Cell, Vol. 115, 851–862, December 26, 2003, Copyright ©2003 by Cell Press

Serotonylation of Small GTPases Is a Signal Transduction Pathway that Triggers Platelet α-Granule Release

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Summary

Serotonin is a neurotransmitter in the central nervous system. In the periphery, serotonin functions as a ubiquitous hormone involved in vasoconstriction and platelet function. Serotonin is synthesized independently in peripheral tissues and neurons by two different rate-limiting tryptophan hydroxylase (TPH) isoenzymes. Here, we show that mice selectively deficient in peripheral TPH and serotonin exhibit impaired hemostasis, resulting in a reduced risk of thrombosis and thromboembolism, although the ultrastructure of the platelets is not affected. While the aggregation of serotonin-deficient platelets in vitro is apparently normal, their adhesion in vivo is reduced due to a blunted secretion of adhesive α-granular proteins. In elucidating the mechanism further, we demonstrate that serotonin is transamidated to small GTPases by transglutaminases during activation and aggregation of platelets, rendering these GTPases constitutively active. Our data provides evidence for a receptor-independent signaling mechanism, termed herein as "serotonylation," which leads to α-granule exocytosis from platelets.

Introduction

Cardiovascular and cerebrovascular diseases are the leading causes of mortality throughout the world. Blood platelets play a central role not only in hemostasis but also in the pathogenesis of thrombosis and atherosclerosis, with platelet adhesion to a damaged blood vessel being the initial trigger for the formation of either an effective hemostatic plug or an intravascular thrombus (Denis et al., 1998; Ni et al., 2000; Jackson et al., 2003). Platelets adhere to the subendothelium through an interaction with von Willebrand factor (vWf), which forms a bridge between collagen within the damaged vessel wall and the platelet receptor glycoprotein GPIb. At in vivo rheological situations where platelets are flowing with high speed in the bloodstream, the only reaction that can initiate thrombogenesis is the interaction of vWf with GPIb. This reversible adhesion allows platelets to roll over the damaged area, which is then followed by a firm adhesion mediated by the collagen receptors α2β1 and GPVI. Therefore, the importance of vWf in promoting both primary adhesion and aggregation under high-shear conditions is now well established. However, the efficiency with which platelets adhere and aggregate at sites of vessel wall injury is dependent on the synergistic action of various adhesive and soluble agonist receptors, and the contribution especially of the soluble agonists remains obscure.

Serotonin (5-hydroxytryptamine; 5-HT), was first isolated fifty-five years ago and identified to be the vasoconstrictor compound contained in serum (Rapport et al., 1948). While its pressor effect has been investigated in great detail (Cohen et al., 1983), only little attention has been paid to the aggregation response of blood platelets upon 5-HT stimulation. In contrast to adenosine diphosphate (ADP), 5-HT is considered only as a weak agonist although ADP and 5-HT are both highly concentrated in platelet dense bodies together with adenosine triphosphate (ATP) and Ca^{2+} cations (653 mM, 65 mM, 436 mM, and 2.2 M, respectively; McNicol and Israels, 1999). The interest in further studies into the role of 5-HT has recently even been diminished by the G protein hypothesis which postulates that platelet aggregation requires the concomitant activation of at least one Gi- and one Gq-coupled receptor (Maayani et al., 2001a). The 5-HT2A receptor (5-HT2AR) has been shown to be the only serotonergic receptor on the platelet membrane (Killam and Cohen, 1991) mediating Gq-dependent intracellular Ca^{2+} mobilization and protein kinase C (PKC) activation (Offermanns et al., 1997). However, characterization of the 5-HT action has been elusive, and moreover, deaggregation is an integral component of the platelet response to ADP (Maayani et al., 2001b) suggesting a degree of complexity to the overall response that is not explained by our current knowledge of platelet physiology.

In addition to the 5-HT2AR, the plasma membrane 5-HT reuptake transporter (SERT) has been found highly expressed in platelets (Lesch et al., 1993). Serotonin specific reuptake inhibitors (SSRI), such as fluoxetine (Flx) attenuate aggregation and dense and α-granule secretion by a yet to be identified mechanism (Serebruany et al., 2001). In contrast, another reuptake blocker, (+)-fenfluramine (Fen) has been proven to have little inhibitory effects on ADP- or collagen-induced shape change and aggregation and elicits no aggregation by itself (Buczko et al., 1975; Barradas et al., 1983). It is common knowledge, that Flx acts directly and specifically on SERT while Fen after being transported into the cytoplasm by SERT also acts on the vesicular monoamine transporter 2 (VMAT2) and reverses the transport

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by VMAT2 as well as by SERT thereby releasing 5-HT by a nonexocytotic mechanism. Explanations for these pharmacological findings are further complicated by the fact that 5-HT2AR antagonists alone attenuate aggregation and platelet-vessel wall interactions (De Clerk et al., 1984) by an unknown mechanism.

As mentioned above, vWf is an essential player in the platelet-vessel wall interaction (Denis et al., 1998). Vascular endothelial cells (ECs) synthesize and release vWf either constitutively or by a regulated pathway triggered by an unknown mechanism from Weibel-Palade bodies at the place of vascular injury. As reported by Palmer et al. (1994) and confirmed later (Schlüter and Bohnensack, 1999), 5-HT enhances vWf secretion from ECs in vitro, raising the question, whether 5-HT could be an effector for vWf secretion, not only from ECs, but also from platelets, in primary hemostasis.

Although platelets only contain 20% of the vWf present in the circulation, they store multimeric forms of vWf that are much larger than those found in plasma (Siess, 1989). In ECs, the same larger multimeric forms are stored in Weibel-Palade bodies. Interestingly, patients with von Willebrand disease type IIA who only lack the larger multimeric forms but apart from that have normal vWf concentrations in the circulation show a prolonged bleeding time (Siess, 1989), indicating that the larger multimeric forms of vWf, stored in platelets and ECs, are predominantly active in primary hemostasis, especially at high-shear stress rates (Schoenwaelder et al., 2002). Platelet α-granules are exocytosed only a few seconds after platelet stimulation whereas Weibel-Palade body release from ECs takes up to 30 min (Palmer et al., 1994). Therefore, platelet-derived, highly multimeric vWf must be considered to be of higher importance in primary hemostasis than vWf from ECs.

It has recently been shown that the platelet transglutaminase (TG) FXIIIa uses 5-HT as a substrate to transamidate vWf and other proaggregatory proteins stored in α-granules in dually collagen and thrombin activated platelets (Dale et al., 2002) thereby enhancing the adhesive properties of these proteins (Szasz and Dale, 2002). Moreover, tissue TG has also been detected in platelets (Puszkin and Raghumaran, 1985). TGs constitute a superfamily of enzymes that catalyze the Ca^{2+}-dependent crosslinking of proteins via the γ-carboximide group of glutamine residues and the ϵ-amino group of lysine residues but are also able to covalently link simple primary amines, such as 5-HT, to glutamine residues (Dale et al., 2002). TGs are not only implicated in the crosslinking of fibrin and extracellular matrix proteins (Greenberg et al., 1991; Citron et al., 2000), but also in synapse stabilization (Citron et al., 2000) and apoptosis (Melino et al., 1994). Moreover, *Bordetella* dermonecrotic toxin (DNT) has been identified as a TG and *E. coli* cytotoxic necrotizing factors (CNF) as deamidases that transamidate and deamidate the small GTPase RhoA (at Gln63), and Rac1 and Cdc42 (at Gln61) (Schmidt et al., 1997, 1999; Aktories et al., 2000; Doye et al., 2002; Masuda et al., 2000). As a consequence the intrinsic GTP-hydrolyzing activity is blocked rendering these small GTPases constitutively active for their respective signaling pathways.

