcium rich environment. Possibly dedi-

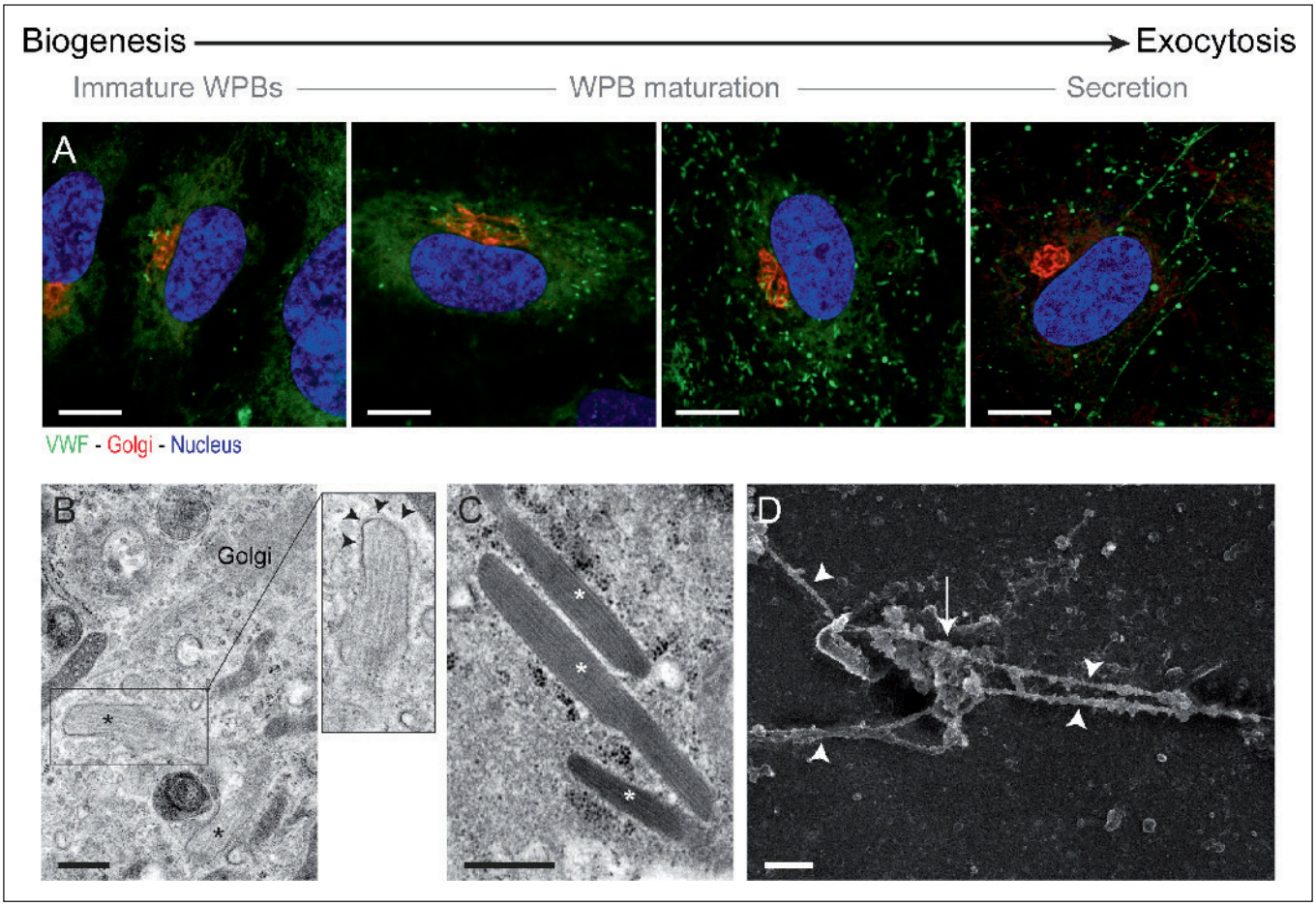

Fig. 1 Lifecycle of Weibel-Palade bodies (scale bar in panel A is 10 µm, scale bar in panels B-D is 500 nm).
The lifecycle of the Weibel-Palade Body (WPB) starts at the trans Golgi network (TGN) and is initiated by von Willebrand Factor (VWF). (**A**) The lifecyle followed by immunofluorescence shows the onset of WPB formation at the Golgi apparatus. Over time the structures at the Golgi become larger and travel towards the periphery of the cell. Upon endothelial stimulation the WPBs undergo exocytosis which is often observed by the formation of VWF strings. (**B**) Electron micrographs of immature WPBs at the TGN reveal small electron lucent granules (black asterisk) that contain spaciously packed VWF tubules. On the membrane of these immature WPBs a clathrin coat is usually observed (black arrowheads). (**C**) After formation at the TGN, the WPBs mature in the periphery of the cell. At this stage, the WPB has a densely packed interior composed of many VWF tubules (white asterisk). (**D**) Upon stimulation of the endothelial cells, the WPB undergo exocytosis to release VWF (white arrow) and many other components. The released VWF forms long adherent strings (white arrowheads) that function to capture platelets from the circulation.

