

REVIEW ARTICLE

Exocytosis of Weibel–Palade bodies: how to unpack a vascular emergency kit

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To cite this article: Schillemans M, Karampini E, Kat M, Bierings R. Exocytosis of Weibel–Palade bodies: how to unpack a vascular emergency kit. *J Thromb Haemost* 2019; **17**: 6–18.

Summary. The blood vessel wall has a number of self-healing properties, enabling it to minimize blood loss and prevent or overcome infections in the event of vascular trauma. Endothelial cells prepackage a cocktail of hemostatic, inflammatory and angiogenic mediators in their unique secretory organelles, the Weibel–Palade bodies (WPBs), which can be immediately released on demand. Secretion of their contents into the vascular lumen through a process called exocytosis enables the endothelium to actively participate in the arrest of bleeding and to slow down and direct leukocytes to areas of inflammation. Owing to their remarkable elongated morphology and their secretory contents, which span the entire size spectrum of small chemokines all the way up to ultralarge von Willebrand factor multimers, WPBs constitute an ideal model system for studying the molecular mechanisms of secretory organelle biogenesis, exocytosis, and content expulsion. Recent studies have now shown that, during exocytosis, WPBs can undergo several distinct modes of fusion, and can utilize fundamentally different mechanisms to expel their contents. In this article, we discuss recent advances in our understanding of the composition of the WPB exocytotic machinery and how, because of its configuration, it is able to support WPB release in its various forms.

Keywords: endothelial cells; exocytosis; von Willebrand disease; von Willebrand factor; Weibel–Palade bodies.

Weibel–Palade bodies: secretory organelles of the endothelium

The main cargo of Weibel–Palade bodies (WPBs) is von Willebrand factor (VWF), which is a large multimeric adhesive protein that mediates platelet adhesion to the endothelium and to the subendothelial matrix [1,2]. VWF also acts as a chaperone for coagulation factor VIII in plasma, and prevents its premature clearance, which is critical for the maintenance of normal circulating FVIII levels. VWF undergoes a complex series of post-translational modifications, including glycosylation, multimerization, and proteolytic processing, and is the driving force behind the formation of WPBs. Mutations in VWF that affect its synthesis or processing and subsequent storage in WPBs constitute the basis of von Willebrand disease (VWD), which is the most common inherited bleeding disorder, and is caused by quantitative or qualitative defects in VWF. The topics of VWF biosynthesis, WPB formation and VWD have been extensively covered in a number of excellent reviews [3–5], and are therefore not included in this review.

Alongside VWF, a considerable number of inflammatory and angiogenic mediators are copackaged into WPBs (Fig. 1C) [6,7]. Their simultaneous release from this vascular emergency package will also direct leukocytes to sites of inflammation and promote vessel repair. To tailor its secretory response to the prevailing vascular condition, the endothelium continuously transduces cues from the local microenvironment into dynamic control over the content of WPBs by selectively including or excluding certain cargoes. Conditions that mimic laminar flow lead to a reduced angiopoietin-2 (Ang-2) content in WPBs [8]. Exposure to proinflammatory cytokines leads to upregulation of chemokines such as interleukin (IL)-8, monocyte chemoattractant protein-1, eotaxin-3, and IL-6, which are packaged into newly synthesized WPBs [9]. Because WPBs are long-lived storage organelles with a turnover of ~ 24 h [10–12], endothelial cells will accumulate distinct populations of granules that differ in their levels of cotargeted WPB cargo [9,13]. Whether the degree of inclusion

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Received: 6 June 2018,

Manuscript handled by: P. H. Reitsma

Final decision: P. H. Reitsma, 15 October 2018

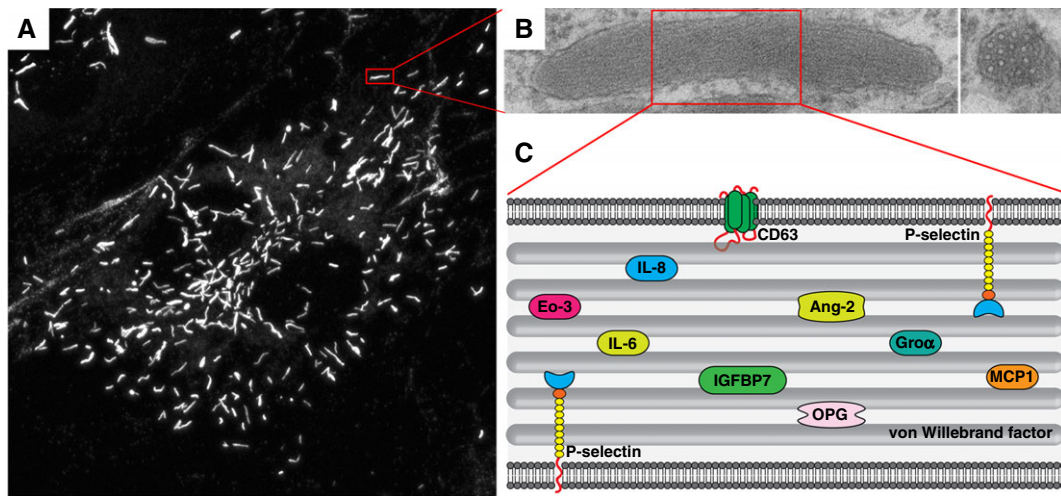


Fig. 1. Weibel–Palade bodies (WPBs), secretory organelles of the endothelium. (A) Endothelial cells containing characteristic elongated WPBs visualized by von Willebrand factor (VWF) immunostaining. (B) WPB ultrastructure, with a longitudinal section (left) showing internal striations, and a cross-section (right) showing bundles, which represent densely packed VWF tubules. (C) Cartoon representation of WPB cargo. Ang-2, angiopoietin-2; Eo-3, eotaxin-3; GRO α , growth regulated oncogene α ; IGFBP7, insulin-like growth factor-binding protein 7; IL-6, interleukin-6; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; OPG, osteoprotegerin.

of cotargeted WPB cargo reflects the endothelial activation state at the moment when the WPB was synthesized, or whether this is a result of stochastic variation following gradual redistribution of contents over the WPB population through content intermixing, as been reported for other long-lived organelles such as lysosomes [14], is currently unclear. Interestingly, it has been suggested that subsets of granules can be subject to differential exocytosis [15], although the mechanism behind this differential release remains elusive.

