Biochemical Pharmacology, Vol. 57, pp. 1191–1197, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00011-8

Serotonin-Induced Secretion of von Willebrand Factor from Human Umbilical Vein Endothelial Cells via the Cyclic AMP-Signaling Systems Independent of Increased Cytoplasmic Calcium Concentration

Thomas Schlüter and Ralf Bohnensack*

INSTITUT FÜR BIOCHEMIE, MEDIZINISCHE FAKULTÄT, OTTO-VON-GUERICKE-UNIVERSITÄT, MAGDEBURG, GERMANY

ABSTRACT. Endothelial cells are able to synthesize von Willebrand factor (vWf) protein, which is then either secreted in a constitutive way or stored within specific cellular secretory granules, the Weibel–Palade bodies. Stimulated secretion of vWf from these organelles is thought to be induced by agonists causing a transient increase in cytoplasmic calcium concentrations. Serotonin (5-hydroxytryptamine, 5-HT), a local transmitter substance released by activated platelets, has also recently been shown to induce the secretion of vWf. In experiments with human umbilical vein endothelial cells (HUVEC), we found that the 5-HT-induced secretion occurred without a significant increase in cellular calcium levels. The 5-HT 1_D subtype-specific receptor agonist sumatriptan also induced the release of vWf without causing a calcium signal in HUVEC. Stimulation of endothelial cells with the adenylate cyclase inhibitor, MDL-12 A330, led to the secretion of vWf as well. Simultaneous addition of submaximal concentrations of histamine and 5-HT to HUVEC potentiated the effects of either agonist. Together, these results suggest that in HUVEC 5-HT-induced secretion of vWf is mediated by a decrease in cyclic AMP levels and is independent of changes in cytoplasmic calcium levels. BIOCHEM PHARMACOL **57**;10:1191–1197, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. endothelial; serotonin; von Willebrand factor; calcium; adenylate cyclase

Inflammatory processes are accompanied by the extravasation of leukocytes from the bloodstream into the affected tissue. Recent studies have demonstrated that a multitude of proteins participate in this thoroughly coordinated inflammatory reaction. The initial "rolling" of leukocytes on the endothelial cell layer is induced by the rapid integration of P-selectin into the plasma membranes of endothelial cells [1]. This glycoprotein, also known as CD 62 p or granule membrane protein (GMP)-140, is stored in the membranes of specific secretory granules, called Weibel–Palade bodies [2]. These granules as a second component also contain large multimers of vWf† within their luminal space [3]. Besides its activity as a member of the coagulation system, this glycoprotein also acts as an adhesion molecule whose main function is the amplification of the contact of leukocytes to the vascular wall, especially under conditions of high shear forces [4]. The degranulation of Weibel–Palade bodies is induced by many agonists, such as thrombin, histamine, adenine nucleotides, leukotrienes, or platelet-activating factor, that also induce a transient rise in cytoplasmic calcium ion concentration $[Ca^{2+}]_i$. It has been stated that this rise is a prerequisite event in the expression of P-selectin and the release of vWf [5].

Recently, it was reported that 5-HT is an agonist also inducing the degranulation of Weibel–Palade bodies [6] by a thus far not entirely characterized mechanism. Basically, two predominant types of signal transduction for 5-HT have been described: (i) a calcium-dependent pathway stimulated by binding of 5-HT to receptors which activate the inositol trisphosphate cascade via phospholipase C (receptor subtype class 2); or (ii) processes regulated by elevating (receptor subtype 4) or reducing the cytosolic level of cAMP (subtype 1 or 3; [7]). In the present study, we investigated the signal transduction pathways in HUVEC using 5-HT as agonist. We describe herein that in HUVEC the degranulation of Weibel–Palade bodies caused by 5-HT is induced independently or rises in $[Ca^{2+}]_i$. We also provide evidence for the participation of an inhibition of adenylate cyclase in this process.

MATERIALS AND METHODS

Materials

Fetal bovine serum, fungizone, L-glutamine, and HEPES were from GIBCO BRL; kanamycin monosulfate, heparin, and histamine were obtained from Sigma; collagenase A

* Corresponding author: Dr. Thomas Schlüter, Institut für Biochemie, Medizinische Fakultät, Otto-von-Guericke-Universität Magdeburg, Leipziger Str. 44, D-39120 Magdeburg, Germany. Tel. +49 391 67 15362; FAX +49 391 67 13050; E-mail: thomas.schlueter@medizin.uni-magdeburg.de

† *Abbreviations:* cAMP, cyclic AMP, adenosine 3′, 5′-cyclic monophosphate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; HBSS, Hanks' balanced salt solution; 5-HT, 5-hydroxytryptamine (serotonin); HUVEC, human umbilical vein endothelial cells; PKA, protein kinase A; vWf, von Willebrand factor; and MDL-12 A330, [*cis-N*-(2-phenylcyclopentyl) azacyclotridec-1-en-2-amine].