cated regions of the TGN create such an environment to facilitate VWF tubule formation thereby initiating the first steps of WPB formation (▶Fig. 1). Interestingly, the multimeric state of the VWF is not important for tubule formation (5, 18).

From VWF tubule to WPB

VWF tubule generation is the major driving force in WPB formation. Expression of VWF in non-endothelial cells, such as CV-1, AtT-20, and HEK293, was shown to result in the formation of morphologically similar granules which are called pseudo-WPBs (16, 18, 27, 28). When observed by electron microscopy these granules are almost indistinguishable from endothelial WPBs, revealing the typical striated pattern of stored tubules of VWF. Moreover, pseudo-WPBs are able to recruit WPB membrane proteins, including P-selectin, Rab27A and CD63, upon co-transfection with VWF (27, 29).

In contrast to these pseudo-WPB producing cell types, there are also several cell types such as COS-1, CHO, and hepatocytes, that produce normal multimers upon VWF expression but fail to form storage granules (30–32). Apparently, there are additional factors involved that initiate VWF storage and WPB formation.

Although it is not yet clear why some cells are able to form pseudo-WPBs upon VWF expression and others not, it is clear that VWF itself plays an important role. To study the contribution of VWF in forming storage granules several studies were conducted in (pseudo-)WPB forming cells. Haberichter et al. (15) showed that expression of the propeptide or the mature VWF alone resulted only in storage of the propeptide. Moreover, the VWF propeptide was also able to traffic other proteins to storage. They showed that the C3 precursor of the complement system could be directed to storage organelles in AtT-20 cells and bovine aortic endothelial cells when fused to the VWF propeptide.

However, similar experiments in CV-1 cells, which can also form pseudo-WPBs, could not confirm the apparent role of the propeptide in storage (16). The observations might be explained by the presence of endogenous storage organelles in AtT-20 cell. Later studies in AtT-20 cells showed that expression of the propeptide alone directed the propeptide to the endogenous storage organelles and did not induce the formation of an independent storage organelle. Only upon expression of the propeptide and the mature VWF, pseudo-WPBs are formed suggesting that the propeptide directs the protein to a storage granule but that VWF is necessary to induces the formation of an independent WPB-like storage organelle in AtT-20 cells (33).

> The tight association between VWF and its propeptide during tubule formation at the TGN thereby provides a mechanism that seems to be important to direct VWF for storage and to initiate WPB biogenesis.

The earliest structures that are formed during WPB biogenesis can be visualised by electron microscopy (▶Fig. 1). Detailed electron microscopy of the Golgi apparatus in endothelial cells has revealed various stages in WPB formation at the TGN (34). The smallest WPB-like granules are typically coated with clathrin and contain very few tubules suggesting that VWF tubules push the Golgi membrane outwards thereby creating the outline of the WPB (34, 35). During WPB formation, the clathrin coat, formed together with adaptor protein-1 (AP-1), was shown to be essential. Knockdown of AP-1 or clathrin stops WPB formation and results in the formation of many small VWF positive puncta (36).

The exact role of clathrin and AP-1 is still not entirely clear, but it is hypothesised to function in scaffolding the elongated shape of the WPB. In more tubulated later staged immature WPBs also conventional clathrin coated pits are typically seen which are proposed to facilitate membrane retrieval to condense the VWF tubules and to remove misdirected proteins (36). From these observations it was however unclear how the relatively short electron lucent immature WPBs transform into long electron dense organelles.

> Changes observed in size and electron density suggested that the WPBs increase their VWF content over time.

The morphological heterogeneity of mature WPBs might suggest that this WPB growth is achieved via homotypic fusion. Their delimiting membrane often display curves and sharp angles that could have been formed upon fusion of several smaller WPBs. Electron tomography on these irregularly shaped WPBs provided additional evidence by revealing the disarray of tubules at sites of presumed fusion (37).