The endothelial secretory pathway

VWF secretion from endothelial cells occurs via three main routes: constitutive secretion and basal secretion, both of which occur in the absence of stimulation, and regulated secretion of WPBs in response to endothelial activation (Fig. 2). Following its journey through the early secretory pathway, VWF undergoes a sorting step at the level of the *trans*-Golgi network (TGN): low molecular weight (LMW) VWF multimers enter the constitutive secretory pathway in small short-lived anterograde carriers, which are immediately released at the plasma membrane [16]. High molecular weight (HMW) VWF multimers enter the storage pathway by condensing into tubules, which are packaged in nascent WPBs that bud off the TGN [17]. This compartment constitutes the releasable pool of HMW VWF that is secreted upon secretagogue stimulation. The proportion of VWF that is sorted into either direction has long been a matter of debate, partly because previous studies considered only constitutive and regulated secretion [16,18]. Careful kinetic monitoring of VWF trafficking has revealed that the storage pathway is also responsible for the majority of

unstimulated VWF release through basal secretion [12]. This most likely reflects gradual, stochastic turnover of WPBs, as VWF retention in this pathway correlates with the reported half-life of these granules [10,12]. It suggests that the secretory machinery of WPBs at baseline is already in a certain degree of readiness that supports low-level, spontaneous release. It also reconciles earlier and recent observations that the bulk of VWF released by resting endothelial cells is of a high multimeric nature [18,19].

Apart from the degree of multimerization, another distinction between the constitutive and basal/stimulated secretion pathways can be made on the basis of the polarity of release. *In vivo*, the apical side of the endothelial cells faces the vascular lumen, which results in VWF secretion directly into the circulation. VWF unfurls and assembles into ultralarge VWF strings that can be up to several millimeters in length. Entanglement of several of these strings leads to the formation of spiderweb-like networks that function as adhesive platforms for platelets [20]. In this way, VWF strings act as polymeric force sensors that, upon flow, expose a shear-dependent binding site for platelet glycoprotein Ib [4]. Basolaterally released VWF is deposited in the subendothelial matrix, where it mediates platelet adhesion when the matrix is exposed following damage to the vessel wall, either directly or via self-association with plasma VWF [21,22]. Over three decades ago, the idea had already arisen that secretion of such functionally distinct pools of VWF may differ in polarity, but previous studies addressing the polarity of endothelial VWF release remained inconclusive because they did not consider the contribution of spontaneous release of regulated secretory cargo via the basal route [23,24]. A recent study has now convincingly shown that

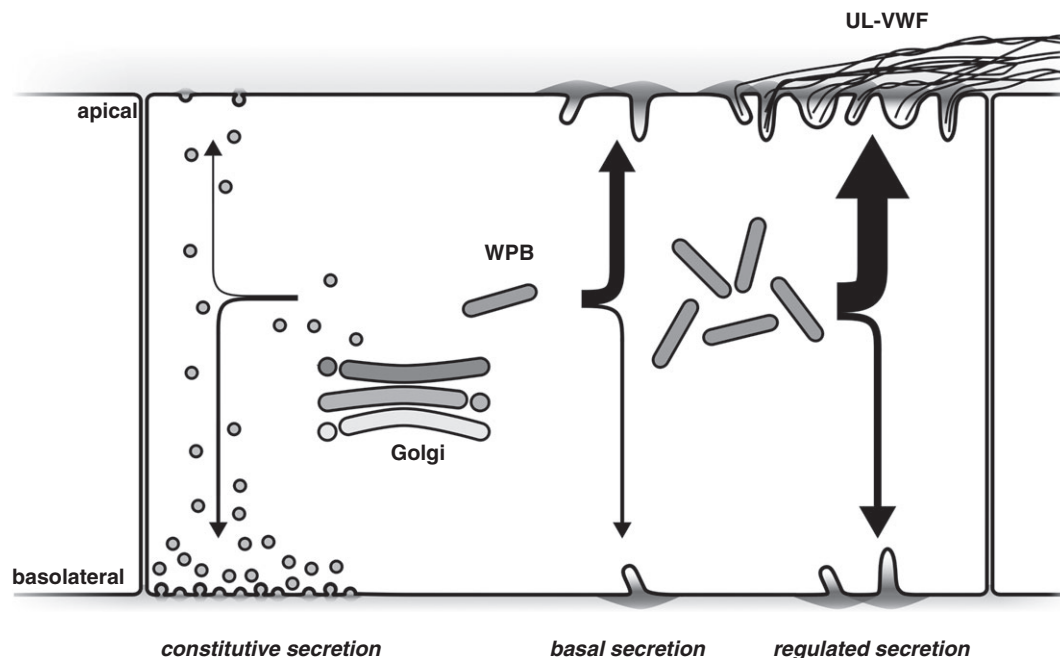


Fig. 2. The endothelial secretory pathway, von Willebrand factor (VWF) secretion occurs via three pathways: (i) constitutive secretion of low molecular weight VWF, which is primarily released at the basolateral side of the endothelium; and (ii) basal and (iii) regulated secretion of high molecular weight VWF from Weibel–Palade bodies (WPBs), which is primarily directed towards the apical surface. From the large number of WPBs that undergo exocytosis upon stimulated release, ultralarge VWF (UL-VWF) multimers emerge that assemble into VWF strings on the apical side of the endothelium.

constitutive release of LMW VWF mainly occurs basolaterally, whereas basal and stimulated release of HMW VWF from WPBs primarily occur at the apical face [19].

