Transglutaminase-dependent RhoA Activation and Depletion by Serotonin in Vascular Smooth Muscle Cells*

Christophe Guilluy[‡], **Malvyne Rolli-Derkinderen**[§1], **Pierre-Louis Tharaux**[¶], **Gerry Melino**[‖], **Pierre Pacaud**[‡2], **and Gervaise Loirand**[‡3]

From the [‡]*INSERM U533 Institut du Thorax, Université de Nantes, 44322 Nantes cedex 3, France,* [¶]*INSERM U702, Hôpital Tenon, Paris, France,* [‖]*Biochemistry Laboratory, University of Rome "Tor Vergata," 00133 Rome, Italy, and* [§]*UMR CNRS 6214-INSERM 771, Faculté de Médecine, 49045 Angers, France*

The small G protein RhoA plays a major role in several vascular processes and cardiovascular disorders. Here we analyze the mechanisms of RhoA regulation by serotonin (5-HT) in arterial smooth muscle. 5-HT ($0.1–10 \mu$M) induced activation of RhoA followed by RhoA depletion at $24–72$ h. Inhibition of 5-HT1 receptors reduced the early phase of RhoA activation but had no effect on 5-HT-induced delayed RhoA activation and depletion, which were suppressed by the 5-HT transporter inhibitor fluoxetine and the transglutaminase inhibitor monodansylcadaverin and in type 2 transglutaminase-deficient smooth muscle cells. Coimmunoprecipitations demonstrated that 5-HT associated with RhoA both *in vitro* and *in vivo*. This association was calcium-dependent and inhibited by fluoxetine and monodansylcadaverin. 5-HT promotes the association of RhoA with the E3 ubiquitin ligase Smurf1, and 5-HT-induced RhoA depletion was inhibited by the proteasome inhibitor MG132 and the RhoA inhibitor Tat-C3. Simvastatin, the Rho kinase inhibitor Y-27632, small interfering RNA-mediated RhoA gene silencing, and long-term 5-HT stimulation induced Akt activation. In contrast, inhibition of 5-HT-mediated RhoA degradation by MG132 prevented 5-HT-induced Akt activation. Long-term 5-HT stimulation also led to the inhibition of the RhoA/Rho kinase component of arterial contraction. Our data provide evidence that 5-HT, internalized through the 5-HT transporter, is transamidated to RhoA by transglutaminase. Transamidation of RhoA leads to RhoA activation and enhanced proteasomal degradation, which in turn is responsible for Akt activation and contraction inhibition. The observation of transamidation of 5-HT to RhoA in pulmonary artery of hypoxic rats suggests that this process could participate in pulmonary artery remodeling and hypertension.

The small G proteins of the Rho family are identified as key signaling molecules in the vasculature. RhoA and its downstream effector Rho kinase have been shown to play a major role in vascular processes such as smooth muscle cell contraction, proliferation and differentiation, endothelial permeability, platelet activation, and leukocyte migration (1, 2). Abnormal activation of the RhoA/Rho kinase pathway has been observed in major cardiovascular disorders such as atherosclerosis, restenosis, hypertension, pulmonary hypertension, and cardiac hypertrophy. The activity of RhoA is under the direct control of a large set of regulatory proteins (3). In the inactive GDP-bound form, RhoA is locked in the cytosol by guanine dissociation inhibitors (4). The guanine nucleotide exchange factors catalyze the exchange of GDP for GTP to activate RhoA (5). In the active GTP-bound form, RhoA translocates to plasma membrane, where it interacts with effectors to transduce the signal downstream. GTPase-activating proteins that hydrolyzed GTP to GDP then turn off activation. In addition to this activation/inactivation cycle, recent reports have proposed that regulation of Rho protein degradation could participate in the regulation of Rho protein functions. Phosphorylation of RhoA by cGMP-dependent protein kinase protects RhoA from ubiquitin/proteasome-mediated degradation in vascular smooth muscle cells (6). In contrast, constitutive activation of Rho proteins by the bacterial toxin cytotoxic necrotizing factor 1 enhances their ubiquitin/proteasome-mediated degradation (7). To date, the relevance of this mechanism of Rho protein depletion in a physiological/pathophysiological context, in response to membrane receptor agonist stimulation, has not been addressed.