In 2014, Ferraro et al. (38) published data indicating a major role for the Golgi in regulating the size of forming WPBs. High-throughput measurements on WPB lengths and super-resolution microscopy on VWF distribution in WPBs revealed that WPBs are formed from several nanoclusters or „quanta“ of about 0.5 µm in length. By unlinking the Golgi stacks they could show that WPB size is determined by the so-called mini-stacks of the Golgi apparatus. Live-cell imaging confirmed that these quanta are able to fuse with each other before budding from the Golgi.

Shortly after this publication, we revealed by correlative light and electron microscopy that forming WPBs stay connected with the Golgi apparatus during their formation. We followed WPB formation over time and observed that even in the late stages, in which the WPBs were up to 2 µm long, still numerous connections existed between the WPB and the TGN. These long WPBs were often irregularly shaped suggesting that they were formed from multiple „quanta“ that have fused before leaving the Golgi. In more early stages we observed partially tubulated VWF in the lumen of forming WPBs suggesting that clusters of VWF are brought together to form WPBs in specialised regions of the TGN (39).

In depth research to underlying mechanisms for WPB formation in the TGN just recently revealed that the type II phosphatidylinositol-4 kinases PI4IIα and PI4IIβ are important for WPB biogenesis. Depletion of these kinases resulted in shorter WPBs with perturbed packaging of VWF while VWF multimerisation was normal (40). Possibly, these kinases play a role in the recruitment of AP-1 or other phosphatidylinositol 4-phosphate (PI4P) binding proteins that facilitate changes in membrane composition and shape. Sur-

prisingly, knockdown of these kinases did not impair WPB exocytosis as normal levels of secreted VWF were measured after stimulation. However, the released VWF from PI4IIα and PI4IIβ depleted cells was less efficient in capturing platelets suggesting that these kinases also influence VWF tubule formation and possibly co-storage of other WPB components as well (40).

A similar effect on WPB size and VWF functioning was recently also discovered upon treating endothelial cells with statins (41). Statins were found to shorten WPB size by unlinking the Golgi ribbon using a mechanism that is largely independent on KLF-2 activation. KLF-2 is a transcription factor that controls WPB size and reduces WPB size upon overexpression in endothelial cells (42). Although statins are able to activate KLF-2, depletion of KLF-2 was not sufficient to fully rescue WPB size to normal control levels (41).

> Statins and the type II PI4 kinases both seem to be able to modulate WPB size and the adhesive capacity of the VWF cargo suggesting that they act on a shared mechanism.

Future investigation may hopefully elucidate even more of the underlying mechanism that regulates WPB size, VWF content and possibly also the co-storage of other WPB components. During WPB biogenesis at the TGN many other proteins are co-stored with VWF. Several WPB components, such as P-selectin, IGFBP-7, interleukin-8, and osteoprotegerin, are possibly directed to the forming WPB by VWF itself as it was shown that these proteins can directly interact with VWF (9, 43–46). The recruitment of membrane protein P-selectin, for example, seems to be driven by targeting tyrosine motifs in the cytoplasmic tail but also by a luminal part of the protein that seems to associate with the structural domains D'D3 of VWF (43, 47).

However, conflicting evidence suggests that the recruitment of P-selectin is not dependent on VWF but occurs upon steric entrapment by the VWF paracrystal formed by VWF tubules (25, 48). Disruption of the VWF paracrystal with ammonium chloride was shown to increase P-selectin mobility while leaving VWF immobile. Moreover, a mutation in P-selectin preventing immobilisation resulted in impaired P-selectin recruitment to WPBs. Possibly the interaction between P-selectin and VWF is pH sensitive and is disrupted upon increasing the luminal pH of the WPB by ammonium chloride treatment. Nevertheless, for most of the WPB components, such as angiopoietin-2 and eotaxin-3, the sorting mechanism is not known and seems to occur independently of VWF.

Sorting at the TGN determines the composition of the WPB. As endothelial cells are very heterogenous cells that show differences in functioning depending on their localisation, it is more than expected that also large differences in WPB content exist. For example, some specialised endothelial cells such as liver sinusoidal endothelial cells are also able to co-store factor VIII in WPBs (12–14). The various proteins that have been found in WPBs can be categorised in processes such as

- inflammation,
- haemostasis, and
- angiogenesis.

In vitro evidence suggests that endothelial cells are able the skew the WPB content depending on external conditions. Exposing endothelial cells to interleukin-1β, for example, induces the *de novo* synthesis of interleukin-8 which is subsequently stored in WPBs (46). Additionally, endothelial cells seem to produce subpopulations of WPBs which have differences in the composition in the co-stored proteins. For the storage of angiopoietin-2 and P-selectin, endothelial cells seem to produce two distinct types of WPBs. Immunocytochemistry on these WPB compounds revealed that they are not stored together in WPBs. Instead, two types of WPBs are produced that either contain P-selectin or angiopoietin-2 (8).