Interestingly, RhoA, Rac1, Cdc42, Rab4, and other small GTPases are directly involved in signaling during platelet aggregation, especially in mediating exocytosis and cytoskeleton rearrangement (Shirakawa et al., 2000; Azim et al., 2000; Schoenwaelder et al., 2002). Therefore, it was tempting to speculate that 5-HT might be rapidly transamidated to small GTPases during platelet activation and aggregation, immediately leading to a substantial increase of constitutively active signaling pathways.

Recently, we have generated mice deficient for the rate-limiting enzyme in the 5-HT biosynthesis, tryptophan hydroxylase (TPH), to elucidate the multitude of physiological effects of this monoamine in primary hemostasis, immune responses, neurophysiology, and embryology (Walther and Bader, 1999; Walther et al., 2003). We discovered a second TPH isoform specifically expressed in neurons, and have named this second isoform TPH2 and the previously known isoform TPH1 (Walther et al., 2003). *Tph1*$^{-/-}$ mice contain normal levels of 5-HT in the brain, but are depleted of the monoamine in peripheral tissues and fluids (Walther et al., 2003), rendering these animals an excellent model for studies on the controversially discussed multiple effects of 5-HT outside the central nervous system, especially in primary hemostasis.

Here, we show that α-granule secretion depends on high cytoplasmic 5-HT and Ca^{2+} concentrations and that 5-HT is then transamidated to small GTPases rendering these GTPases constitutively active. Thus, we have identified a receptor-independent intracellular signaling pathway for 5-HT.

Results

Bleeding Time Prolongation and Platelet Function in *Tph1*$^{-/-}$ Mice

Survival is identical in *Tph1*$^{-/-}$ and wild-type mice, and blood platelet counts do not differ between wild-type mice (730,000 $\pm$ 70,000/μl; n = 5) and TPH1-deficient mice (790,000 $\pm$ 200,000; n = 5). The platelets of *Tph1*$^{-/-}$ mice contain only minute amounts of 5-HT (Walther et al., 2003; Höltje et al., 2003). Their morphology is normal as evaluated by electron microscopy, and they are able to take up 5-HT by SERT and to pack it into dense granules by VMAT2 by a Gαq-modulated mechanism (Höltje et al., 2003). Therefore, we could detect no storage pool deficiency (SPD) in *Tph1*$^{-/-}$ mice. However, the consequence of platelets lacking only 5-HT is a major bleeding diathesis leading to a four times prolonged bleeding time of experimentally severed tail tips, as compared to control animals (Figure 1A). This phenotype was completely reversed by application of 5-HT 15 min prior to severing the tail tips (Figure 1B). Conversely, a similar phenotype and its rescue have been described for beige mice, which are a model for the mostly lethal human Chediak-Higashi syndrome (CHS) defined by a prominent SPD (Holland, 1976).

Beige mice platelets lacking 5-HT exhibit reduced aggregability, suggesting that reduced platelet aggregability could be one reason for the impaired primary hemostasis also in *Tph1*$^{-/-}$ mice. But surprisingly, *Tph1*$^{-/-}$ platelets exhibited normal aggregation responses to collagen, thrombin, and the thromboxane A2 mimetic U46619 in vitro (Figure 1C). Our data and the bleeding time reversal by 5-HT application alone in the CHS model

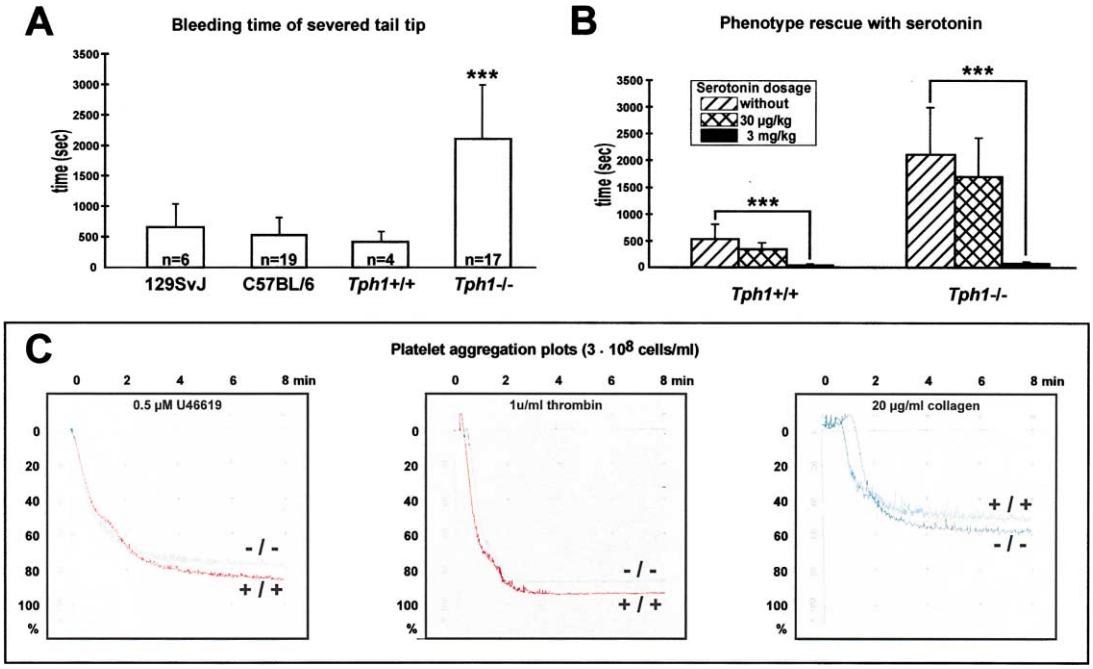


Figure 1. Impaired Primary Hemostasis in *Tph1*$^{-/-}$ Mice

(A) Prolonged bleeding time of *Tph1*$^{-/-}$ mice.

(B) Phenotype rescue by intracardial 5-HT-application. The indicated 5-HT amounts correspond to 1/10 and 10 times the normal blood concentration after application. ***, $p < 1 \times 10^{-5}$ ($n = 4–8$).

(C) Representative platelet aggregation plots in Tyrode-HEPES buffer with the thromboxane A_2 mimetic U46619 (0.5 μM), thrombin (1 u/ml), and collagen (20 μg/ml) as proaggregatory substances. *Tph1*$^{-/-}$ and *Tph1*$^{+/+}$ platelets aggregate to the same extent under the investigated conditions.

(Holland, 1976) suggest a not yet characterized central role of 5-HT in the primary hemostasis process. However, an odd of in vitro aggregation studies is that irreversible and reversible aggregation (agglutination) is hardly distinguishable. In vivo, irreversible aggregation rather than agglutination leads to mortal thromboembolism and to vessel occlusion in thrombosis. Prompted by these contradictory data, we hypothesized that *Tph1*$^{-/-}$ platelets might have reduced adhesive properties.

5-HT-Induced Release of α-granular vWf from Platelets

Since vWf is a major factor in irreversible platelet aggregation and adhesion, we hypothesized that 5-HT normally triggers its release, resulting in a blunted secretion of vWf from α-granules of *Tph1*$^{-/-}$ platelets lacking 5-HT. In plasma, steady-state levels of vWf and of blood coagulation factor FVIII, which is bound and stabilized by vWf, were not significantly different between *Tph1*$^{-/-}$ mice and their wild-type littermates (vWf: *Tph1*$^{+/+}$: 100 $\pm$ 11%, *Tph1*$^{-/-}$: 89 $\pm$ 30%; n = 6; FVIII: *Tph1*$^{+/+}$: 100 $\pm$ 20%, *Tph1*$^{-/-}$: 86 $\pm$ 29%; n = 6). Thus, at least the synthesis and constitutive secretion of vWf is unaffected in *Tph1*$^{-/-}$ animals.

A significant reduction of about two thirds of the pathologically prolonged bleeding time was obtained by infusion of vWf, which was without effect in wild-type mice (Figure 2A). Since a direct in situ measurement of the rapid processes at the site of vascular injury is technically not possible, we confirmed our hypothesis in vitro using washed wild-type platelets. 5-HT alone induced a 20% release of α-granular vWf contents and a collagen release of 45%, as detected in the supernatants of aggregated platelets by a vWf ELISA (Figure 2B). Although ADP potentiated the 5-HT-induced release, no significant concentrations of vWf could be detected in the supernatants of platelets, induced with ADP alone.