Stimuli and signaling cascades

WPB exocytosis is triggered by a wide range of physiological (stress hormones, e.g. epinephrine; proteases, e.g. thrombin; biogenic amines, e.g. histamine and 5-hydroxytryptamine; and shear stress) and pathological (e.g. bacterial toxins) signals (reviewed in [6]) that use Ca^{2+} or cAMP as second messengers (Fig. 3). As part of an integrated response to vascular injury, Ca^{2+} -mediated secretagogues such as histamine and thrombin locally promote a prothrombotic, proinflammatory state by instantaneously causing release of large quantities of VWF and other WPB constituents, while simultaneously increasing endothelial permeability and vascular tone [25,26]. In contrast, cAMP-mediated stimuli such as epinephrine and vasopressin act systemically, increase endothelial barrier function, and, when applied to cultured endothelial cells, induce a slow but sustained release of WPBs [27–30]. This pathway can be clinically exploited to correct prolonged bleeding times in patients with mild hemophilia A or VWD through administration of the vasopressin analog DDAVP, which mobilizes VWF from its endothelial stores following activation of vasopressin-2 receptor (V2R) [28,31]. Despite their distinct kinetic and physiological profiles, Ca^{2+} -dependent and cAMP-dependent

pathways converge at the same effector pathways, which control actin remodeling and tethering and fusion of WPBs, albeit in some cases with different outcomes (Fig. 3).

Increases in the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) occur after activation of phospholipase C (PLC) by ligand-bound G-protein-coupled receptors (GPCRs) [32]. Dose-dependent, subsecond release of WPBs in response to ionophores or caged Ca^{2+} implies that sustained elevation of $[\text{Ca}^{2+}]_i$ is a sufficient transduction signal to drive WPB fusion independently from additional receptor-triggered signaling [26,33]. In nearly every regulated secretory system, a vesicle-associated Ca^{2+} sensor is responsible for coupling transient elevations in $[\text{Ca}^{2+}]_i$ to the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) fusion machinery, but, so far, such a WPB-localized Ca^{2+} sensor has not been identified [34]. However, a number of other Ca^{2+} -sensing mechanisms have been reported to couple cytosolic Ca^{2+} to the endothelial exocytotic response. When Ca^{2+} is associated with the Ca^{2+} -sensor calmodulin (CaM), the Ca^{2+} –CaM complex binds to the N-terminus of the guanine nucleotide exchange factor (GEF) RalGDS, thereby unleashing its GEF activity towards the small GTPase RalA [35]. Activated RalA most likely coordinates a tethering step by promoting Arf6-dependent phospholipase D1 (PLD1) activity [36]. Local modification of phospholipids by PLD1 generates plasma membrane microdomains that recruit the Ca^{2+} -binding and phospholipid-binding

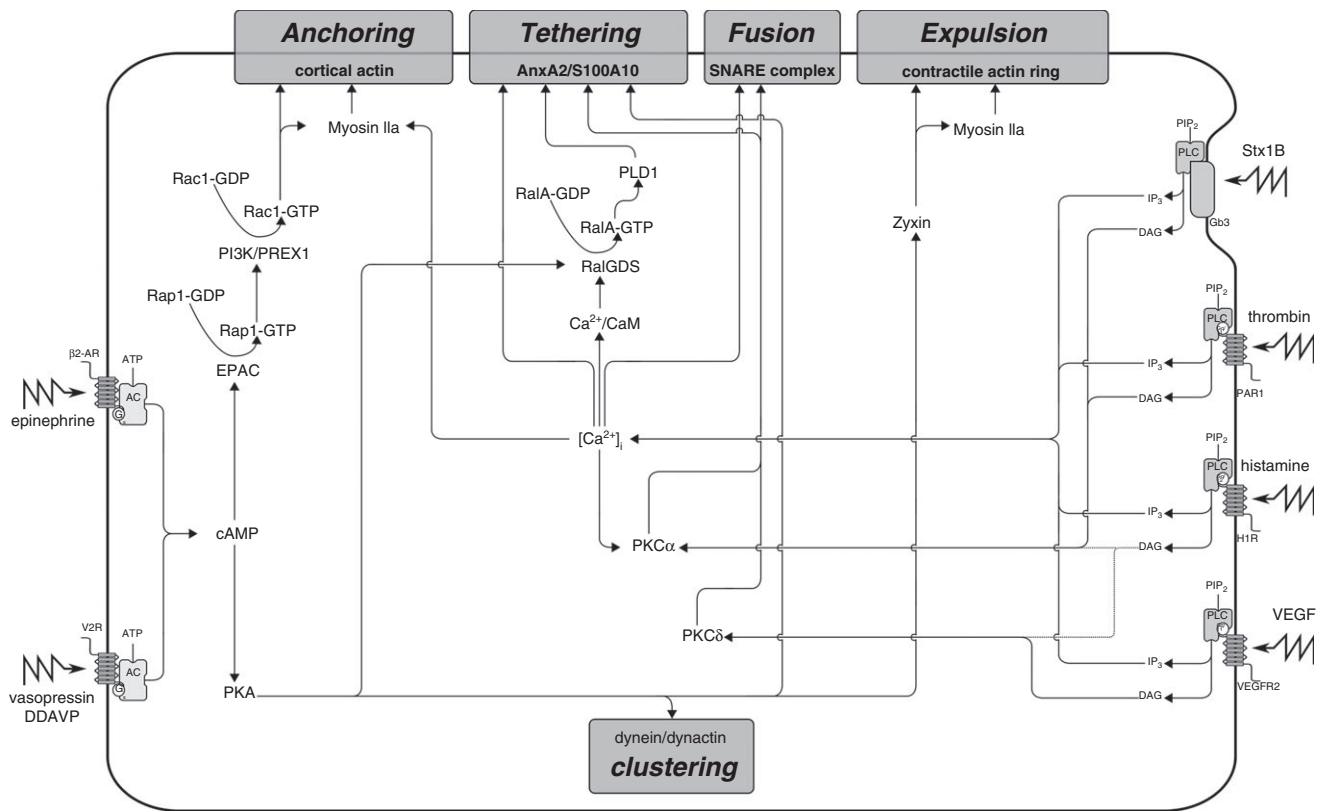


Fig. 3. Signaling cascades in Weibel–Palade body (WPB) exocytosis. Ca^{2+} -mediated and cAMP-mediated secretagogues that trigger WPB exocytosis use distinct and common signaling circuits that converge at effector pathways that control anchoring, tethering, vesicle fusion, and actin contractility. AC, adenyl cyclase; ATP, adenosine triphosphate; CaM, calmodulin; $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; DAG, diacylglycerol; EPAC, exchange protein that is directly activated by cAMP; Gb3, ceramide trihexoside; H1R, histamine H1 receptor; IP_3 , inositol 1,4,5-triphosphate; PAR1, protease-activated receptor 1; PI3K, phosphatidylinositol 3-kinase; PIP_2 , phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PLD1, phospholipase D1; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; Stx1B, Shiga toxin 1B; V2R, vasopressin-2 receptor; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; β_2 -AR, β_2 -adrenergic receptor.