Apart from sorting proteins at the TGN for co-storage with VWF, the forming WPB also recruits the machinery for regulated exocytosis. The presence of AP-1 in the membrane of the forming WPB does not only facilitate the scaffolding clathrin coat but it also recruits the effectors aftiphilin and γ-synergin. Aftiphilin and γ-synergin direct the WPB into the regulated secretory pathways and prevent premature exocytosis. Depleting cells of these proteins results in normal WPBs that are insensitive for secretory stimuli and are secreted following the constitutive secretory pathway instead (49).

After formation at the TGN, the lumen of the WPB has adopted an acidic pH of 5.5 to keep VWF in its tubular conformation (5, 50). At this stage, the VWF tubules are densely packed in para-crystalline composition, which was revealed by cryo-electron tomography in frozen hydrated endothelial cells (25). Also additional proteins are recruited to the WPB which are considered to be the final steps in WPB maturation which occur in the periphery of the cell. One of these proteins that is added to the WPB membrane is the tetraspanin CD63 (51). CD63 is transported from endosomal organelles to the WPB and functions upon WPB exocytosis as a cofactor for P-selectin to facilitate leukocyte binding (47, 52). Depletion of CD63 was shown, both in cells and in mice, to strongly perturb leukocyte binding to P-selectin (52). During maturation, also the small GTPases Rab3D and Rab27A are recruited to the WPB. Especially the recruitment of Rab27A is considered to be the final step in maturation because of its key role in regulating WPB exocytosis. Surprisingly, Rab27A is also present on pseudo-WPBs upon heterologous VWF expression in HEK293 cells suggesting that its recruitment mechanism is remarkably content and maturation driven, occurring independent on the type of cell (29).

Mature WPBs move around in the periphery of the cell and are characterised by their dense packaging of VWF (▶Fig. 1). Electron tomography on the VWF tubules revealed that the tubules are packaged in a slightly twisted conformation indicating a spring loaded mechanism to facilitate exocytosis (37). Amongst the mature WPBs the well-known cigar shaped granule are found but also the irregularly shaped WPB that show kinks or bends (34, 37). Live-cell imaging data suggested that these hinge and flex points facilitate WPB movement (34). However, they may also be a reminiscent feature that was created upon WPB formation (38, 39).

In contrast to the rigid appearance of the VWF tubules, they were found to have

some flexibility. Cryo-electron tomography on vitrified endothelial cells showed the presence of continuous tubules at hinge points (25). The data suggests that the length of the tubules and the arrangement thereof are the main determinant to create flexibility at hinge points (25).

Exocytosis of WPBs

Endothelial cells produce several secretory granules that are either released via

- constitutive secretion,
- basal secretion, or
- regulated secretion (53).

Basal release of WPBs occurs spontaneously under resting conditions (54) whereas the regulated secretion pathway is triggered upon various stimuli including inflammation, shear stress, and vessel injury (1). Interestingly, these different secretion routes also seem to have an apical/basolateral polarity. Several studies revealed that exocytosis of WPBs under stimulated conditions mainly occurs at the apical side of the endothelium (17, 23, 55). Apical release of WPBs enables large VWF multimers to form VWF strings which function to fish platelets from the circulation. In addition, apical release also allows the distribution of other WPB cargo. Interestingly constitutive secretion does not seem to be polarised although some studies find it to be more directed to the basal side of the endothelium (23, 55).

The most studied pathways that activate regulated secretion of WPBs are induced by agonists that activate G-protein coupled receptors which either increase intracellular calcium or induce the rise of cyclic AMP (cAMP) levels (56, 57). Thrombin and histamine are agonists that induce a calcium influx and are known to promote extensive WPB exocytosis (57–59). Epinephrine and vasopressin on the other hand act via cAMP (56, 60) and induce the secretion of a selected population of WPBs releasing VWF but little or no P-selectin (61). As shown in ▶Fig. 2, part of the signalling cascade of both activation routes has been elucidated (58, 62–65). These signalling cascades probably represent the most basic steps required for WPB exocytosis as they do not fully explain the differences in cellular response that are observed upon treating cells using various agonists. The signalling involved in WPB secretion appears to be complex and is not yet fully understood.