Received 17 March 1998; accepted 22 October 1998.

(EC 3.4.24.3) and endothelial cell growth supplement were from Boehringer Mannheim. Fura-2-acetoxymethyl ester was purchased from Molecular Probes. MDL-12 A330 hydrochloride was from Calbiochem. Sumatriptanhydrogensuccinate (Imigran®) was obtained from Glaxo. Cell culture ware was purchased from Greiner (25-cm^2 flasks; ELISA grade 96-well plates) and Sarstedt (24-well dishes).

Cell Culture

HUVEC were isolated by collagenase digestion of human umbilical cord veins as described by Jaffe *et al.* [8]. Briefly, cells were detached by incubation for 10 min at 37° with 10–20 mL HBSS containing 0.1 mg/mL of collagenase A and 1 mM of $CaCl_2$, then collected by centrifugation at 100 *g* for 10 min and resuspended in Medium 199 supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 15 mM HEPES, 25 μg/mL of endothelial cell growth supplement, 100 μg/mL of kanamycin monosulfate, 0.25 μg/mL of fungizone, and 100 units/mL of heparin. Cells were cultured in 25-cm^2 tissue culture grade flasks at 37° in a humidified atmosphere of air/CO_2 (19:1). The medium was renewed the following day and then every second day. Confluent cells (4–5 days) were subcultured 1:4 after detachment with 0.02% EDTA in HBSS. HUVEC were used for experiments when confluent at passage 2–3.

Measurement of vWf Secretion

Confluent HUVEC were removed from culture flasks as described above and subcultured in 24-well plastic dishes (1 flask/dish) for a further 48 hr. Unless otherwise stated, the endothelial cells were washed twice immediately prior to the experiments with HBSS (without Ca^{2+} and Mg^{2+}) and then equilibrated in HBSS (1 mM Mg^{2+}, 2.5 mM Ca^{2+}) for 15 min at 37°. After the subsequent addition of agonists, cells were incubated for the indicated time periods. The supernatant was withdrawn and centrifuged at 2000 *g* to remove any residual suspended cells. Supernatant fractions were stored frozen at −20° and analyzed within 1 week according to the protocol indicated below.

Secretion of vWf into the cell culture supernatants was determined with an ELISA using unlabeled and peroxidase-labeled polyclonal anti-vWf antibodies as described by the manufacturer's general instructions for ELISA (DAKO). The vWf concentration was calibrated for each test by comparing the optical densities of cell culture supernatant samples with that of a serial dilution of pooled human plasma in HBSS. Under the standard experimental conditions, HUVEC released a basal level of vWf which was about 1.35 ± 0.29% of the concentration found in pooled plasma.

Measurement of Changes in Intracellular Ca^{2+} Concentrations

Basically, the procedure described [9] was applied. After dissociation of HUVEC with 0.02% EDTA in HBSS, the single cell suspension was washed twice with complete medium and the fluorescent dye was added to a final concentration of 4 μM. The cells were incubated for 30 min at room temperature, washed twice with buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, and 1% BSA) and resuspended in the same buffer with 0.1% BSA to a concentration of 1×10^6 cells/mL. There followed an additional incubation period of 60 min, which is required for hydrolysis of the fura-2-acetoxymethyl ester. Stained cells were kept at room temperature in the dark. Determinations of $[Ca^{2+}]_i$ were performed within the following 3 hr. The content of free intracellular fura-2 acid was determined by addition of $MnCl_2$ (1 mM) in the presence of 10 μM ionomycin, which causes quenching of the free acid [10]. Aliquots of 2 mL of the cell suspension were transferred to a continuously stirred quartz cuvette (maintained at 22°) in a Sigma ZWS dual-wavelength spectrophotometer (Eppendorf) equipped with a KV-500 cut-off filter (Schott). The changes in $[Ca^{2+}]_i$ were determined by calculating the ratios between the fluorescence intensities at the excitation wavelengths of 340 nm and 380 nm [10].

The Petri dishes were made nominally calcium-free by washing the cell monolayer three times with HBSS with Mg^{2+} (1 mM) and without Ca^{2+}. No calcium buffer such as EGTA was added to the incubation medium to avoid detachment of cells from the dishes. The concentration of Ca^{2+} ions under these nominal "calcium-free" conditions was determined as 1.7 ± 0.25 μM by comparing the fura-2 ratio of HBSS (no CA^{2+} added, 1 mM Mg^{2+}) with the fluorescence of buffers containing defined concentrations of Ca^{2+} (0.017 to 39.5 μM; Molecular Probes).

Measurement of Adenosine 3′,5′-Cyclic Monophosphate

The determination of cAMP was performed with a competitive enzyme immunoassay (R&D Systems) according to the manufacturer's instructions.