AnxA2–S100A10 complex, which, in its turn, links WPBs to membrane fusion sites through the WPB-localized tethering factor Munc13-4 [37–39]. Ca^{2+} -mediated VWF secretion is also dependent on rapid changes in the actin cytoskeleton that lead to the formation of parallel stress fibers, a process that is primarily regulated by GTPases of the Rho family [29,40,41].

Triggering of the GPCR-coupled V2R and β_2 -adrenergic receptors activates cAMP-dependent protein kinase A (PKA) after conversion of ATP to cAMP [27,28]. PKA is critically involved in VWF secretion by activating a number of effector pathways [42], such as tethering of WPBs through the RalGDS–RalA pathway and the AnxA2–S100A10 tethering complex [30,35,38], and via phosphorylation of zyxin during contractile ring assembly [43]. During cAMP-mediated stimulation, a subset of WPBs avoid release by clustering together around the microtubule organizing center, as a result of dynein–dynactin-dependent retrograde transport activated by PKA [42,44]. Whether this is a stochastic process that serves to limit the secretory response or whether these WPBs represent a

specific subset of granules that are actively set aside, e.g. according to their recruited membrane components, state of maturation, or localization [15], is still unclear. The exchange protein that is directly activated by cAMP is also activated during cAMP-mediated WPB release, and catalyzes the activation of the small GTPase Rap1 [45,46]. Via a complex of its downstream effectors phosphatidylinositol 3-kinase and the Rac exchange factor PREX1, activated Rap1, in its turn, promotes activation of the Rho GTPase Rac1 [47]. The Rap1–Rac1 pathway is thought to promote secretion by rearranging actin into thin cortical bundles in close apposition to the plasma membrane and/or by regulating the formation or contractility of actin on the fusing WPB membrane [43].

Protein kinase C (PKC) is a key signaling mediator for a number of endothelial agonists that trigger secretion of VWF. Phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), are potent (non-physiological) stimulators of VWF secretion that directly activate PKC by mimicking the action of diacylglycerol [25]. Histamine, vascular endothelial growth factor (VEGF) and Shiga toxin 1B

(Stx1B) elevate $[Ca^{2+}]_i$ in a PLC-dependent manner, and simultaneously either signal through the Ca^{2+} -independent PKC δ (VEGF and histamine) or utilize the Ca^{2+} -dependent PKC α (Stx1B and histamine) [32,48–51]. Among its targets are components of the SNARE machinery, the assembly of which is partly regulated through PKC-dependent phosphorylation events [52]. Nevertheless, several ambiguities remain that suggest that PKC is not always essential for VWF secretion. Both VEGF and histamine activate PKC δ ; however, broad-range PKC inhibition or specific depletion of PKC δ significantly reduced secretory responses after VEGF but not after histamine administration [48]. PKC α is activated by Stx1B as well as by histamine, but is only essential for Stx1B-induced release; inhibition of PKC α did not block histamine-induced VWF secretion and, in some studies, even enhanced it [48,51,53–55]. Paradoxically, three independent studies showed that blocking Ca^{2+} signaling, which entirely abolishes VWF release induced by both histamine and PKC α -dependent Stx1B, was not sufficient to fully inhibit VEGF-induced VWF secretion [32,48,50,53]. The difference in requirement for PKC and Ca^{2+} raises intriguing questions about how common signaling pathways activated by different agonists are differentially utilized and integrated to control secretion.

Exocytotic machinery of WPBs

After emerging from the TGN, immature WPBs initially are secretion-incompetent because they have yet to acquire (parts of) the exocytotic machinery [56]. In a maturation-dependent manner, WPBs recruit several GTPases of the Rab family, including Rab27A, several Rab3 isoforms, and Rab15 [37,56–58]. Rab GTPases are molecular switches that cycle between a GDP-bound ‘off’ state and a membrane-associated, GTP-bound ‘on’ state, and generally contribute to defining organelle identity [59]. In their active GTP-bound state, the Rabs are responsible for the subsequent recruitment of a set of effector proteins (MyRIP, Munc13-4, and Slp4-a) to the WPB. This process correlates with the acquisition of secretion competence [56], and enables WPBs to interact with the cytoskeleton and/or plasma membrane (Fig. 4) [56,58,60]. MyRIP is a Rab27A-specific effector that binds actin, directly and via the actin motor protein myosin Va, and tethers WPBs to the actin cytoskeleton in the cell periphery [41,60,61]. The actin cytoskeleton plays opposing roles in the release of WPBs. On the one hand, it is necessary for peripheral distribution of WPBs, which is important for releasability and depends on myosin IIa [62]. On the other hand, actin acts as a barrier [29,40], and, by anchoring WPBs to the actin cytoskeleton, MyRIP acts as a brake during exocytosis [41,56,60]. Munc13-4 binds both Rab27A and Rab15, and promotes exocytosis by tethering WPBs to release sites on the plasma membrane, which contain the annexin A2–S100A10 complex [39,58].

Slp4-a interacts with Rab3 isoforms and Rab27A, in an activity-insensitive manner with the latter, and promotes WPB exocytosis [56] by providing the link between the WPB and members of the SNARE complex [63]. From this all, it becomes clear that, during acquisition of secretion competence, WPBs recruit a cocktail of Rabs and Rab effectors that individually perform opposing functions during secretion. Upon exocytosis, they engage in a tug of war in which the balance of effectors, their levels of activation by upstream signaling events and the efficacy of their downstream mechanisms decide the probability of release [56].