Low 5-HT Protects against Thrombosis and Thromboembolism

Tph1$^{-/-}$ animals are protected against thrombosis in a well-established in vivo model. $FeCl_3$ induces vascular injury and thrombus formation by removal of the endothelium and exposure of the subendothelial matrix (Ni et al., 2000). In wild-type animals, two minutes after $FeCl_3$-application, numerous fluorescence-labeled, adherent platelets were visible in the injured areas by using intravital microscopy (Figure 3A). Moreover, formation and growth of thrombi proceeded all over the recorded time culminating in vessel occlusion. However, in the *Tph1*$^{-/-}$ mice, platelet interactions with the vessel wall were unstable and thrombi in these mice failed to resist high shear stress and eventually detached from the subendothelial matrix (Figure 3A).

When thromboembolism is induced by the injection of collagen and epinephrine the survival rate of *Tph1*$^{-/-}$ mice is increased and the survival time of the animals which died, prolonged (Figure 3B), also indicating reduced stability of formed thrombi, in accordance to our

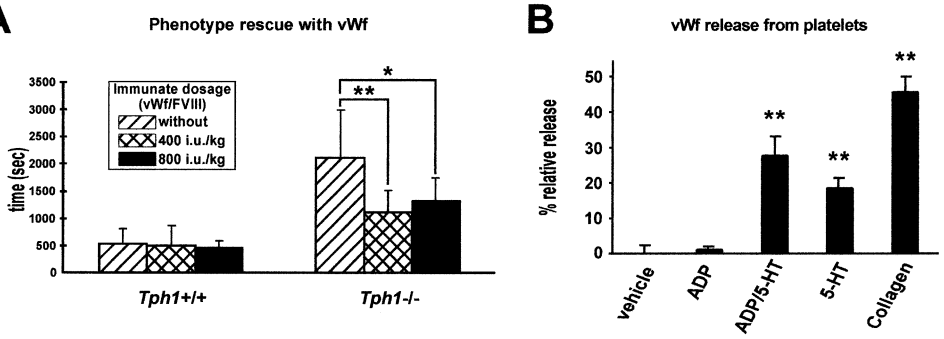


Figure 2. Rescue of the Bleeding Phenotype with vWf Infusion and vWf Secretion in Vitro

(A) Partial phenotype rescue by intracardial vWf-application in the indicated doses. The effect is specific for the *Tph1*$^{-/-}$ mice. *, $p < 0.05$; **, $p < 0.01$ ($n = 4–8$).

(B) Secretion of α-granular contents from aggregating wild-type platelets in vitro using vWf as a marker. Collagen-induced (30 μg/ml) vWf release is more effective than the induction with the downstream mediators. Vehicle and ADP (14 μM) alone do not induce the release of α-granular contents, but a significant 5-HT-induced (12 μM) release can be detected. The 5-HT-induced vWf secretion is potentiated if ADP is added together with 5-HT (14 μM and 12 μM, respectively). *, $p < 0.01$ ($n = 3$).

proposal that 5-HT is the crucial effector of the regulated secretion of vWf in primary hemostasis in vivo.

Cytoplasmic 5-HT and Ca^{2+} Are a Prerequisite for Platelet Aggregation

Collagen stimulation induces a pronounced shape change and culminates in aggregation in both wild-type and *Tph1*$^{-/-}$ platelets (Figures 1C and 4A). An initial dense body secretion, liberating 5-HT, ADP, and Ca^{2+}, drives this reaction, which in turn induces α-granule release (Siess, 1989; Shirakawa et al., 2000). The synergistic action of ADP, Ca^{2+}, and 5-HT may explain the preserved aggregation of *Tph1*$^{-/-}$ platelets in vitro, given that they contain about 5% of wild-type 5-HT amounts (Höltje et al., 2003), which appears to be still enough to support the reaction together with the other secretagogues.

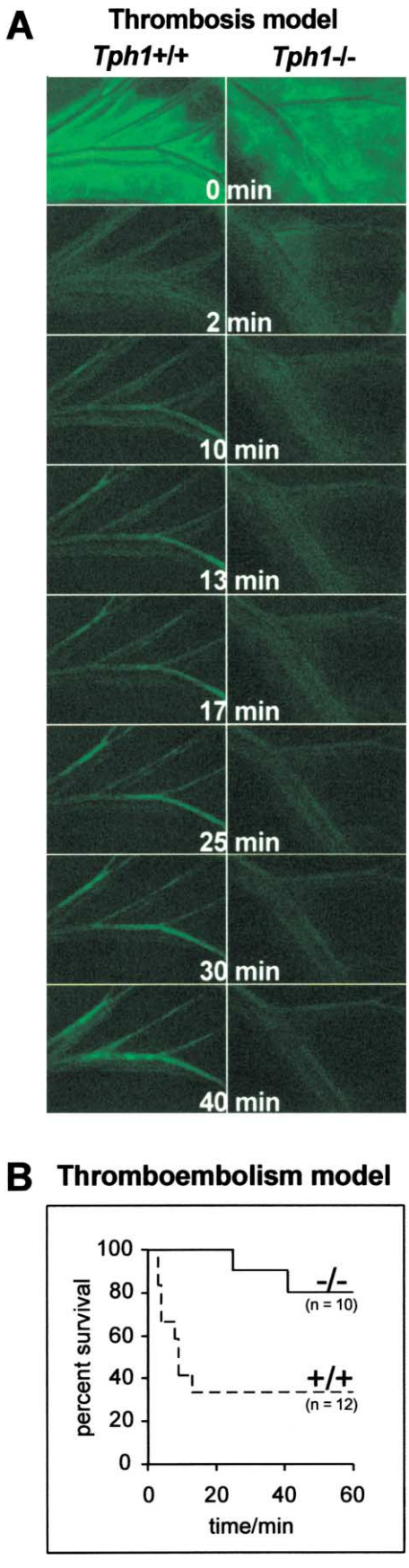
However, exogenously added ADP elicits a completely reversible aggregation in platelet-rich plasma (Figure 4B), meeting our finding that ADP does not induce α-granule release from washed platelets (Figure 2B). Although it has been reported previously that the response to ADP is not accompanied by granule secretion (Maayani et al., 2001a, 2001b; Mohammad et al., 1975) the underlying mechanism is still unknown. However, even isolation procedures for native platelets have been developed based on these findings (Mohammad et al., 1975). Conversely, apyrase added in excess to break down all secreted ADP leads to a delayed platelet aggregation without a preceding shape change after collagen stimulation, and the requirement for 5-HT of the remaining reaction is now discernable in *Tph1*$^{-/-}$ platelets as compared to wild-type platelets (Figure 4C). However, since *Tph1*$^{-/-}$ platelets contain only about 5% of the wild-type 5-HT, the small difference in this response suggests a 5-HT-mediated signaling mechanism that is largely potentiated by downstream effectors. Prompted by these findings, we designed a pharmacological approach to prove whether a cytosolic rise in the 5-HT concentration ($[5\text{-HT}]_i$) is involved in α-granule secretion (Figure 4D). Albeit contradictory to the G protein hypothesis, Fen + Flx-pretreated platelets immediately aggregate upon stimulation with the 5-HT2AR agonist α-methyl-serotonin (α-ms; Figures 4E and 4F) at concentrations that elicit no response (180 nM) or only a weak, reversible aggregation on untreated platelets (1.8 μM; Figure 4G). The dependency on cytosolic $[5\text{-HT}]_i$ can be appreciated by the delayed aggregation of *Tph1*$^{-/-}$ platelets compared with wild-type platelets (Figures 4E-4H) and the delay of Fen-treated versus Fen + Flx-treated *Tph1*$^{-/-}$ platelets (Figures 4E and 4F). Since 5-HT2AR stimulation leads to Ca^{2+} mobilization (Offermanns et al., 1997), high cytoplasmic $[5\text{-HT}]_i$ and $[Ca^{2+}]_i$ must be considered a prerequisite for platelet aggregation.