Analysis of Data

All of the data obtained in this study are shown as means ± SD of at least three independent experiments. The amounts of vWf released by stimulated cells were standardized by calculating the difference between stimulated samples and controls, expressed as % of vWf, exaggerating the level of untreated control cells (set to 100%). Statistical significance was determined by one-way analysis of variance followed by Dunnett's *t*-test. Differences are considered significant if P (two-sided) < 0.05 (SAS/STAT 6.12 software, SAS Institute) [11]. EC_{50} doses were calculated by hyperbolic curve (Sigmaplot 2.0 software, Jandel).

RESULTS

Serotonin-enhanced vWf Secretion in HUVEC

The incubation of HUVEC with 5-HT led to an enhanced secretion of vWf into the cell culture supernatant (Fig. 1).

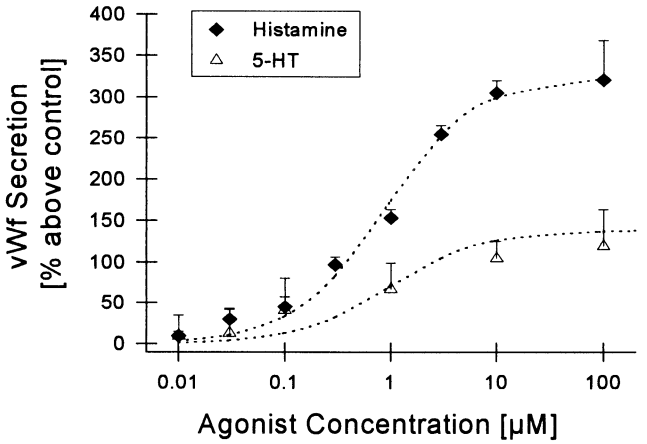

FIG. 1. Effect of different concentrations of histamine (◆) or 5-HT (△) on vWf release by HUVEC. Cells were incubated with the indicated concentrations of agonists for 30 min. Release of vWf from unstimulated controls was set as 100%. Data are shown as % of control exceeding the basal secretion of 1.35% of the concentration found in pooled human plasma. Results are presented as the means ± SD of 4 measurements.

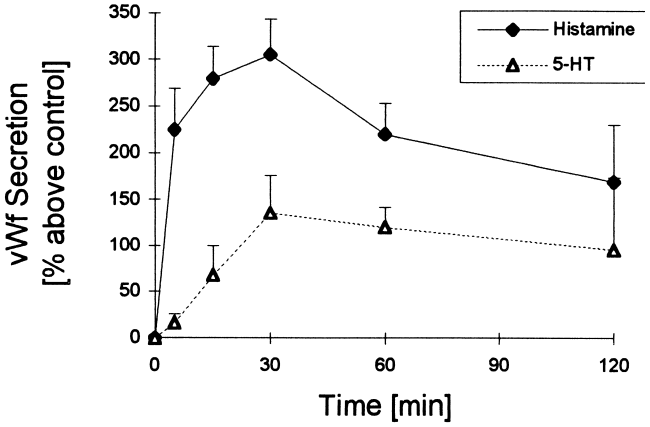

FIG. 2. Time–course of vWf secretion after stimulation of HUVEC with histamine or 5-HT. Cells were incubated for the indicated time periods with 10 μM histamine (◆) or 100 μM 5-HT (△). Release of vWf from unstimulated controls was set as 100%. Data are shown as % of control exceeding the basal secretion of 1.35% of the concentration found in pooled human plasma. Results are presented as means ± SD of 3 measurements.

The amount of vWf exaggerating the basal release was between 43 (0.1 μM) and 112% (100 μM) of the basal vWf concentration. Compared with cells stimulated with histamine, the secretion was lower for each 5-HT concentration, although the EC_{50} concentration inducing half-maximal effect for both agents appeared to be about 1 μM (0.86 for histamine and 0.91 for 5-HT, respectively). An agonist saturation was achieved above concentrations of 10 μM. The ability to respond to a stimulation with 5-HT was dependent on the cell culture conditions: cells with passage numbers greater than 3–4 rapidly lost their capability for enhanced vWf secretion upon 5-HT stimulation (not shown).

HUVEC treated with either 10 μM histamine or 100 μM 5-HT exhibited a different behavior within the early phase of stimulation (Fig. 2): Histamine induced a rapid release of vWf within the first 5 min, which then increased only slightly up to a maximum after 30 min. The release of vWf upon incubation with 5-HT was more sustained. After starting with a nearly linear increase, it also peaked 30 min after the addition of the agonist. For both agonists, the concentrations of vWf in the cell culture supernatants diminished within 60–90 min, probably due to a reassociation of vWf with the cells [6].

Serotonin-induced Release of vWf by the Regulated Pathway of Secretion

To demonstrate whether the 5-HT-induced effect was based on degranulation of Weibel–Palade bodies or on a raised direct secretion of newly synthesized vWf protein, we investigated the release of vWf in the presence of the known inhibitor of protein synthesis, cycloheximide. Cycloheximide, added 20 min prior to the stimulation with histamine or 5-HT, caused only a slight but not significant decrease in vWf secretion to the supernatants' one which was similar in unstimulated controls and histamine- or 5-HT-treated samples (not shown). Obviously, the constitutive secretion was inhibited by cycloheximide, whereas the extent of the stimulated vWf release was not affected.