In the final phase of exocytosis, the WPB fuses with the plasma membrane; this is catalyzed by a ternary complex of SNARE proteins that are positioned on the two opposing membranes. SNARE proteins can be functionally classified as t-SNAREs, which are found on the target membrane, and v-SNAREs, which are found on the donor/vesicle membrane. The exocytotic SNARE complex consists of a v-SNARE of the vesicle-associated membrane protein (VAMP) family on the donor (i.e. WPB) membrane, and two t-SNAREs (one SNARE helix from a syntaxin, and two SNARE helices from a SNAP25 homolog) on the acceptor (i.e. plasma) membrane (Fig. 5). Together, they assemble into a four-helix bundle that, when it rolls up, brings donor and acceptor membranes together in a zipper-like motion, thereby largely overcoming the energy barrier that normally prevents fusion of membranes. WPBs contain two v-SNAREs, VAMP3 and VAMP8, of which only VAMP3 has so far been shown to support stimulus-induced WPB release [64,65]. t-SNAREs that take part in WPB release include the SNAP25 homolog SNAP23, syntaxin-3, syntaxin-4, and most likely also syntaxin-2 [52,63,66,67]. Owing to their promiscuous nature, various combinations of these SNAREs exist: in endothelial cells, syntaxin-4 engages with SNAP23 and VAMP3 or VAMP8 [65,67], and syntaxin-3 primarily interacts with VAMP8 and SNAP23 [67]. The SNARE partners of syntaxin-2 are still unknown. This suggests that endothelial cells can employ at least three (syntaxin-4–SNAP23–VAMP3, syntaxin-4–SNAP23–VAMP8, and syntaxin-3–SNAP23–VAMP8) and possibly more distinct SNARE complexes for release of WPBs. Interestingly, whereas syntaxin-4 is localized on the plasma membrane, syntaxin-3 is found on WPBs [67], suggesting that, in certain cases, WPBs can also function as the acceptor compartment during fusion.

The formation of SNARE complexes is controlled by syntaxin-binding proteins (STXBPs), several of which take part in WPB release. The Slp4-a interactor STXBP1 promotes VWF secretion and interacts with syntaxin-2 or syntaxin-3 [63], potentially controlling two separate downstream mechanisms for exocytosis. Munc18c (also known as STXBP3) controls the assembly of syntaxin-4-containing SNARE complexes. Depending on a (de) phosphorylation switch controlled by PKC α and protein

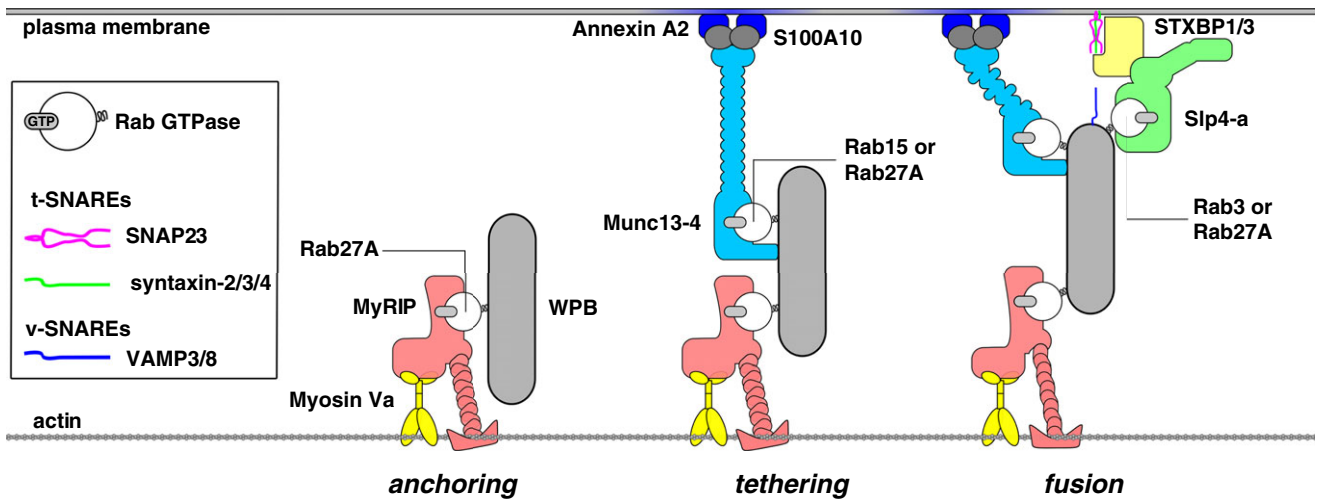


Fig. 4. The Weibel–Palade body (WPB) exocytotic machinery. Rab effector complexes mediate anchoring of WPBs to the cytoskeleton, tethering to the plasma membrane, and interactions with the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) fusion machinery. The Rab27A–MyRIP–myosin Va complex anchors WPBs to the actin cytoskeleton. Munc13-4 can be recruited by Rab GTPase-dependent (Rab15/Rab27A) or by Rab-independent mechanisms, and tethers WPBs to membrane fusion sites via the annexin A2–S100A10 complex. The Rab27A–Slp4-a complex docks WPBs and forms the link between the WPB and the SNARE complex via members of the syntaxin-binding protein (STXBP) family. VAMP, vesicle-associated membrane protein.

phosphatase 2B, phosphorylated Munc18c releases its grip on syntaxin-4 and allows the latter to engage with VAMP3 [52,68]. STXBP5 was initially implicated in the WPB exocytotic machinery as a genetic modifier of circulating VWF: a polymorphism encoding the non-synonymous N436S substitution in STXBP5 is linked to lower VWF plasma levels [69]. In endothelial cells, STXBP5 is a negative regulator of WPB release, and interacts with syntaxin-4 but not with SNAP23 [70]. Possibly, the C-terminal VAMP-like domain acts as a decoy for syntaxin-4, producing a non-fusogenic dead end, thereby lowering the number of syntaxin-4-containing SNARE complexes that can support exocytosis. Interestingly, the N436S variant of STXBP5 attenuates VWF secretion in endothelial cells [70], although the mechanism through which this substitution inhibits release is still unclear. The SNARE machinery is therefore highly dynamic and, according to its configuration, may promote or attenuate secretion, but exactly how all of these individual components together orchestrate WPB release remains unclear. Moreover, it raises the question of why endothelial cells utilize so many different SNARE complexes for exocytosis of the same secretory organelle.