In vascular physiology, Akt signaling has been shown to mediate survival signals of many angiogenic factors and plays central roles in the regulation of vascular homeostasis and angiogenesis (8). Recent reports also reveal that Akt signaling plays important roles in vascular smooth muscle cells. Akt signaling mediates cell survival, proliferation, and migration of vascular smooth muscle cells induced by angiotensin II, serotonin 5-hydroxytryptamine (5-HT),[4] and several growth factors (9–12). Akt signaling pathway is suggested to play critical roles in pathological accumulation of vascular smooth muscle cells observed in various types of vascular lesions (11, 13). Recently, it has been shown that Akt is negatively regulated by the RhoA/Rho kinase pathway in endothelial cells (14). Accord-

[4] The abbreviations used are: 5-HT, serotonin; 5-HTT, 5-HT transporter; TG, transglutaminase; PTEN, phosphatase and tensin homolog deleted on chromosome 10; MDC, monodansylcadaverin; siRNA, small interfering RNA; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

ingly, Rho kinase inhibition leads to the activation of Akt (15). Indirect inhibition of the RhoA/Rho kinase activity could also account for the activation of Akt induced by statins (16). Indeed, by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase, statins prevent the formation of geranylgeranylpyrophosphate required for membrane translocation and activation of RhoA, which finally leads to the reduction of Rho kinase activity (17).

Although both 5-HT receptors and 5-HT transporter (5-HTT) could participate in the effect of 5-HT on vascular smooth muscle cell growth, the signaling pathways involved are not clearly established. Here we demonstrate that 5-HT, internalized through the 5-HTT, is transamidated to RhoA by transglutaminase (TG). Transamidation of RhoA leads to its activation and its rapid proteasomal degradation, which in turn is responsible for Akt activation.

EXPERIMENTAL PROCEDURES

Cell Culture—Rat and mice (C57BL/6 and type 2 TG (TG2)-deficient (TG2$^{-/-}$) mice (18)) aortic smooth muscle cells were isolated by enzymatic dissociation as previously described (19).

siRNA—The siRNA were introduced into vascular smooth muscle cells by electroporation (Nucleofector, Amaxa) according to the manufacturer's instructions. The sense and antisense strands of siRNAs (Eurogentec, Seraing, Belgium) used were: RhoA, sense 5′-GAAGUCAAGCAUUUCUGUCd-TdT-3′, antisense 5′-GACAGAAAUGCUUGACUUCdTdT-3′; scrambled, sense 5′-GCCUGUGAUGACUACAGACd-TdT-3′, antisense 5′-GUCUGUAGUCAUCACAGGCdTdT-3′. Efficiency and specificity of RhoA-siRNA have been validated by real-time quantitative PCR performed in the iCycler iQ^{TM} detection system (Bio-Rad) using Sybr green detection (Molecular Probes). PCR primers were synthesized by Sigma Genosys and Titanium^{TM} *Taq* DNA polymerase (Clontech) according to the manufacturer's recommendations. The scrambled siRNA had no effect on RhoA mRNA. In contrast, RhoA-siRNA (1.2 μg/ml each strands) decreased the RhoA mRNA level by 80 ± 2 and 70 ± 5% at 24 and 48 h (*n* = 4), respectively, without effect on other members of the Rho family: RhoB, Rac1, and Rnd3.