Combination of Histamine and Serotonin in vWf Release

Based on the results of the time dependency of the vWf release, we hypothesized that histamine and 5-HT might use different mechanisms of signal transduction. Therefore, we incubated HUVEC simultaneously with submaximal concentrations of histamine (1 μM) and serotonin (10 μM). As can be seen in Fig. 3, the secretion of vWf was potentiated when both agents were present in the incuba-

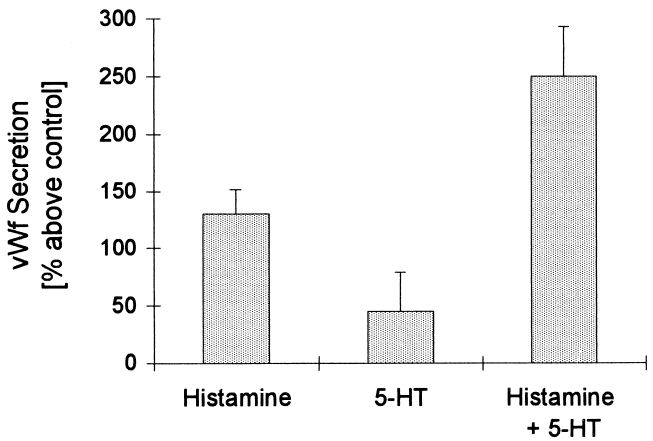

FIG. 3. Effect of combined incubation of HUVEC with histamine and 5-HT. Cells were incubated with 1 μM histamine or 0.3 μM 5-HT, or both agonists together for 30 min. Results are the means ± SD of 4 experiments.

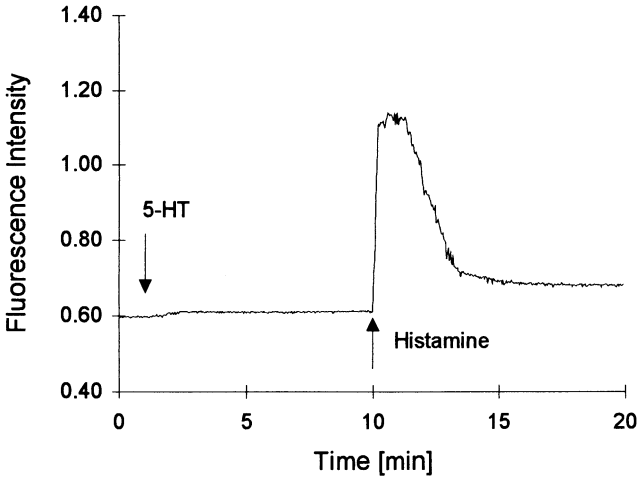

FIG. 4. Changes in cytosolic free calcium ion concentrations of HUVEC after subsequent stimulation with 5-HT (100 μM) and histamine (10 μM). Agonists were added to 1 × 10^6 fura-2-loaded cells suspended in 2 mL HBSS (2.5 mM Ca^{2+}, 1 mM Mg^{2+}) at the indicated time.

tion medium. The response was apparently larger than the sum of the concentrations derived with either agonist alone.

Calcium Dependency of 5-HT Stimulation

To investigate the calcium dependency of the 5-HT stimulation, we challenged fura-2-loaded HUVEC with either histamine or 5-HT (Fig. 4). After the addition of histamine, the cells showed a rapid increase in the 340/380 nm fluorescence intensity ratio, indicating a large increase in the cytosolic free calcium ion concentration. For concentrations of histamine which caused vWf release, the signal exhibited the typical biphasic shape of receptor-operated calcium metabolism: within seconds, it rose to its maximum, declined slightly after 1–2 min, and then reached a plateau phase which usually extended for more than 10 min without returning to the basal level. On the contrary, when the cells were incubated with 5-HT in concentrations from 10 nM to 1 mM, no significant change in $[Ca^{2+}]_i$ could be detected (Fig. 4).

Release of vWf by 5-HT: Independent of Extracellular Calcium

In order to determine the role of calcium in the secretion of vWf in serotonin- and histamine-stimulated cells, we treated HUVEC either in the presence or absence of extracellular calcium ions. The removal of calcium from the incubation buffer led to a marked reduction (75%) in the amount of vWf released by histamine-stimulated cells (Fig. 5A). The fact that the release of vWf under these conditions was not completely abolished may be primarily due to the fact that the rise in intracellular calcium concentration caused by histamine is known to be biphasic, i.e. first there is an instant release of calcium from stores within the cell which is independent of the presence of extracellular calcium. This rise is followed by a more sustained response caused by a calcium-induced influx of calcium ions from outside the cell. The early response may already be sufficient to induce a minor vWf release. The serotonin response was clearly less affected by this treatment, indicating that the 5-HT-dependent secretion was not induced by an influx of calcium ions through the cytoplasm membrane (Fig. 5B).