Secretory modes of WPB exocytosis

Several different modes of stimulus-induced WPB exocytosis have been reported (Fig. 5A). Initially, a lipidic fusion pore is formed at the interface of vesicle and plasma membrane that establishes an aqueous channel between the WPB lumen and the extracellular space [71]. Recordings of fusion pore dynamics of serotonin-loaded WPBs by the use of amperometry revealed that this

transient structure exists for only a limited time (usually < 200 ms) before it rapidly expands within tens of milliseconds [72]. With its pore expanded fully, the WPB undergoes full fusion, resulting in the delivery of soluble granule cargo into the lumen and collapse of the vesicle membrane together with its associated membrane components into the plasma membrane [26,73]. In a subset of cases, the WPB fusion pore does not expand fully, but the pore lingers in a restricted state for some seconds before closing prematurely. The restricted diameter of the pore during this ‘lingering’ mode of fusion, so-called lingering-kiss exocytosis, acts as a molecular size filter: small cargoes, such as chemokines or the integral membrane protein CD63, are able to traverse the narrow pore that prevents the passage of larger cargo proteins such as VWF, VWF propeptide, and P-selectin. After resealing of the unexpanded fusion pore, these proteins are selectively retained in spherical WPBs, which probably owe their rounded morphology and the disruption of the orderly bundling of VWF tubules to the hydration of their paracrystalline interior [74].

Apart from these heterotypic fusion modes (fusion between different compartments, i.e. WPB and plasma membrane), homotypic fusion modes (i.e. WPB–WPB) have also been described. During compound fusion, several individual WPBs coalesce intracellularly prior to release, and then collectively undergo exocytosis as a single entity. The first evidence for this mode of fusion in endothelial cells was provided by measurements of membrane capacitance, which is proportional to cell surface area, stepwise increases of which are attributable to the addition of membrane during the fusion of secretory organelles. A significant proportion of discrete membrane