The available data so far suggests that the calcium elevating pathway and the cAMP pathway share at least parts of the signalling route that ultimately leads to exocytosis. Both cascades were found to signal RalGDS to activate the GTPase RalA, the common activator of exocytosis that associates with WPBs (64,66). RalA can stimulate soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) mediated membrane fusion at the plasma membrane by the activation of phospholipase D (1). In this process, alpha-synuclein appears to regulate RalA activity and can prevent SNARE mediated vesicle fusion (67). Upstream of RalA several other regulating pathways may also influence WPB exocytosis. For example, it was recently shown that dephosphorylation of annexin A2 by protein kinase A (PKA) is also important of cAMP mediated WPB exocytosis (68, 69).

One of the factors important for WPBs release is the interaction with the cytoskeleton. WPBs emerging from the Golgi, interact with microtubules and travel towards the periphery of the cell. After recruitment of Rab27A, the effector protein MyRIB anchors the WPB to the actin cytoskeleton to prevent exocytosis (70). In this anchoring step myosin Va (MyoVa) might also play a role as previous data indicated that MyRIB can also recruit MyoVa to facilitate WPB binding to actin (71). The importance of the cytoskeleton in exocytosis is reflected in the effects that occur upon interfering with the microtubule and actin network. Disruption of the microtubules was shown to have an inhibitory effect on calcium mediated exocytosis, whereas actin destabilisation enhanced WPB secretion (59, 72, 73).

Interestingly, this effect is not seen in cAMP mediated exocytosis. Calcium mediated exocytosis probably stimulates WPB transport to the cell membrane since almost all WPBs are released. In contrast, cAMP targets only a subset of WPBs for exocytosis (56, 74, 75). These differential needs for the cytoskeleton in WPB release indicate distinct signaling pathways for calcium and cAMP mediated secretion.

> Both signalling pathways may modulate the cytoskeleton. However, it is still unclear how this subsequently translates into the differences that are observed upon stimulating cells with different agonist.

Apart from anchoring WPBs to the actin cytoskeleton, Rab27A also regulates WPB exocytosis by binding different effector proteins (72). Recruitment of synaptotagmin-like-protein-4a (Slp-4a) to Rab27A was shown to have a positive effect on of WPB exocytosis (76). Moreover, Rab27A was also found to recruit Munc14–3 which may facilitate the assembly of the SNARE complex between WPB and plasma membrane (77). In addition to Rab27A, also Rab15, Rab3B and Rab3D have been implicated to regulate WPB exocytosis (68, 77). These Rab-GTPases are hypothesised to regulate the recruitment of Rab27A effector proteins. Rab15 has been suggested to regulate Munc14–3, as it shares this effector protein with Rab27A (77), whereas Rab3B and Rab3D seem to regulate the availability of Slp-4 (76). As suggested by Bierings et al. (76) switching of the Rab between the GTP or GDP bound state may regulate the affinity for differential effector proteins which may determine a resting or a secretory condition.

For the final step in WPB exocytosis, fusion with the plasma membrane occurs (▶Fig. 2). During this process, SNARE proteins of the plasma membrane (target SNAREs [t-SNAREs]) interact with SNAREs on the WPB membrane (vesicle SNAREs [v-SNAREs]) to bring the membranes together for fusion (1). For WPB exocytosis, the SNARE machinery appears to be complicated as different candidates have been proposed. So far, the SNAREs that have been implicated to function in WPB fusion are

- synaptosomal-associated protein 23 (SNAP23),
- syntaxin 2,
- syntaxin 3,
- syntaxin 4 (v-SNAREs), and
- vesicle-associated membrane protein 3 (VAMP3, t-SNARE) (78–81).

For a long time, syntaxin 4, VAMP3 and SNAP23 were considered to form the core machinery for WPB exocytosis. Several studies showed 25–75 % decreased VWF exocytosis upon treating permeabilised cells with inhibitory antibodies against syntaxin 4, VAMP3 and SNAP23 or by ablating protein expression using siRNA (78, 80, 81).

However, the investigation of Rab27A effector proteins identified a different set of SNARE proteins that also seem to be involved in WPB secretion. A pull-down of the Rab27A effector Slp-4a, revealed syntaxin binding protein 1 (STXBP1 or Munc18–1) as binding partner (▶Fig. 2) which upon knock-down reduced secretion with about 40 % for both calcium and cAMP mediated exocytosis. In addition, it was found that STXBP1 associates with syntaxin 2 and 3, but not with syntaxin 4, thereby providing evidence for a second SNARE protein assembly that is involved in WPB secretion (79). Syntaxin 4 was found to associate with STXBP3 (Munc18c) which is hypothesised to interact with other effector proteins of the regulatory Rabs, such as Munc14–3 (▶Fig. 2) (77, 80).