Mediation of HUVEC Stimulation by the 1_D Receptor Subtype

Sumatriptan (Imigran®), a 1_D subtype-specific serotonin receptor agonist [12], induced a vWf release in concentra-

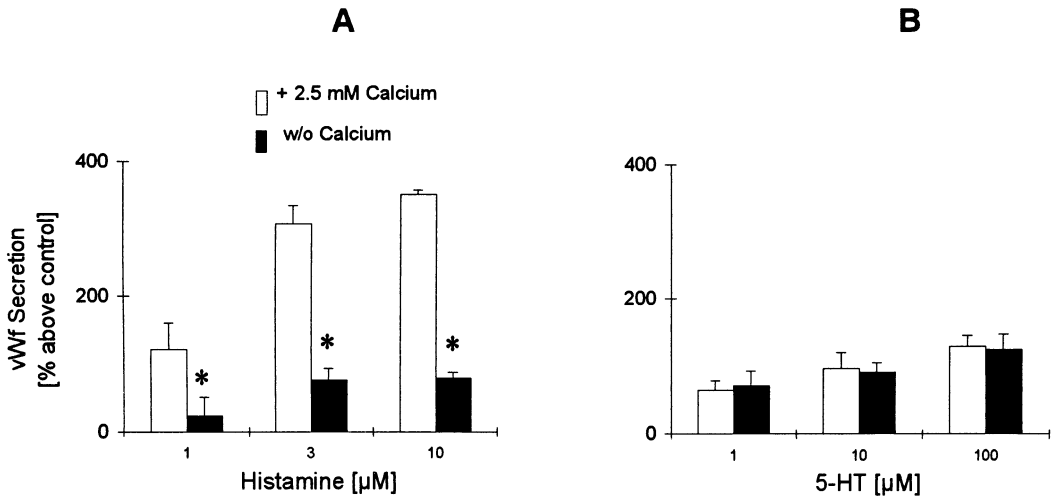

FIG. 5. Influence of the omission of extracellular calcium ions from the incubation buffer. The Petri dishes were made nominally calcium-free by aspiring the normal medium and washing the cell monolayer three times with HBSS with Mg^{2+} and without Ca^{2+}. HUVEC were stimulated with the indicated concentrations of histamine (A) or 5-HT (B) for 30 min in the presence (2.5 mM, open columns) or absence (filled columns) of Ca^{2+} in HBSS with Mg^{2+} (1 mM). Results are the means ± SD of 3 measurements; * = $P <$ 0.01.

TABLE 1. Release of vWf after incubation of HUVEC with different agonists for 30 min with the indicated concentrations

Agonist	Concentration [μM]	vWf released [% above control]
5-HT	10	91 ± 14
MDL-12, A 330	1	105 ± 9
Sumatriptan	1	89 ± 11

Results are the means ± SD of four experiments.

tions between 0.1–10 μM. The amount of vWf released by HUVEC after treatment with sumatriptan was similar to that found with 5-HT (Table 1). It is known that 1_D receptor subtypes are linked to cellular responses by a G-protein exerting its effect by inhibiting the activity of adenylate cyclase [13]. Therefore, we investigated the influence of the adenylate cyclase inhibitor MDL-12 on induced vWf release. The amount of vWf found in the cell culture supernatants was significantly enhanced by MDL-12 (Table 1). When added in combination with either serotonin or histamine, MDL-12 induced a release of vWf that was additive for 5-HT and more than additive for histamine to the release by the agonists alone.

To further demonstrate that the stimulation of HUVEC by serotonin was transduced via a 5-HT 1 receptor subtype, we investigated the cytosolic content of cAMP after the addition of agonists. As shown in Fig. 6, the addition of histamine caused no significant changes in the cellular cAMP level, whereas the stimulation of HUVEC with serotonin, sumatriptan, or MDL-12 led to a reduction in cytosolic cAMP concentrations of 48 to 64% ($P < 0.01$). The use of adenylate cyclase activator forskolin as a positive control (10 μM) was able to elevate cytosolic cAMP by 195 ± 7%.

DISCUSSION

It was previously thought that excretion of Weibel–Palade bodies was always connected with increased cytoplasmic calcium concentrations [5]. This notion was derived from the fact that the known activators of secretion were all agonists which cause a receptor-operated increase in $[Ca^{2+}]_i$. Two lines of evidence support the hypothesis that calcium might be responsible for the secretory response of endothelial cells: (i) Exocytosis stimulated by thrombin is diminished after preincubation with the cell-permeant calcium chelator BAPTA acetoxymethyl ester, whereas treatment of the cells with calcium ionophores such as A 23187 leads to an increased secretion [14, 15]; and (ii) For thrombin, it has also been shown that inhibition of myosin light chain kinase, a calcium/calmodulin-dependent enzyme, led to a considerable decrease in vWf release by endothelial cells [16].