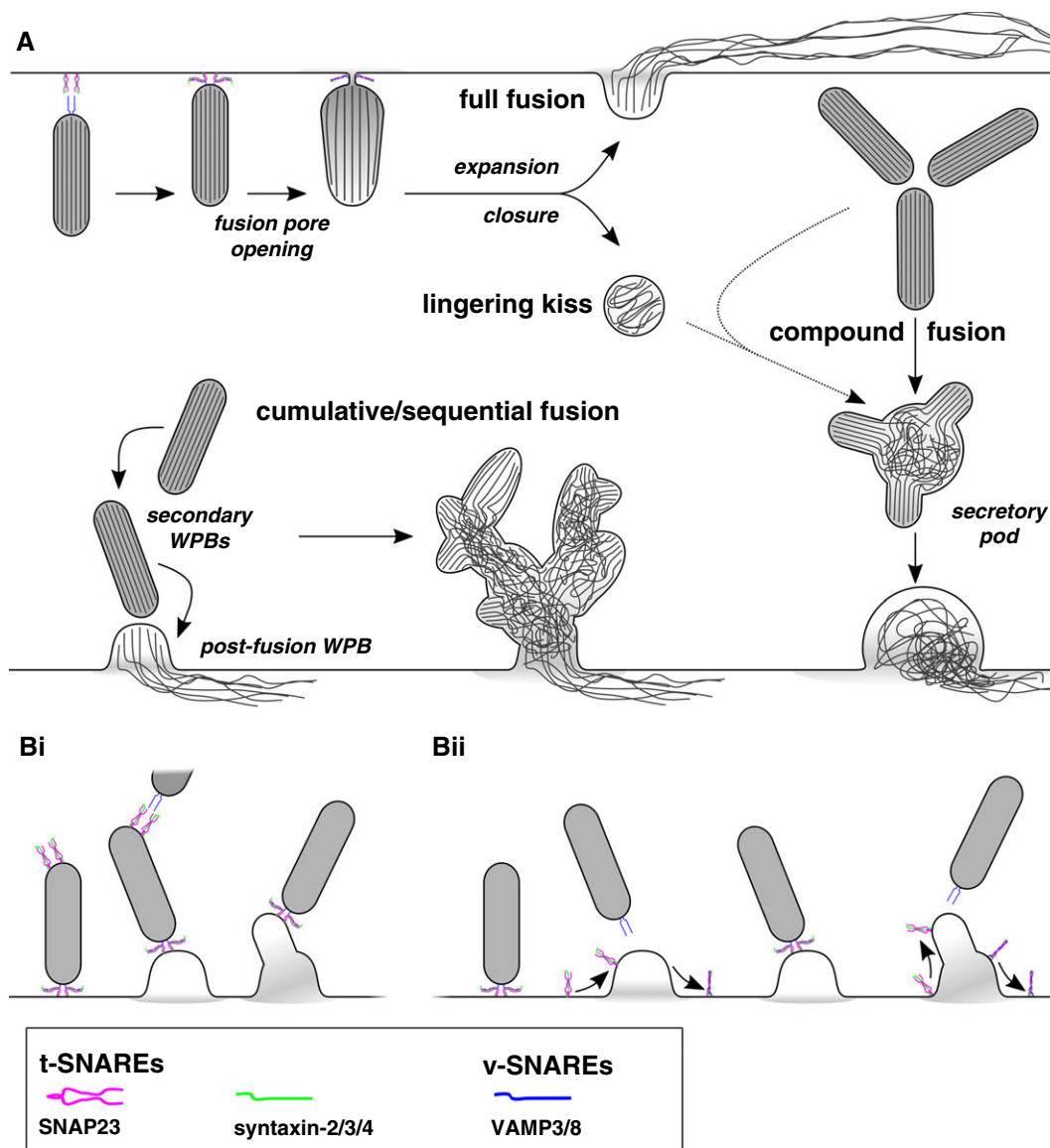


Fig. 5. Secretory modes of Weibel–Palade body (WPB) exocytosis. (A) Cartoon of a WPB with WPB-localized v-SNAREs (blue) engaging with target membrane-localized t-SNAREs (pink and green), thereby bringing together donor and acceptor membranes. Top: a narrow fusion pore is formed that permits release of small cargo. Lingering-kiss fusion resulting from premature closure of the fusion pore results in large cargo (von Willebrand factor [VWF]) being retained in a collapsed granule, whereas expansion of the fusion pore results in full fusion followed by explosive release of ultralarge VWF strings. Top right: compound fusion of WPBs, possibly including lingering-kiss end-products, leads to the formation of an enlarged secretory pod that eventually undergoes fusion at the plasma membrane. Bottom left: cumulative or sequential fusion, in which a primary granule undergoes fusion at the plasma membrane, which is followed by secondary fusion events in the postfusion WPB, ultimately leading to extensive, ginger root-like fusion structures. (B) Two models of SNARE-mediated cumulative fusion. (Bi) Sequential fusion events are supported by cognate SNARE assemblies on individual, prefusion WPBs. (Bii) Cumulative release is supported by rapid membrane mixing, recruiting plasma membrane SNARE components into the postfusion WPB. SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; VAMP, vesicle-associated membrane protein.

capacitance increases observed after evoking of WPB exocytosis with high levels of $[Ca^{2+}]_i$ were too large to be accounted for by the fusion of a single WPB, and could only be explained by fusion of an accumulated structure made up of several WPBs [33]. Morphological studies of such intermediate structures have shown that they consist of enlarged, rounded structures, termed secretory pods, which contain disordered VWF tubules [75]. Secretory

pods are prominent features following extended stimulation with PMA, but the occurrence of similar structures has also been demonstrated *in vivo* in response to activated FX or histamine in toads and rats, respectively [76,77]. Despite the loss of tubular organization of VWF multimers in the spherical secretory pods, VWF strings can eventually emerge from the extruded material [78]. Finally, by monitoring of the redistribution of EGFP–

CD63 through the membranes of fusing granules, a related but mechanistically distinct mode of homotypic WPB fusion, termed sequential or cumulative exocytosis, was observed [79]. In this mode, a postfusion WPB is used as a membrane fusion site for subsequent cumulative fusion of additional WPBs, culminating in the ginger root-like fusion structures that were observed in ultrastructural studies [76,80].

A number of questions remain. First, what is the molecular basis for all of these different release mechanisms? The specificity of membrane fusion during exocytosis is mediated by the interaction of SNAREs on secretory organelles with their cognate SNAREs on the target membrane. In cases of homotypic fusion, such as during secretory pod or cumulative exocytosis, this would require the formation of a *trans*-complex consisting of v-SNAREs and t-SNAREs that are positioned on opposing WPBs (Fig. 5Bi). Within the extensive set of SNAREs that have, in one way or another, been implicated in the WPB exocytotic machinery, the pair composed of VAMP8 and syntaxin-3 has been shown to sustain homotypic fusion modes in cells in which both were found on secretory organelles [65,67,81,82]. An alternative explanation is offered by the rapid transfer of plasma membrane proteins to fusing WPBs through membrane mixing, such as was observed for Rab35 [79]. Possibly, this confers plasma membrane identity to the postfusion WPB, including the acquisition of components of the fusion machinery from the plasma membrane, such as the cognate SNAREs for WPB-localized VAMPs (Fig. 5Bii). This also raises the possibility that secretory pods represent an intermediate state during cumulative fusion into WPBs that have previously undergone lingering-kiss exocytosis.

Second, what is the use of all these distinct modes of exocytosis? Indirect or incomplete fusion modes are, in certain cases, energetically favorable. Homotypic fusion modes can serve to concentrate cargo release at hotspots when access to fusion sites on the membrane becomes limiting. It may also augment the secretory response by providing indirect access to the plasma membrane to a pool of WPBs situated deeper into the cytoplasm [80]. Lingering-kiss fusion not only saves vesicle reformation and retrieval of vesicle membrane proteins, but may also help in preserving a delicate balance between endocytosis and exocytosis by limiting membrane expenditure. Exocytosis–endocytosis coupling is pertinent to the choice of secretory mode, as pharmacological blocking of compensatory endocytosis resulted in a shift from full fusion towards cumulative exocytosis, possibly because of the inability to retrieve membrane components from the postfusion WPBs [80]. Distinct fusion modes also allow the endothelium to fine-tune its secretory response to specific vascular conditions: lingering-kiss fusion releases only a subset of molecules, such as chemokines, whereas prothrombotic cargo is not released [74]. Postfusion entanglement of VWF tubules in both compound and cumulative

fusion structures does not preclude the eventual formation of VWF strings, but results in shorter strings when cumulative fusion is promoted [78,80].

Content expulsion

Secretory cargo expulsion is dependent on lateral tension in the plasma membrane; relaxation of this tension through the addition of new membrane from fusing granules opens the fusion pore and provides a force to drive secretory cargo out; this is followed by full collapse of the vesicle into the plasma membrane [83]. However, the near-solid VWF paracrystal presents a significant barrier to release, as its enormous size and state will naturally resist discharge and membrane collapse. Recent studies have shown that endothelial cells, depending on their type of activation, can utilize fundamentally different modes of secretory content expulsion.

The rapid process of content expulsion during Ca^{2+} -mediated full fusion of WPBs bears the hallmarks of a jack-in-the-box mechanism that is often observed when condensed, polyionic molecules such as VWF need to undergo a phase transition before release [84]. Exchange of ions and entry of water molecules into the vesicle interior through the stalk-shaped fusion pore leads to hydration of the vesicle core and rapid neutralization of the acidic milieu of the WPB [26]. Dissolution of the proton gradient and other cationic species such as Ca^{2+} that are crucial for the condensed tubular packing of VWF [85,86] will result in loss of shielding of negative charges in VWF. The consequential charge repulsion within and between VWF multimers leads to decondensation of cargo, as shown by the swelling of the granule and increased separation between VWF tubules [71]. This is followed by an explosive discharge of ultralarge VWF multimers and other cargo on a subsecond timescale, consistent with a mechanism in which VWF tubules act as electrostatic springs [26,73,87,88].