Moreover, we found that under our experimental conditions about 40% of preloaded ^{14}C-5-HT remain in a nonreleasable pool in the platelet aggregates after complete aggregation. Homogenization of the aggregates liberates almost all ^{14}C-5-HT into the soluble fraction, while only a small but significant amount remains in the insoluble fraction (Figure 4I). Similar pools of "unreleasable" 5-HT have been reported previously (Shirakawa et al., 2000), although without explanations for this phenomenon. Interestingly, the retained 5-HT can be roughly estimated to reach very high cytoplasmic concentrations, of up to 500 to 600 μM.

5-HT-Transamidation of Small GTPases

The secretion of α- and dense granules has been dissected showing that α-granule release is regulated by Rab4 (Shirakawa et al., 2000) and that extensive cytoskeletal rearrangement occurs during shape change and aggregation via RhoA signaling (Nishioka et al., 2001; Bodie et al., 2001).

Prompted by our findings that high $[5\text{-HT}]_i$ and $[Ca^{2+}]_i$ are a prerequisite for secretion and aggregation, we formed our hypothesis that platelet TGs could be mechanistically involved, since 5-HT is a substrate and Ca^{2+} an activating signal for many of these enzymes (Dale et al., 2002). Streptolysin O-permeabilized wild-type platelets lose over 80% of their cytosol and can be induced to secrete about 40% of their α-granular contents upon Ca^{2+} addition when reconstituted with brain cytosol (Fig-



ure 5A), as has been reported previously (Shirakawa et al., 2000). Interestingly, brain cytosol (BC) is very rich in TGs (Maggio et al., 2001; Tucholski et al., 2001; Citron et al., 2000). In contrast, skeletal muscle cytosol (SMC), which in comparison to BC is poor in TGs (Lee et al., 2000), does not support α-granule secretion even after addition of exogenous 5-HT (Figure 5A). Moreover, exogenously added TG together with SMC leads to an extensive secretion of α-granular contents (Figure 5A), revealing the indispensable need for TG in the release reaction, which cannot be potentiated by further addition of 5-HT (Figure 5A). This lack of potentiation may be explained by the fact that dense body 5-HT is rapidly secreted from permeabilized platelets upon Ca^{2+} addition (Shirakawa et al., 2000), reaching effective concentrations by itself.

Secretion from permeabilized *Tph1*$^{-/-}$ platelets reconstituted with BC and SMC resembles the wild-type reactions (Figure 5B), and again, 5-HT addition without TG does not lead to any α-granule release. Only the combination of SMC with 5-HT and TG leads to the vWf secretion (Figure 5B). Moreover, the addition of TG to *Tph1*$^{-/-}$ platelets reconstituted with SMC does not lead to increased exocytosis (Figure 5B), reflecting the lack of 5-HT, and demonstrating the need for the enzyme and 5-HT as the substrate for activation and secretion.

The sequences of known small GTPase substrates for bacterial TGs RhoA, Rac1, and Cdc42 allow delineating a TG substrate consensus within the GTP hydrolyzing domain that can be found also in Rab GTPases (Figure 5C), suggesting that Rab4 and other Rab GTPases might be suitable substrates as well. *Tph1*$^{-/-}$ platelets preloaded with ^{14}C-5-HT and aggregated using Fen, Flx, and α-ms present many ^{14}C-5-HT labeled bands in SDS-PAGE as expected, especially in the high molecular range (data not shown). This is not surprising, since 5-HT is transamidated to several high-molecular proteins, such as vWf and fibrinogen (Dale et al., 2002). However, also some bands in the range of 20–25 kDa appear, and immunoprecipitation of RhoA and Ras isoforms reveal that RhoA, but not Ras isoforms, were covalently labeled with ^{14}C-5-HT (Figure 5D). However, labeled Rab4 could not be detected by this method due to low signal/noise ratio, but native Rab4 isolated from skeletal muscle by immunoprecipitation serves as a substrate for TG in vitro, incorporating ^{14}C-5-HT (Figure 5E).

Tph1$^{-/-}$ thrombocytes provide an excellent possibility to offer other substrates to TG, avoiding competition with the endogenous substrate 5-HT. Addition of various concentrations of the alternative fluorescent TG substrate monodansylcadaverine (MDC) together with the enzyme and SMC mimics the action of 5-HT, although

Figure 3. *Tph1*$^{-/-}$ Mice Are Less Prone to Succumb from Thromboembolism and Thrombosis

(A) Fluorescent thrombus formation is detectable by intravital microscopy soon after $FeCl_3$ injury of endothelial cells in *Tph1*$^{+/+}$ animals, whereas platelet deposition is blunted in *Tph1*$^{-/-}$ mice. The few thrombi formed in *Tph1*$^{-/-}$ usually detach spontaneously from injured arterioles.

(B) Intravenous application of 200 μg/ml collagen together with 10 μM epinephrine is well tolerated by *Tph1*$^{-/-}$ mice, whereas most of the treated *Tph1*$^{+/+}$ mice succumb in less than 15 min.

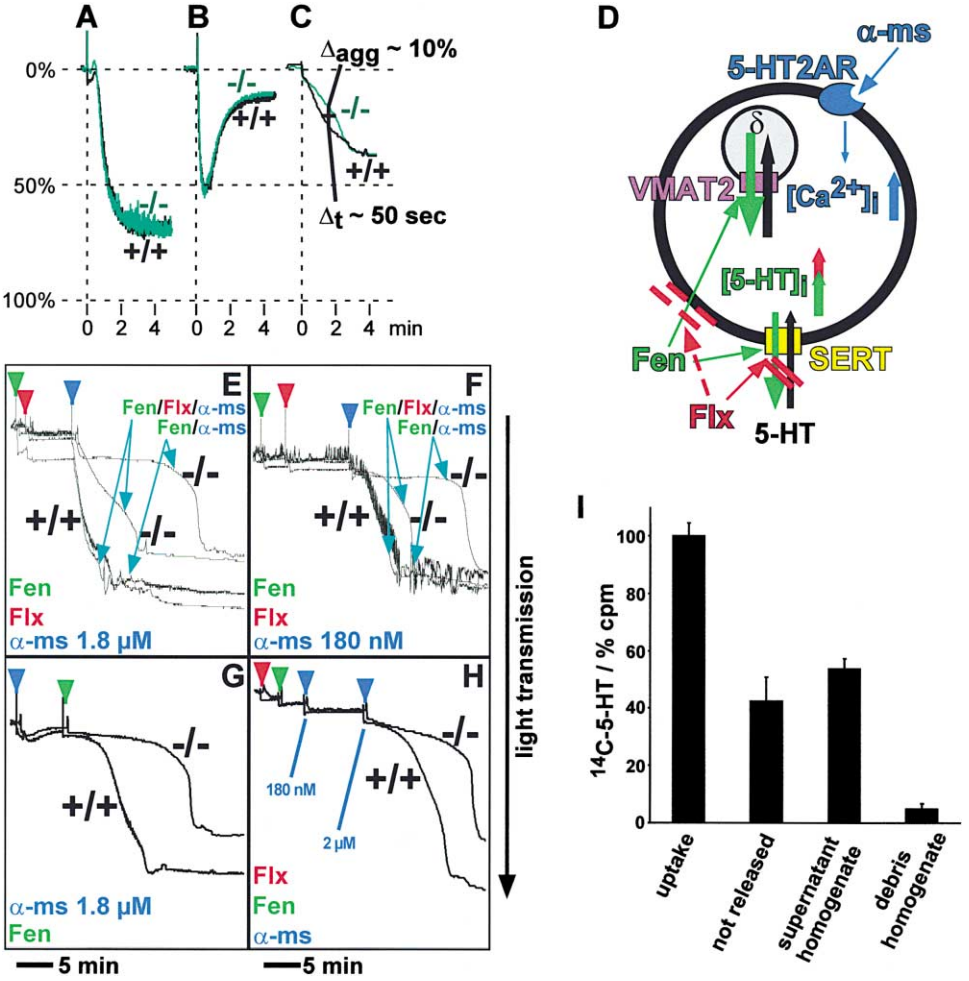


Figure 4. G Protein Hypothesis Contradictory Platelet Aggregation

(A) After a short lag phase, collagen (20 μg/ml) induces a pronounced shape change during which 5-HT and ADP become secreted, then culminating in the aggregation response (compare with Figure 1C). The initial shape change is thought to depend on secreted ADP.