In addition to the cellular approaches, a genome wide association study may have uncovered a third player in regulating the SNAREs required for WPB exocytosis. Analysis of single-nucleotide polymorphisms (SNPs) associated with VWF plasma levels identified STXBP5 (tomosyn) and syntaxin-2 as two significant determinants (82, 83). The contribution of STXBP5 in WPB exocytosis was found to be inhibitory and thereby seems to counteract on STXBP 1 and 3 that both support WPB secretion. Surprisingly, STXBP5 was found to associate with syntaxin 4 and thereby seems to be a regulator of syntaxin 4 mediated WPB fusion by competing with STXBP3 for the binding of syntaxin 4.

> Taken together, the current data indicate that multiple sets of SNARE protein are involved in WPB secretion which allows flexibility in the fusion machinery. Often redundancy in SNARE protein function allows this flexibility and also allows a complex regulatory mechanism that is required to respond accurately on different stimuli.

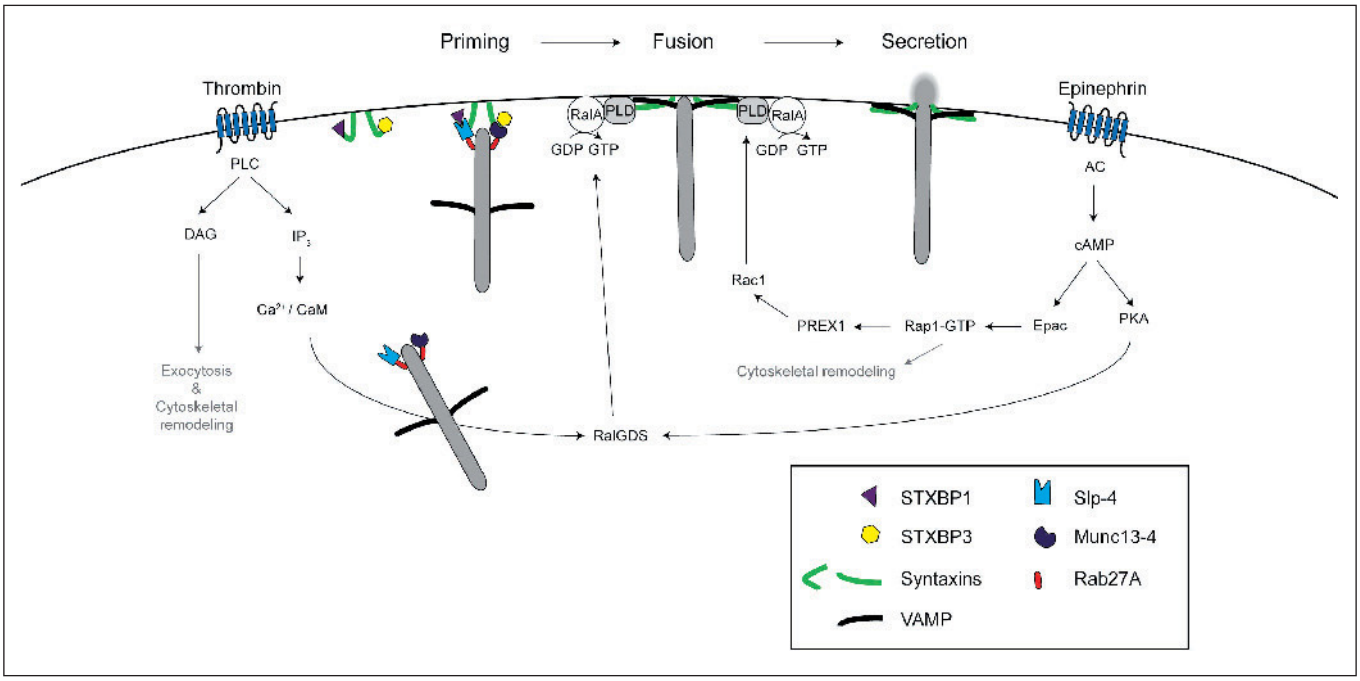

Fig. 2 Signaling and regulation of WPB exocytosis.
The two major signaling routes that trigger WPB exocytosis are both activated via G-protein coupled receptors (GPCR) that either raise intracellular calcium (Ca^{2+}) or cyclic adenine monophosphate (cAMP). Agonists such as thrombin and histamine trigger activation of phospholipase C (PLC) upon activating their GPCR which induces the production of diacylglycerol (DAG) and inositol-triphosphate (IP_3). IP_3 subsequently triggers the opening of calcium channels and increases intracellular Ca^{2+} which targets calmodulin (CaM) to activate RalGDS. RalGDS then translocates to the plasma membrane to activate RalA. The other pathway, activated by compounds such as epinephrine and vasopressin, also acts via a GPCR and stimulates adenylate cyclase (AC) for the production of cAMP. cAMP then activates protein kinase A (PKA) and Epac which both stimulate WPB release by enhancing SNARE mediated fusion. PKA also enhances RalA activity via RalGDS and Epac was shown to signal via Rac-1 which promotes phospholipase D (PLD) activation (1, 58, 62–65). On the WPB membrane secretion is mainly regulated by Rab27A and its effector proteins. Recruitment of Slp-4 was shown to stimulate WPB secretion by binding to syntaxin binding protein 1 (STXBP1 or Munc18–1) which is present on the plasma membrane. Munc13–4 is also an effector protein of Rab27A and has been suggested to interacts with STXBP3 (Munc18c). STXBPs are regulatory proteins that are in complex with syntaxins which function as target membrane SNARE proteins (t-SNAREs). Complex formation of Syntaxins with VAMPs, the vesicle-SNAREs (v-SNAREs) on the WPB, can establish membrane fusion. The steps leading to fusion usually start with priming of the SNARE complex which upon activation lead to activation of the complex, leading to fusion and subsequent exocytosis of the WPB (76–80).

Modes of WPB secretion

In response to different types of stimuli, endothelial cells can modulate WPB exocytosis. The most characterised secretion mode is the exocytosis of single WPBs in which the complete WPB content is expelled from the storage granule. In histamine stimulated cells a short transient burst of exocytosis occurs for 10–30 seconds. In this period, single WPBs are rapidly released in less than a second (50).