Known protein kinase C activators such as phorbol myristyl acetate are potent inducers of Weibel–Palade body release; however, inhibition of protein kinase C was not sufficient to inhibit substantial amounts of thrombine- or histamine-induced secretion of vWf [16]. Schorer *et al.* showed that regulated secretion of Weibel–Palade bodies can be induced by interleukin-1β [17], an agonist known not to significantly induce increased calcium levels in endothelial cells. Carew *et al.* reported that adenosine triphosphate, which efficiently elevates $[Ca^{2+}]_i$, in HUVEC, is only a very poor effector of vWf release [18]. Thus, whether there were other ways of action by which endothelial cells could be driven to release their secretory granules remained to be elucidated. Some authors have postulated that calcium may be a prerequisite, albeit not sufficient, for a regulated endothelial secretion. Tranquille and Emeis found that in rat hindleg bradykinin alone was a bad secretagogue [19]. When they treated the cells simultaneously with bradykinin and the adenylate cyclase activator forskolin or a non-hydrolyzable analog of cAMP, the release of vWf was markedly improved [19]. Recently, it was demonstrated that a release of vWf by HUVEC may occur solely by increased cytosolic concentrations of cAMP (either receptor-mediated subsequent to activation of adenylate cyclase by norepinephrine or by inhibition of phos-

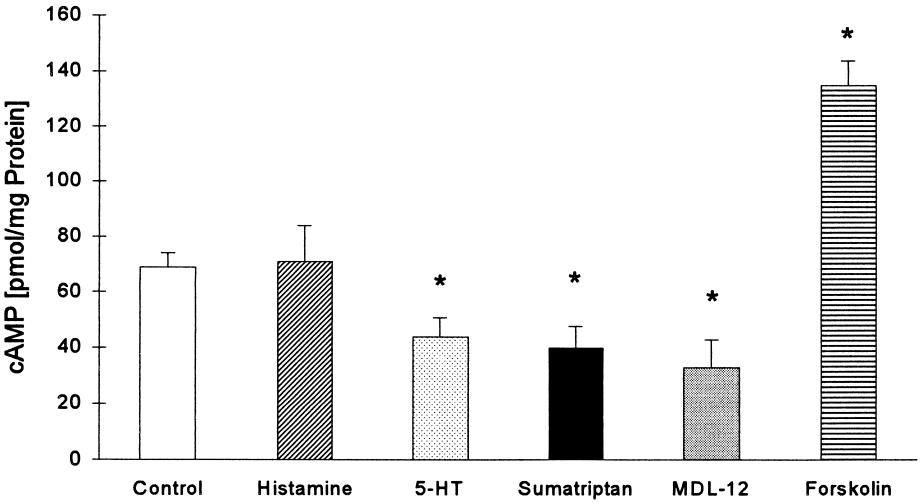

FIG. 6. Cytosolic concentrations of cAMP after incubation of HUVEC with histamine (10 μM), 5-HT (10 μM), sumatriptan (1 μM), MDL-12 (1 μM), or forskolin (10 μM) for 10 min. The results are shown as the means ± SD of 3 to 5 experiments. * = $P <$ 0.01.

phodiesterase) without a detectable change in $[Ca^{2+}]_i$ [20]. Thus, a calcium-dependent change in protein phosphorylation or in the polymerization status of cytoskeleton proteins is not the only mechanism by which endothelial cells may fuse their secretory granules with their plasma membrane.

The present study was performed to investigate the mechanisms of early signal transduction towards the exocytosis of Weibel–Palade bodies after stimulation of endothelial cells by another vasoactive substance, serotonin. Our data demonstrate that this process can indeed be induced without significant changes in cytoplasmic calcium concentrations: Incubation of HUVEC with serotonin did not induce any change in $[Ca^{2+}]_i$ although there was a significant enhancement of the vWf release. Three of 5-HT's known receptor subtypes were found by molecular biological methods in HUVEC [21]. The presence of a functional phosphatidyl inositol-specific phospholipase C-dependent subtype 5-HT 2 receptor, as described recently [22], would implicate the appearance of a detectable calcium signal. Gill *et al.* [23] found that serotonin causes a 5-HT2_A receptor-operated uptake of ^{45}Ca in HUVEC. This uptake was obviously not correlated with a net increase in cytosolic free calcium concentration, but rather to an enhanced exchange of Ca^{2+} ions through the plasma membrane. From our data, we can exclude the possibility that there is a 5-HT 2 receptor abundantly expressed in *in vitro* cultivated HUVEC and that this receptor is not connected to the phosphatidyl inositol pathway. However, as a serotonin-caused secretion was also seen in the absence of extracellular Ca^{2+} (Fig. 5), we conclude that 5-HT 2 receptor subtypes are not involved in the observed release of vWf. The two other receptor subtypes detected in HUVEC thus far are of the 5-HT 1_D or the 5-HT 4 type, with both being correlated to the cAMP level via G-proteins acting either stimulatory (5-HT 4) or inhibitory (5-HT 1_D) on adenylate cyclase activity [7].