(B) Without collagen, ADP alone (20 μM) elicits a completely reversible aggregation response without granule secretion in presence of plasma protein, demonstrating that agglutination and aggregation cannot be distinguished using in vitro experiments.

(C) In presence of an excess of apyrase that immediately breaks down all secreted ADP, collagen stimulation (20 μg/ml) still leads to an irreversible although less efficient aggregation response without a preceding shape change. This response is supported by 5-HT as can be appreciated by a delayed time (Δ_t ~50 s) and magnitude (Δ_{agg} ~10%) of the 5-HT deficient *Tph1*$^{-/-}$ platelets. All plots are representative for at least four independent experiments.

(D) Besides 5-HT, platelets contain three serotonergic components: the 5-HT2A receptor (5-HT2AR), the plasma membrane serotonin reuptake transporter (SERT), and the vesicular monoamine transporter 2 (VMAT2). Intact platelets take up 5-HT by the SERT and store the monoamine in vesicles by the VMAT2 (black arrows). Fenfluramine (Fen) is SERT-dependently transported into the cytoplasm and reverses the VMAT2 transport, increasing the cytoplasmic 5-HT concentration $[5\text{-HT}]_i$ to levels that reverse the SERT activity, resulting in a continuous slow release of 5-HT (green arrows). After Fen, fluoxetine (Flx) addition completely blocks SERT, thereby trapping 5-HT in the platelet cytoplasm accelerating the increase of $[5\text{-HT}]_i$ (red arrows). Flx before Fen blocks the entry of Fen into the platelets, thereby avoiding Fen-induced VMAT2 reversal and increased $[5\text{-HT}]_i$ (dashed red lines). 5-HT2AR-specific agonists increase free calcium $[Ca^{2+}]_i$ via Gαq (blue arrows). According to the G protein hypothesis, neither Fen, Flx, or 5-HT2AR-dependent signaling nor combinations of these compounds should elicit any aggregation responses.

(E) Fen (1 μM) followed by Flx (1 μM) does not elicit any platelet responses without concomitant 5-HT2AR stimulation. Addition of the 5-HT2AR agonist α-methyl-serotonin (α-ms; 1.8 μM) rapidly leads to extensive aggregation. Tph1$^{-/-}$ platelets containing only about 5% of wild-type 5-HT levels exhibit a delayed response. Moreover, while omission of Flx has no influence on the response of wild-type platelets, *Tph1*$^{-/-}$ platelets allow to resolve the $[5\text{-HT}]_i$-dependent response. Obviously, a critical $[5\text{-HT}]_i$ must be reached for the aggregation response.

(F) As in (E), even 180 nM α-ms leads to complete aggregation after Fen + Flx or Fen alone and again, only the *Tph1*$^{-/-}$ response is resolved in a $[5\text{-HT}]_i$-dependent manner.

(G) 5-HT2AR stimulation with α-ms (1.8 μM) induces an almost not detectable agglutination (data not shown: no response with 180 nM α-ms). Addition of Fen (1 μM) leads to a complete aggregation response, which is delayed for more than 10 min in *Tph1*$^{-/-}$ platelets compared with wild-type platelets.

(H) Blockade of SERT with Flx (1 μM) prior to Fen (1 μM) avoids Fen entry into the cytoplasm and its action on VMAT2. The dependence of the aggregation response on increased $[5\text{-HT}]_i$ is obvious from the insensitivity of the platelets to 180 nM α-ms, a concentration that after Fen + Flx regimen induces extensive spontaneous aggregation. Only an excessive addition of α-ms (2 μM) competing for SERT binding is able to induce a response, although delayed for more than 5 min in wild-type and for more than 15 min in Tph1$^{-/-}$ platelets. All plots are representative for at least four independent experiments.

(I) After aggregation of *Tph1*$^{-/-}$ platelets preloaded with ^{14}C-5-HT, about 40 to 50% of the ^{14}C-5-HT remain in the aggregates collected by centrifugation and washing 3 × with Tyrode-HEPES buffer (100% = 3 × washed resting platelets). Supernatants of sonicated aggregates also contain about 40 to 50% of ^{14}C-5-HT, while a 3 × washed insoluble fraction retains about 5 to 8% of ^{14}C-5-HT (results of one representative experiment are shown; the sum of supernatant homogenate and debris homogenate is not significantly different from the not released value).

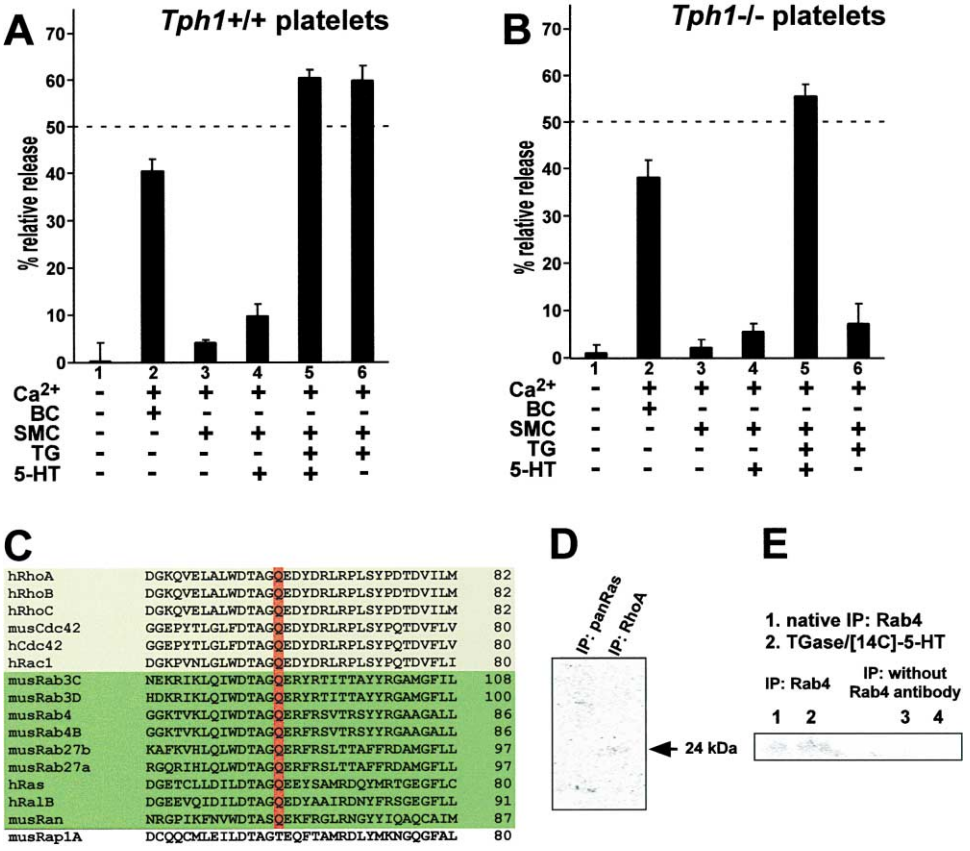


Figure 5. Serotonylation of Small GTPases in Platelets

(A) Permeabilized wild-type platelets incubated at 37°C while shaking with 1000 rpm after buffer addition do not release more vWf than resting permeabilized platelets (column 1). In presence of 3 mg/ml brain cytosol (BC) protein, about 40% of the α-granular contents become released (column 2). Skeletal muscle cytosol (SMC) protein (3 mg/ml) does not induce significant α-granule release (column 3), even if 5-HT (15 μM) is added (column 4). Addition of 25 mU guinea pig liver TG lead to about 60% release of vWf in presence and absence of 15 μM added 5-HT (columns 5 and 6).