Upon fusion with the plasma membrane the WPB changes from an elongated organelle to a round vesicle which is induced upon the rapid pH shift that happens upon fusion: Before fusion, the lumen of the WPB has a pH of about 5.5 which is required to keep VWF in a tubular conformation (5, 50, 84). Once the lumen of the organelle comes into contact with the neutral pH of the circulating blood, the VWF tubules unfold and are released into the circulation (50, 84). The propeptide of VWF then also dissociates from the mature VWF and diffuses away rapidly (85). Amperometry measurements to study the dynamics of the fusion pore revealed that WPBs first form an initial narrow pore which is then further expanded to release WPB content (86). This initial fusion pore was previously surprisingly well imaged by cryo-electron tomography in vitrified endothelial cells (25).

In addition, it was shown that the formation of the fusion pore is influenced and maybe regulated by cholesterol. Depleting cholesterol from endothelial cells slowed WPB exocytosis whereas supplementing cells with cholesterol resulted in increased VWF secretion. This relationship between cholesterol and WPB exocytosis may therefore contribute to mechanisms that influence the risk of vascular disease as it is known that elevated VWF plasma levels are observed together with chronically elevated plasma cholesterol levels (86).

Conversely, long chain omaga-3 polyunsaturated fatty acids that were shown to provide cardiovascular benefits appeared to protect against WPB degranulation (87). Possibly, this inhibition of WPB release contributes to the anti-inflammatory and vasoprotective effects of omega-3 fatty acids (87).

Also other environmental conditions can modulate WPB secretion. Local acidosis associated with inflammation possibly results in partial WPB exocytosis. Stimulated exocytosis in a more acidic extracellular environment was shown to shift secretion to a more inflammatory profile as VWF is unable to unfold (88). Interestingly, a similar effect is also seen in endothelial cells that are treated with low levels of calcium elevating agonist. This so-called „lingering kiss exocytosis“ results in partial release of the WPB and occurs upon temporary fusion with the plasma membrane (89). In contrast to acidosis, the VWF multimers unfold in this secretion mode but are retained in the cell by a 12 nm fusion pore that only allows proteins up to 3 kDa in size to leave the WPB (89). Among these proteins are for example CD63 and interleukin-8 (89).

Apart from single WPB exocytosis also multigranular exocytosis has been described in endothelial cells in which multiple WPBs fuse with each other before exocytosis.

> The occurrence of multigranular exocytosis was mainly observed in cells containing over 100 WPBs and seems to function in the release of high levels of VWF which are required for the formation of platelet adhering strings.

In addition, multigranular exocytosis may also contribute to differential release of WPB cargo (90, 91). During multigranular exocytosis, fusion of WPBs induces large VWF containing structures, which are called secretory pods (91). Live-cell imaging of this process suggests that contact with the plasma membrane is made during this process as the fluorescent signal increases upon WPB rounding and fusion (90). Previous studies to WPB exocytosis has revealed that this increase in fluorescence is induced by a rise in pH which also occurs upon fusion with the plasma membrane (50).