An alternative mechanism of VWF expulsion has been observed in response to Ca^{2+} -independent secretagogues, such as cAMP-mediated agonists or phorbol esters. Prior to fusion, local rearrangements of cortical actin lead to the formation of actin frameworks that appear to encage WPBs. The formation of such frameworks is dependent on the tension-sensitive focal adhesion protein zyxin following its phosphorylation by PKA, and positions WPBs close to the membrane [43]. Then, shortly after opening of the fusion pore, an actomyosin ring is assembled coating the distal end of the fusing granule, a process that involves *de novo* actin polymerization [43,89]. Inhibition of the myosin IIa-dependent contractility of the actomyosin ring prevented VWF release from fusing WPBs, while not affecting the secretory fate of P-selectin [89] (but not in [62]), leading to the hypothesis that contraction of such rings provides the necessary physical force to squeeze VWF out of the fusing granule. It is currently

unclear what initiates actomyosin ring formation on the postfusion WPB, but the involvement of the tension sensor zyxin [43] suggests that changes in local (membrane) tension, such as on a swelling granule, may be an important factor in this process. Actomyosin ring contractility is regulated by phosphorylation of myosin IIa, which has been shown to involve zyxin-dependent recruitment of casein kinase II [62]. Interestingly, zyxin depletion did not impair VWF release by thrombin or histamine, which is consistent with the observation that actomyosin rings do not play an important role in expulsion of VWF during Ca^{2+} -mediated WPB exocytosis [43,87].

WPBs in health and disease

Circulating levels of VWF vary widely across the human population [90]. The clinical manifestations at the far ends of this distribution highlight the pivotal role that VWF plays in vascular health. Low levels of plasma VWF lead to bleeding, such as in the inherited bleeding disorder VWD [5]. In some cases, this is the result of mutations in *VWF* that lead to altered WPB morphology and decreased releasability, such as is evident in VWD blood outgrowth endothelial cells [91]. Increased levels of circulating VWF are associated with cardiovascular morbidities, such as coronary heart disease, ischemic stroke, and venous and arterial thrombosis [92,93], which are pathologies for which the risks are reduced in VWD patients [94]. The variation in VWF plasma levels across the population is largely genetically determined [95]. Population-based linkage analyses in healthy individuals have identified a number of genetic loci that are associated with VWF levels, including the *ABO* locus and the clearance receptors *CLEC4M* and *STAB2*, but also genes encoding several components of the SNARE machinery (*STX2* and *STXBP5*) [69,96]. One of the SNPs in *STXBP5* that was linked with higher VWF levels is also associated with venous thrombosis [97]. Variants in both *STX2* and *STXBP5* are also associated with VWF levels and disease severity in VWD patients [98]. Together, these findings show that SNARE-mediated secretion from WPBs is an important determinant of VWF plasma levels. This fits with the current consensus that most circulating VWF, which primarily consists of high-multimer VWF, originates from the storage pathway rather than from constitutive secretion [12,18,19]. Whether this arises from the basal compartment or stimulated compartment may be less evident. Several of the Rab-associated and SNARE-associated regulators of stimulated WPB exocytosis also affect the basal secretion of VWF [56,63,67], suggesting that the same SNARE fusion machinery is also responsible for basal release. Basal secretion accounts for the bulk of VWF that endothelial cells produce and release over time. How reflective this is of the situation *in vivo*, in which some areas of the vasculature may be under the continuous influence of low-level stimulation by

hormones such as epinephrine or vasopressin, is unclear. However, on the basis of extrapolation of the ratio of basal to stimulated release from cultured endothelial cells, the area of vascular bed that needs to be to be maximally activated at any given time to match what is secreted basally makes it unlikely that stimulated release contributes significantly to steady-state VWF levels [19].

Another pathology that may arise from disturbed release from WPBs is angiodysplasia, which is a common complication in VWD patients that causes recurrent gastrointestinal bleeding through small vascular malformations in the gut. Recent studies have shown that VWF regulates angiogenesis, extracellularly by controlling the cell surface expression of $\alpha_v\beta_3$ integrin and/or indirectly by influencing the release of one of its WPB coresidents, the angiogenic mediator Ang-2 [99]. In the absence of its storage compartment, such as occurs in severe type 3 VWD, Ang-2 is secreted constitutively, promoting vessel destabilization. Apart from Ang-2, a number of other angiogenic mediators, such as insulin-like growth factor-binding protein 7, galectin-1, and galectin-3, are found in WPBs [7,100], and may be subject to similar dysregulated release in the absence of VWF, potentially further contributing to pathological angiogenesis.

Conclusions and future directions

Endothelial cells control secretion from their WPBs by using a remarkably complex exocytotic machinery, the outlines of which are now starting to become clear. However, our knowledge of the exact composition of this machinery is incomplete and, importantly, we are still a long way from understanding how all of these components are able to direct the different modes of WPB fusion. Also, we currently do not understand how expansion of the fusion pore is regulated or what drives content expulsion in response to different endothelial activation states. To obtain a deeper understanding of these processes, we will need to determine the changes to the characteristic ultrastructure of WPBs during fusion and content expulsion, and to link these changes to individual components of the exocytotic machinery. Understanding the mechanism and purpose of the endothelium's ability to fine-tune its secretory response to vascular events would potentially allow us to exploit this mechanism for therapeutic benefit in the treatment of hematological and cardiovascular diseases.

Acknowledgements

We apologize to all authors whose work has been omitted owing to space restrictions, and for not always citing primary literature. Work in our laboratory is supported by grants from the Landsteiner Stichting voor Bloedtransfusie Research (LSBR-1244 and LSBR-1707), the Netherlands Ministry of Health (PPOC-2015-24P and PPOC-

2018-21), the Dutch Thrombosis Foundation (TSN-56-2015 and TSN-2017-01), and a Research Fellowship from the European Hematology Association (EHA).

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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