(B) The same treatment of permeabilized *Tph1*$^{-/-}$ platelets as in (A) shows similar reactions for all conditions (columns 1 to 5), excepting for omission of 5-HT (column 6). No significant release can be detected from permeabilized *Tph1*$^{-/-}$ platelets in presence of SMC (3 mg/ml) and TG (25 mU) in absence of exogenous 5-HT (column 6).

(C) Alignment of a selection of Ras-related small GTPases. The glutamine residue of the catalytic site (red), which has been shown to be modified by bacterial TGs (light green) with concomitant constitutive activation, is conserved in most of the other shown GTPases (dark green), excepting Rap1A (not colored).

(D) Aggregated wild-type platelets as described in Figure 4I were washed and sonicated in presence of a protease inhibitor cocktail followed by immunoprecipitation with anti-Ras (all isoforms) and anti-RhoA. Only in the RhoA precipitate emerges a ^{14}C-5-HT labeled signal of 24 kDa.

(E) Rab4-rich skeletal muscle cytosol was used for native immunoprecipitation of Rab4, and then treated with 25 mU TG and ^{14}C-5-HT in duplicate (1 and 2). Mock immunoprecipitations served as negative control (3 and 4).

to a lower extent (Figure 6A). Similar to ^{14}C-5-HT, MDC is transamidated to many high-molecular weight proteins upon platelet stimulation, but also to proteins of about 24 kDa, presumably the small GTPases (Figure 6B), therefore demonstrating that this synthetic monoamine can functionally replace the endogenous substrate 5-HT.

Transamidation reactions with GST- and 6× His-tagged GTPases with MDC and guinea pig liver TG reveal that Rab GTPases are excellent substrates (Figure 6C), while RhoA, one of the accepted substrates for bacterial TGs, is less efficiently transamidated by the mammalian enzyme (Figure 6D). However, 6× His-RhoA transamidated in vitro with either MDC or 5-HT reveals that the modification leads to strong effector binding of the GTPase even in the GDP bound form in rhotekin pull-down assays (Figure 6F).

Discussion

Primary hemostasis is a complex self-preserving reaction preventing major blood loss upon injury. The active half-life of almost all platelet agonists is extremely short. Inhibitors or enzymatic breakdown rapidly inactivate thrombin, thromboxanes, adrenalin, and adenine nucleotides. In contrast, secreted 5-HT is scavenged by active transport into platelets, thereby leading to a transiently elevated cytoplasmic concentration of this monoamine. We have recently shown that Gαq stimulation drastically reduces VMAT2-dependent vesicular uptake (Höltje et al., 2003), probably resulting in accumulation of cytoplasmic 5-HT to levels higher than previously expected. The functional importance of this increase in $[5\text{-HT}]_i$ for platelet aggregation was not appreciated previously.

It is not surprising that *Tph1*$^{-/-}$ mice lacking 5-HT in

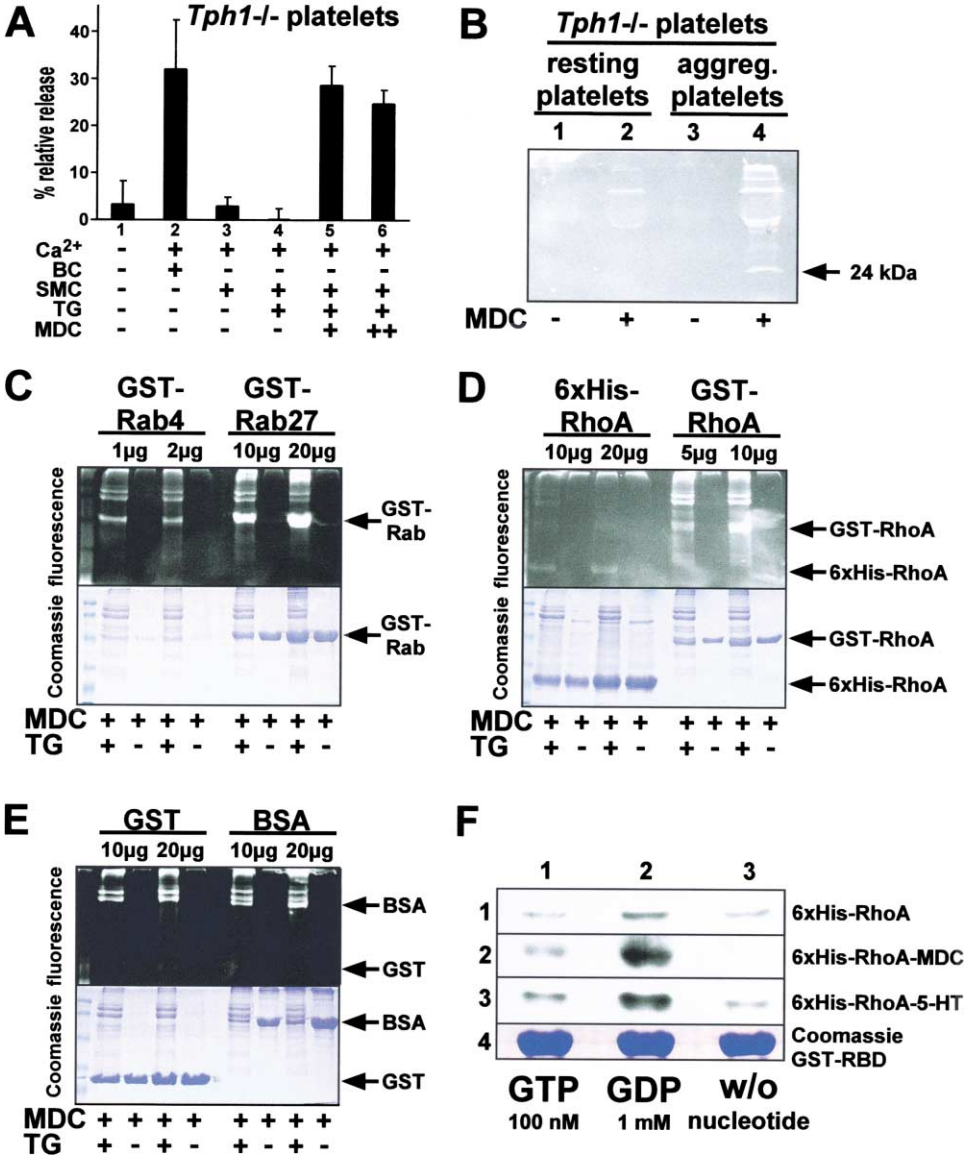


Figure 6. Synthetic Monoamines Can Functionally Replace Endogenous Substrates for Constitutive Small GTPase Activation

(A) Permeabilized *Tph1*$^{-/-}$ platelets were treated under the conditions described in Figure 5B (columns 1 to 3). Again, skeletal muscle cytosol and TG without addition of a monoamine exhibits no release reaction [column 4; 6 in Figure 5B]. However, the addition of the synthetic monoamine monodansylcadaverine (MDC) under the conditions of column 4 in Figure 5B leads to the release of α-granular contents at various concentrations (500 μM and 1 mM; columns 5 and 6, respectively).

(B) Resting and aggregated *Tph1*$^{-/-}$ platelet samples without MDC are completely fluorescence inactive (lanes 1 and 3). Resting *Tph1*$^{-/-}$ platelets incubated with MDC exhibit two unknown labeled bands of about 43 and 110 kDa (lane 2), while MDC incubated platelets exhibit many labeled proteins after aggregation, including bands at about 24 kDa (lane 4).