That endothelial cells may be activated in principle by decreased levels of cyclic AMP has been described previously. Charles *et al.* showed that the release of a neutrophil chemoattractant factor by human endothelial cells is mediated by a 5-HT 1 receptor subtype [24]. Recently, it was reported that hypoxia induces exocytosis of Weibel–Palade bodies in endothelial cells [25] and that chronic hypoxia PC12 cells may lead to a reduction in PKA activity [26]. In addition, there is evidence that in endothelial cells cytoskeletal reorganization leading to increased monolayer permeability can be caused by decreased concentrations of cytosolic cAMP [27].

The release of secretory granules by HUVEC after 5-HT stimulation as described in the present paper is also mediated by a decreased adenylate cyclase activity, supported by the following findings: (i) The 5-HT 1_D specific receptor agonist sumatriptan was able to mimic the 5-HT effect (Table 1); (ii) inhibition of adenylate cyclase led to the release of vWf (Table 1); and (iii) Neither 5-HT nor sumatriptan caused increased levels of cAMP but, on the contrary, significantly decreased these levels (Fig. 6). Obviously, the calcium-independent secretion of Weibel–Palade bodies may be induced not only by activation of the cAMP pathway [20] but also by inhibition of adenylate cyclase. It is possible that an activation of PKA by high levels of cAMP may cause a phosphorylation pattern of proteins (e.g. myosin light chain) that would allow the fusion of secretory granules with the plasma membrane in the presence of basal concentrations of free calcium ions. On the other hand, an inhibition of PKA may result in dephosphorylated enzymes (e.g. myosin light chain kinase or kinesin light chain), increasing their affinity to calmodulin to a degree that permits the binding of the cofactor with subsequent activation of exocytosis [28–30]. However, the exact substrates for dephosphorylation remain to be identified.

The presence of at least three alternative pathways for regulated endothelial granule secretion may play a role in enhancing the cellular response to simultaneous administration of submaximal concentrations of either agonist *in vivo*. This hypothesis is supported by our finding that histamine and 5-HT act synergistically in stimulating the release of vWf (Fig. 3). When histamine or any other compound from among the calcium-mediated agonists is sensed by endothelial cells simultaneously with 5-HT, the activation of calmodulin-dependent enzymes by decreased PKA activity may result in a stronger response to the first agonist.

We wish to thank Mrs. G. Jacob for her excellent technical assistance in performing the experiments.

References

1. Hattori R, Hamilton KK, Fugate RD, McEver RP and Sims PJ, Stimulated secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. *J Biol Chem* **264:** 7768–7771, 1989.
2. Weibel ER and Palade GE, New cytoplasmic components in arterial endothelia. *J Cell Biol* **23:** 101–112, 1964.
3. Hattori R, Hamilton KK, McEver RP and Sims PJ, Complement proteins C5b-9 induce secretion of high molecular weight multimers of endothelial von Willebrand factor and translocation of granule membrane protein GMP-140 to the cell surface. *J Biol Chem* **264:** 9053–9060, 1989.
4. Weiss HJ, von Willebrand factor and platelet function. *Ann NY Acad Sci* **614:** 125–137, 1991.
5. Newby AC and Henderson AH, Stimulus–secretion coupling in vascular endothelial cells. *Annu Rev Physiol* **52:** 661–674, 1990.
6. Palmer DS, Aye MT, Ganz PR, Halpenny M and Hashemi S, Adenosine nucleotides and serotonin stimulate von Willebrand factor release from cultured human endothelial cells. *Thromb Haemost* **72:** 132–139, 1994.
7. Martin GR, Vascular receptors for 5-hydroxytryptamine: Distribution, function and classification. *Pharmacol Ther* **62:** 283–324, 1994.
8. Jaffe EA, Nachman RL, Becker CG and Minick CR, Culture of human endothelial cells derived from umbilical veins.