Interestingly, the secretory pod does not show the release of small compounds such as CD63 during its formation and therefore seems to have a different onset when compared to lingering kiss exocytosis. Formation of the secretory pod is possibly regulated or influenced by tiny interposing vesicles (nanovesicles) that were found to associate with WPBs and secretory pods (91).

Multigranular exocytosis is triggered upon stimulating cells with thrombin but seems to be more pronounced upon stimulating cells using the diacylglycerol (DAG) analogue phorbol 12-myristate 13-acetate (PMA) (91). PMA signaling acts via protein kinase C (PKC) and results in a secretion profile that has a slow onset but releases WPBs over a longer, more persistent, period when compared to histamine (50, 74, 92). Currently, the physiological role of this secretory route is not yet known but it might follow a similar stimulation pathway as induced by vascular endothelial growth factor (VEGF), another agonist which was shown to induce WPB exocytosis. VEGF stimulates WPB exocytosis via PKCδ using a signalling pathways that is less dependent on calcium. Interestingly, PKCδ inhibition by staurosporine decreased both VEGF and PMA stimulated VWF release but did not influence histamine evoked secretion (93).

Compared to VEGF, PMA is a much more potent agonist in stimulating WPB exocytosis and is, in contrast to histamine, dependent on actin to facilitate WPB release. In PMA stimulated cells, a contractile ring of actin and myosin II was observed around fusing WPBs which appeared to function to squeeze the WPB content out of the granule into the extracellular environment. Stabilising or dissociating the actin ring did not inhibit fusion but prevented VWF release from the WPB (94). Apparently, a different mechanism acts during PMA induced WPB secretion to release VWF from the granule when compared to histamine or thrombin evoked exocytosis. Additionally, it indicates that the pH shift, which occurs upon WPB fusion with the plasma membrane, is not the only mechanism that contributes to complete degranulation of the WPB.

WPB related deficiencies

Deficiencies in WPB formation and secretion can affect several processes including haemostasis and angiogenesis. Von Wille-

brand's disease (VWD) is probably the most well-known deficiency that can influence the lifecycle of WPBs. This hereditary bleeding disorder leads to prolonged bleeding and is caused by genetic defects in VWF. VWD is categorised into three types:

- type 1 VWD with a mild quantitative reduction of normal VWF,
- type 2 with a qualitative defect leading to a dysfunctional VWF protein, and
- type 3 VWD with a complete lack of the protein in the plasma.

In type 3 VWD no WPBs are formed due to the absence of the prerequisite driving protein VWF. It was shown that upon transfection and expression of VWF the WPB formation was re-established (95). The mutations causing type 1 and type 2 VWD are mainly missense mutations with a dominant-negative effect. Whether those defects will lead to no or abnormal WPB formation is highly dependent on the pathophysiologic mechanism of the specific mutation (7).

Some mutations in type 1 VWD are mainly characterised by a very fast clearance of VWF from the circulation; however, those mutations do not influence the WPB formation and exocytosis. Other mutations may lead to retention of the mutant VWF in the ER leading to aberrant WPB formation (96–98). In other cases, WPB are formed, but seem not to be released properly (96, 97). Many mutations in VWF interfere with dimerisation and multimerisation of VWF, and may have a negative impact on WPB formation, but multimerisation itself is not a prerequisite for normal WPB formation (98). The effect of different VWF mutations on morphology and secretion of WPBs has been reviewed before in detail (7).

Apart from defects in VWF also mutations in WPB regulatory proteins may affect the WPB lifecycle. Loss-of-function mutations in the STXBP1 gene are known to cause early infantile epileptic encephalopathy type 4 (EIEE4), a very rare disease which is characterised by severe epileptic seizures (99,100). The defects in STXBP1 impair neurotransmitter release (100), but also seem to inhibit WPB release (79). Endothelial cells cultured from the peripheral blood from a EIEE4 patient showed reduced WPB secretion when compared to healthy control cells. Interestingly, the VWF plasma levels of the EIEE4 patient were low but still within the normal range (79). Possibly, the *in vivo* effect of reduced WPB secretion becomes more prominent upon endothelial stimulation. However, it is also possible that the plasticity in the secretory machinery compensates for the partial defect.

> Despite the discovery in recent years of many factors involved in the formation, maturation and exocytosis of WPB, still, the picture of signalling pathways needs higher resolution to fully understand this important organelle involved in haemostasis, inflammation and angiogenesis.

Conflict of interest

The authors declare that there is no conflict of interest.

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