(C–E) In vitro transamidation of tagged GTPases with MDC reveal dose-dependent monoaminylation of GST-Rab4, GST-Rab27 (C), 6$\times$ His-RhoA, and GST-RhoA (D) but no transamidation of GST alone (E; with bovine serum albumin as positive control). Shown are the UV-fluorescence (upper images) and the Coomassie stainings of the same polyacrylamide gels (lower images).

(F) Rhotekin pull-down of reaction products as shown in (D), preincubated with nucleotides at the indicated concentrations, and anti-RhoA detection of the active GTPase by immunoblotting. 100 nM GTP (about 50$\times$ molar excess) is hydrolyzed by Gln63 RhoA (lane 1, image 1) but not by RhoA-MDC nor RhoA-5-HT (lane 1, images 2 and 3). GDP at inactivating 1 mM surprisingly leads to strong effector binding of RhoA-MDC and RhoA-5-HT but not of Gln63 RhoA (lane 2, images 1–3). In negative controls without nucleotides, only slight background or no effector binding occurs (lane 3, images 1–3). Image 4 shows equal loading control of the RhoA binding GST-fusion of rhotekin.

platelets exhibit a significant prolongation of experimental bleeding times (Figure 1A), and that this phenotype can be rescued by 5-HT application (Figure 1B). But it was surprising that *Tph1*$^{-/-}$ platelets did not fail to aggregate in vitro upon stimulation with several proaggregatory compounds (Figure 1C). We confirmed our hypothesis, that other mediators such as vWf and other constituents of platelet α-granules, are responsible for the impaired primary hemostasis in vivo. In vitro we have demonstrated that 5-HT triggers the release of vWf, a

representative protein of α-granules, from platelets and that ADP as the second agonist in dense core vesicles only potentiates the 5-HT-induced release reaction (Figure 2B). Moreover, the prolonged bleeding time in *Tph1*$^{-/-}$ mice was rescued by infusion of commercial preparations of vWf (Figure 2A).

Interestingly, injection of higher doses of vWf did not completely normalize the prolonged bleeding time of *Tph1*$^{-/-}$ mice, indicating that the remaining prolonged bleeding time was either due to the absence of 5-HT or due to the difference in the multimeric forms of vWf found in plasma and platelets. Although the latter explanation is supported by the finding that patients with von Willebrand disease type IIA who lack the larger multimeric forms but have normal vWf concentrations in the circulation show a prolonged bleeding time (Siess, 1989), but this explanation is contradictory to the fact that infusion of high doses of commercial plasma-derived vWf can be used to cure von Willebrand disease patients.

Therefore, other explanations are more probable: 5-HT is known to constrict endothelium-denuded arteries, an important mechanism for the closure of vessels at sites of tissue injury (Cohen et al., 1983). Moreover, vWf is not the only protein in α-granules important for hemostasis and blood coagulation, and we only have chosen vWf as a representative marker of α-granular release due to its easy detection by ELISA. In *Tph1*$^{-/-}$ mice, the release of fibrinogen, vitronectin, fibronectin, the prothrombin activating factor V, and several membrane bound glycoproteins identified as collagen- and vWf-receptors exteriorized after platelet activation (Siess, 1989) should be decreased. In addition, 5-HT is not only responsible for the release of proteins from α-granules as indicated by our data, but also for their efficient binding to the platelet surface. It has recently been shown, that 5-HT is covalently bound by a TG-catalyzed process to vWf, fibrinogen, factor V, and other α-granular proteins after secretion and then binds to thrombospondin and fibrinogen on the surface of highly activated platelets (Dale et al., 2002; Szasz and Dale, 2002). Therefore, the substrate for this TG-dependent binding enhancement is deeply decreased in *Tph1*$^{-/-}$ platelets, and the absence of all mentioned effects in *Tph1*$^{-/-}$ mice may explain why exogenously added vWf cannot completely rescue the prolonged bleeding time of these animals (Figure 2A). Concordant with this multifactorial view, vWf knockout mice exhibit a reduced, albeit persistent experimental thrombus formation (Denis et al., 1998), since all other α-granular proteins can be released by 5-HT in this model. The phenotype of our 5-HT-deficient mice (Figure 3) resembles more that of fibrinogen/vWf double-knockout mice, in which thrombus instability and detachment has been demonstrated (Ni et al., 2000).

Our data show that *Tph1*$^{-/-}$ animals are less prone to succumb from vessel occlusion in thromboembolism and thrombosis due to the described thrombus instability (Figure 3). From a clinical point of view, this beneficial effect renders a therapeutic 5-HT reduction useful, even taking into account the observed prolonged bleeding as a side effect of 5-HT-absence. These findings might open new avenues to ameliorate vascular disease and its complications in thrombotic diseases by specific inhibition of TPH1 thereby impeding peripheral 5-HT synthesis (Walther et al., 2003). Since thrombosis in coronary or cerebral arteries is the major cause of morbidity and mortality worldwide, further understanding of the specific hormonal role of 5-HT in hemostasis and thrombosis is important to possibly prevent and treat deleterious hemorrhagic and cardiovascular disorders.

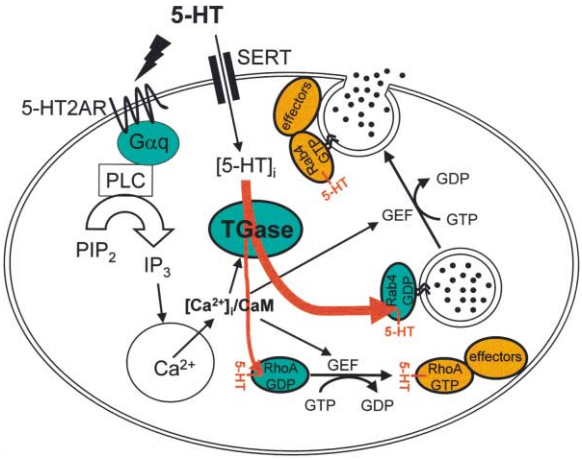


Figure 7. Machinery of 5-HT-Induced Exocytosis of α-Granules from Platelets during Irreversible Aggregation

Upon stimulation of platelets by 5-HT, the phosphatidyl inositol pathway is activated, resulting in a rise of cytoplasmic Ca^{2+}. 5-HT signaling is mediated by the Gαq protein-coupled 5-HT2AR, and moreover, by transport into the cytoplasm by SERT. Calcium activates TG, which uses 5-HT to transamidate small GTPases, such as cytoplasmic RhoA and α-granule-associated Rab4, thereby blocking the GTP-hydrolyzing activity of these proteins. Moreover, calcium in complex with calmodulin (CaM) activates guanine nucleotide exchange factors (GEF), which aid the exchange of GDP for GTP on RhoA and Rab4. The lack of GTP hydrolysis renders the serotonylated small GTPases constitutively active. RhoA and Rab4 in their GTP bound conformation interact downstream with effector molecules, resulting in cytoskeleton rearrangement and exocytosis of α-granules. The contents of α-granules are released into the circulation (for example vWf, black circles) or exposed at the plasma membrane (for example glycoprotein IIb/IIIa and P-selectin), thereby supporting irreversible aggregation.

Likely to be of greater importance is our finding that elevated cytoplasmic 5-HT concentrations are a crucial component of small GTPase activation in rapid exocytotic processes from platelets (Figures 4 and 5) and possibly also in other vesicular trafficking processes. Furthermore, we could show that this activation occurs through a rapid covalent TG-mediated binding of 5-HT to at least the small GTPases Rab4 and RhoA (Figures 5 and 6), suggesting a potential receptor independent mechanism of 5-HT signaling in the periphery (Figure 7). This mechanism assigns a central role to TGs during physiologic processes that depend on active monoamine transport into target cells.