Identification by morphologic and immunologic criteria. *J Clin Invest* **52:** 2745–2756, 1973.
9. Jacob R, Agonist-stimulated divalent cation entry into single cultured human umbilical vein endothelial cells. *J Physiol (Lond)* **421:** 55–77, 1990.
10. Thomas AP and Delaville F, The use of fluorescent indicators for measurement of cytosolic free calcium concentration in cell populations and single cells. In: *Calcium: A Practical Approach* (Eds. McCormack JG and Cobbold PH), pp. 1–54. IRL-Press, Oxford, 1991.
11. Sachs L, Angewandte Statistik. Springer Verlag, Berlin–New York, 1984.
12. Schoeffter P and Hoyer D, How selective is GR 43175? Interactions with functional 5-$HT1_A$, 5-$HT1_B$, 5-$HT1_C$ and 5-$HT1_D$ receptors. *Naunyn Schmiedebergs Arch Pharmacol* **340:** 135–138, 1989.
13. Schoeffter P, Waeber C, Palacios JM and Hoyer D, The 5-hydroxytryptamine 5-$HT1_D$ receptor subytpe is negatively coupled to adenylate cyclase in calf substantia nigra. *Naunyn Schmiedebergs Arch Pharmacol* **337:** 602–608, 1988.
14. Hamilton KK and Sims PJ, Changes in cytosolic Ca^{2+} associated with von Willebrand factor release in human endothelial cells exposed to histamine. Study of microcarrier cell monolayers using the fluorescent probe indo-1. *J Clin Invest* **79:** 600–608, 1987.
15. de Grott PG, Gonsalves MD, Loesberg C, van Buul-Wortelboer MF, van Aken WG and van Mourik JA, Thrombin-induced release of von Willebrand factor from endothelial cells is mediated by phospholipid methylation. Prostacyclin synthesis is independent of phospholipid methylation. *J Biol Chem* **259:** 13329–13333, 1984.
16. Ewenstein BM, Jacobson BC and Birch KA, Regulated secretion in vascular endothelium. *Adv Exp Med Biol* **314:** 141–157, 1991.
17. Schorer AE, Moldow CF and Rick ME, Interleukin-1 or endotoxin increases the release of von Willebrand factor from human endothelial cells. *Br J Haematol* **67:** 193–197, 1987.
18. Carew MA, Paleolog EM and Pearson JD, The roles of protein kinase C and intracellular Ca^{2+} in the secretion of von Willebrand factor from human vascular endothelial cells. *Biochem J* **286:** 631–635, 1992.
19. Tranquille N and Emeis JJ, The role of cyclic nucleotides in the release of tissue-type plasminogen activator and von Willebrand factor. *Thromb Haemost* **69:** 259–261, 1993.
20. Vischer UM and Wollheim CB, Epinephrine induces von Willebrand factor release from cultured endothelial cells: Involvement of cyclic AMP-dependent signalling in exocytosis. *Thromb Haemost* **77:** 1182–1188, 1997.
21. Ullmer C, Schmuck K, Kalkman HO and Lubbert H, Expression of serotonin receptor mRNAs in blood vessels. *FEBS Lett* **370:** 215–221, 1995.
22. Schoeffter P, Ullmer C, Gutierrez M, Weitz-Schmidt G and Lubbert H, Functional serotonin 5-$HT1_D$ receptors and 5-$HT1_D$ beta receptor mRNA expression in human umbilical vein endothelial cells. *Naunyn Schmiedebergs Arch Pharmacol* **352:** 580–582, 1995.
23. Gill JK, Stansby G, Shukla N, Hamilton G, Barradas MA and Jeremy JY, 5-Hydroxytryptamine stimulates $^{45}Ca^{2+}$ uptake by human umbilical vein endothelial cells in culture: mediation by 5-HT2 receptor subtypes. *Eur J Pharmacol* **214:** 269–272, 1992.
24. Charles A, Rounds S and Farber HW, Neutrophil chemoattractant production by cultured serotonin-stimulated bovine and human endothelial cells. *Am J Physiol* **261:** L133–139, 1991.
25. Pinsky DJ, Naka Y, Liao H, Oz MC, Wagner DD, Mayadas TN, Johnson RC, Hynes RO, Heath M, Lawson CA and Stern DM, Hypoxia-induced exocytosis of endothelial cell Weibel–Palade bodies. A mechanism for rapid neutrophil recruitment after cardiac preservation. *J Clin Invest* **97:** 493–500, 1996.
26. Beitner-Johnson D, Leibold J and Millhorn DE, Hypoxia regulates the cAMP and Ca^{2+}/calmodulin signaling systems in PC12 cells. *Biochem Biophys Res Commun* **242:** 61–66, 1998.
27. Hastie LE, Patton WF, Hechtman HB and Shepro D, H_2O_2-induced filamin redistribution in endothelial cells is modulated by the cyclic AMP-dependent protein kinase pathway. *J Cell Physiol* **172:** 373–381, 1997.
28. Saitoh M, Naka M and Hidaka H, The modulatory role of myosin light chain phosphorylation in human platelet activation. *Biochem Biophys Res Commun* **140:** 280–287, 1986.
29. Abdel-Latif AA, Cross talk between cyclic AMP and the polyphosphoinositide signaling cascade in iris sphincter and other nonvascular smooth muscle. *Proc Soc Exp Biol Med* **211:** 163–177, 1996.
30. Matthies HJ, Miller RJ and Palfrey HC, Calmodulin binding to cAMP-dependent phosphorylation of kinesin light chains modulate kinesin ATPase activity. *J Biol Chem* **268:** 11176–11187, 1993.