Thus, our data provide evidence for a receptor-independent signaling pathway for 5-HT that we named "serotonylation," which leads to α-granule exocytosis from platelets. Since TGs also accept other biogenic monoamines, such as histamine and catecholamines as substrates, and even synthetic monoamines, such as MDC (Figure 6), it can be expected that histaminylation and catecholaminylation could be involved in analogous signaling mechanisms, and therefore "monoaminylation"

should be the most suitable term for all of these processes. Interestingly, monoaminylation leads to active GTPases even in the GDP bound form, uncoupling nucleotide binding and hydrolysis from their activity in signaling (Figure 6F). It will be of major importance to elucidate not only the stability of the monoaminylated GTPases in the GTP- and GDP bound forms but also the dissociation rates of the nucleotides in follow-up studies.

Our data helps to understand numerous pathophysiological effects, such as the surprising mitogenic effect of 5-HT in hyperplasia of pulmonary artery smooth muscle cells (PA-SMCs) in primary pulmonary hypertension (PHH), which has been shown to depend exclusively on SERT activity and cytosolic 5-HT accumulation (Eddahibi et al., 2001). Mitogenesis of PA-SMCs induced by 5-HT appears to occur by way of signaling through a mitogen-activated protein kinase (MAPK)-dependent pathway and phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2; Eddahibi et al., 2002). Interestingly, it has been recently established that retinoic acid-mediated TG overexpression leads to cytoskeletal rearrangement and activation of MAPK and ERK1/2 in a RhoA-dependent pathway, in which RhoA is activated by transamidation with radioactive putrescine, without elucidation of the endogenous amino donor (Singh et al., 2003). Collectively these data suggest that constitutive activation of RhoA by transamidation with 5-HT and consecutive downstream signaling via MAPK and ERK1/2 may be responsible for the observed hyperplasia of PA-SMCs in PPH. Furthermore, high cytosolic 5-HT concentrations are likely to be the crucial event leading to the mitogenic response, by analogy to the high cytosolic 5-HT concentration that we have elucidated to be essential in platelet aggregation (Figures 5 and 6). Thus, serotonylation of small GTPases rather than the controversially discussed effect of superoxide anions resulting from 5-HT internalization (Eddahibi et al., 2002) might be responsible for the observed pathophysiological effect of enhanced SERT-mediated 5-HT transport.

It is difficult to predict which other signaling events might depend on monoaminylation and which of the more than 60 known small GTPases might become activated by transamidation. This is further complicated by the fact that eight mammalian TGs have been identified to date (Griffin et al., 2002) and that little is known about their tissue-specific expression and substrate preference. However, it is likely that specific mammalian TGs preferentially act in restricted signaling mechanisms that are still to be elucidated. Further support for this hypothesis comes from the observation that bacterial TGs display preference for different GTPases (Aktories et al., 2000; Masuda et al., 2000; Doye et al., 2002). It has been established that TGs and 5-HT both act in signaling that leads either to differentiation or apoptosis (Singh et al., 2001, 2003; Azmitia, 2001). Interestingly, it has recently been reported that 5-HT induces apoptosis in biopsy-like Burkitt lymphoma cells, an effect that can be completely reversed by SSRIs (Serafeim et al., 2002). Based on our data it is tempting to speculate that this apoptotic effect might be mediated by TG-dependent serotonylation of small GTPases. Of course, many intriguing questions about the control of TG-dependent monoaminylation of small GTPases remain. For instance, it will be important to learn how cells handle constitutively active GTPases to avoid constant signaling through their downstream effectors (Doye et al., 2002). Now that we recognize the importance of the TG/monoamine-dependent activation of small GTPases, it is a matter of time before we explore the full extent of the biological implications of this finding.

Experimental Procedures

General Materials and Methods

All chemicals were purchased from Sigma (Deisenhofen, Germany) or obtained from the otherwise indicated companies. *Tph1*$^{-/-}$ and wild-type animals were bred as described (Walther et al., 2003). Antibodies were obtained from Sigma and Santa Cruz Biotechnology (CA 95060, USA). GST-GTPase fusions were cloned and purified using standard procedures. RhoA cDNA was kindly provided by F. Hofmann (Hannover, Germany). TG reactions with MDC were performed as previously described (Case and Stein, 2003) using guinea pig liver TG (Sigma). The rhotekin binding kit for the pull-down of active RhoA was used according to the manufacturer's instructions (Pierce, Germany).

Determination of Bleeding Times

Bleeding times were determined basically as previously described (Holland, 1976). Briefly, test animals were anaesthetized with ketamine and the terminal 2 mm portion of the tail was severed using a razor blade. Bleeding time was measured by a stopwatch to the nearest second using a filter paper intermittently applied to the adherent drop of blood, without touching the wound.

Determination of Hematological Parameters and Plasma vWf and FVIII

vWf was determined in citrated plasma using an enzyme immunoassay (Diagnostic International, vWf Antigen ELISA Kit, Schriesheim, Germany) according to the manufacturers' indications. The mean value of control animals was arbitrarily considered as 100% and used as reference.

Standard hematological parameters and plasma factor VIII levels were determined double-blinded by technical staff of the Franz-Volhard-Clinic in Berlin-Buch, using a Coulter counter and a STA Factor VIII kit (Boehringer Mannheim, Germany), respectively.

Platelet Aggregation and Secretion Studies

Blood (9 vol.) was collected from the vena cava inferior in 300 units/ml Heparin (1 vol.). After addition of half a volume of Tyrode-HEPES buffer (TH: 134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$; [pH 7.3]), the samples were centrifuged 7.5 min at room temperature and 200 $\times$ g. Platelets were then sedimented by a 5 min centrifugation at room temperature and 1000 $\times$ g. Platelet pellets were resuspended in TH at a density of 3 $\times$ 10^8 platelets/ml. After recalcification of 220 μl portions with 5 μl 50 mM $CaCl_2$ in siliconized glass cuvettes, aggregation reactions were started by addition of proaggregatory compounds in 25 μl TH to the indicated final concentrations, and the time-courses of the transmission-increments were recorded in a platelet aggregation profiler (Mölab, Germany).

For analysis of vWf secretion, the platelets were centrifuged and resuspended two times more with TH to wash away plasma vWf and fibrinogen. Final density was adjusted to 6 $\times$ 10^8 platelets/ml, and aggregation was performed as described above, using physiologically effective concentrations of the proaggregatory substances ADP (14 μM), 5-HT (12 μM), and collagen (30 μg/ml). Ten min after addition of the proaggregatory substances, the aggregates were centrifuged, and vWf concentrations in the supernatants were determined using the ELISA kit, as described above.

Permeabilization of platelets with streptolysin-O and secretion from (6–7) $\times$ 10^7 permeabilized platelets was done basically as described (Höltje et al., 2003 and Shirakawa et al., 2000, respectively). Supernatants of resting and sonicated resting platelets were used as 0% and 100% reference, respectively.

Thromboembolism Test

In mice anaesthetized with sodium pentobarbital (80 mg/kg), a collagen/epinephrine mixture (5 ml/kg of a saline-based solution containing 200 μg/ml collagen and 10 μM adrenaline) was injected into the tail vein. Survival was monitored up to 1 hr after injection as reported (Offermanns et al., 1997).

In Vivo Thrombosis Model

Mice were anaesthetized with sodium pentobarbital (80 mg/kg) for intravital microscopy of mesenteric microvessels in vivo, as described for the rat (Katayama et al., 2000), using the nonfluorescent fluorochrome-precursor carboxyfluorescein diacetate succinimidyl ester, which is converted to carboxyfluorescein succinimidyl ester specifically in platelets. Induction of vascular injury and thrombus formation with $FeCl_3$ (30 μl of a 250 mM solution; Sigma) and intravital microscopy were performed as described (Denis et al., 1998).

Acknowledgments

We thank Maria Gerstäcker, Susanne Werner, and Elisa Lusian for experimental support, and the excellent technical assistance of Gerhild Böttger is appreciated. The study was funded in part by grants of the Deutsche Forschungsgemeinschaft (INK21) and the Verbund Klinische Pharmakologie Berlin/Brandenburg.

Received: June 12, 2003
Revised: November 19, 2003
Accepted: November 20, 2003
Published: December 24, 2003

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