Western Blot Analysis—Cells were harvested in lysis buffer (50 mM Tris-Cl, pH 7.2, 500 mM NaCl, 10 mM MgCl$_2$, 0.5% sodium deoxycholate, 0.1% SDS) containing 1% Triton, protease inhibitors, and phosphatase inhibitor mixture (Sigma). Nuclei and unlysed cells were removed by low speed centrifugation. Total protein samples were heated at 95 °C for 5 min in Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with primary monoclonal antibody against RhoA (1:200), polyclonal antibody against phosphoserine 473 of Akt (1:1000), polyclonal antibody against Akt (1:1000), polyclonal antibody against phosphatase and tensin homolog deleted on chromosome 10 (PTEN; 1:1000) or polyclonal antibody against phospho-PTEN (serine 380/threonine 382/383; 1:1000) (P-PTEN). β-Actin antibody was used as loading control. Signals from immunoreactive bands were revealed with horseradish peroxidase-conjugated secondary antibody,

detected by ECL (Amersham Biosciences), and quantified using QuantityOne (Bio-Rad).

Coimmunoprecipitation—Cells were harvested in NETF buffer (100 mM NaCl, 2 mM EGTA, 50 mM Tris-Cl, pH 7.4, and 50 mM NaF) containing 1% Nonidet P-40, 2 mM orthovanadate, protease inhibitors, and phosphatase inhibitor mixture (Sigma). Samples were precleared with 40 μl of protein A- or G-Sepharose beads (which have been washed in NETF buffer to give a 50% slurry), and immunoprecipitations were carried out with monoclonal anti-RhoA antibody or polyclonal anti-Smurf1 antibody preadsorbed on protein A- or G-Sepharose beads. The protein A/G-Sepharose-bound immune complexes were washed twice in NETF buffer containing Nonidet P-40 (1% w/v) and once in NETF without detergent. Pellets from the immunoprecipitations were heated at 95 °C for 5 min in 70 μl of Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis and analyzed by immunoblot for 5-HT or RhoA. Signals from immunoreactive bands were detected by ECL (Amersham Biosciences) and quantified using QuantityOne.

Measurement of RhoA Activity—RhoA activity was assessed in homogenized cell samples by pulldown assays using the Rho binding domain of the Rho effector Rhotekin as described previously (20). Precipitated GTP-bound RhoA and total RhoA were then analyzed by Western blotting. Signals from immunoreactive bands were detected by ECL (Amersham Biosciences) and quantified using QuantityOne.

Two-dimension Electrophoresis—Vascular smooth muscle cells were washed 3 times with 25 mM Tris, pH 7.4 and scraped directly in UTC buffer (8 M urea, 2 M thiourea, 4% CHAPS, 150 μl per 2 × 10^6 cells). Protein concentration was determined by commercial Bio-Rad protein assay, and aliquots of 100 μg were stored at −80 °C until use. Samples were applied on a 7-cm IPG strip, pH 3–10, linear gradient (Immobiline Dry Strip, GE Healthcare) for 12 h of passive rehydration in Ettan IPGphor3 Manifold (GE Healthcare). After isoelectric focusing (Ettan IPGphor3, GE Healthcare), the strips were equilibrated in 50 mM Tris, 6 M urea, 30%glycerol, 2% SDS, and 1% dithiothreitol for 15 min and the same buffer in which DTT was replaced by 2.5% iodoacetamide. The equilibrated IPG strips were transferred on 12% acrylamide gel for second dimension and Western blot analysis as described above.

Hypoxia—The hypoxic rats were housed in a hypobaric chamber at 380 mm Hg (Vacucell 111 L, Medcenter, Munich, Germany) for 2 days. Fluoxetin (20 mg/kg/day) was administrated by gavage for 2 days in control (normoxic) and hypoxic rats. At completion of the exposure to hypoxia, aorta and pulmonary arteries were removed and prepared as indicated for immunoprecipitation and Western blot analyses.

Contraction Measurements—The aorta was collected in physiological saline solution (130 mM NaCl, 5.6 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 11 mM glucose, 10 mM Tris, pH 7.4 with HCl) cleaned of fat and adherent connective tissue and cut in rings. The endothelium was carefully removed by gently rubbing the intimal surface with the tip of small forceps. Rings were then incubated for 48 h in serum-free Dulbecco's modified Eagle's medium at 37 °C in an incubator in the absence or presence of 10 μM 5-HT and in the presence of 1 μM GR 127936 and 10 μM mianserin. Arterial rings were then suspended under
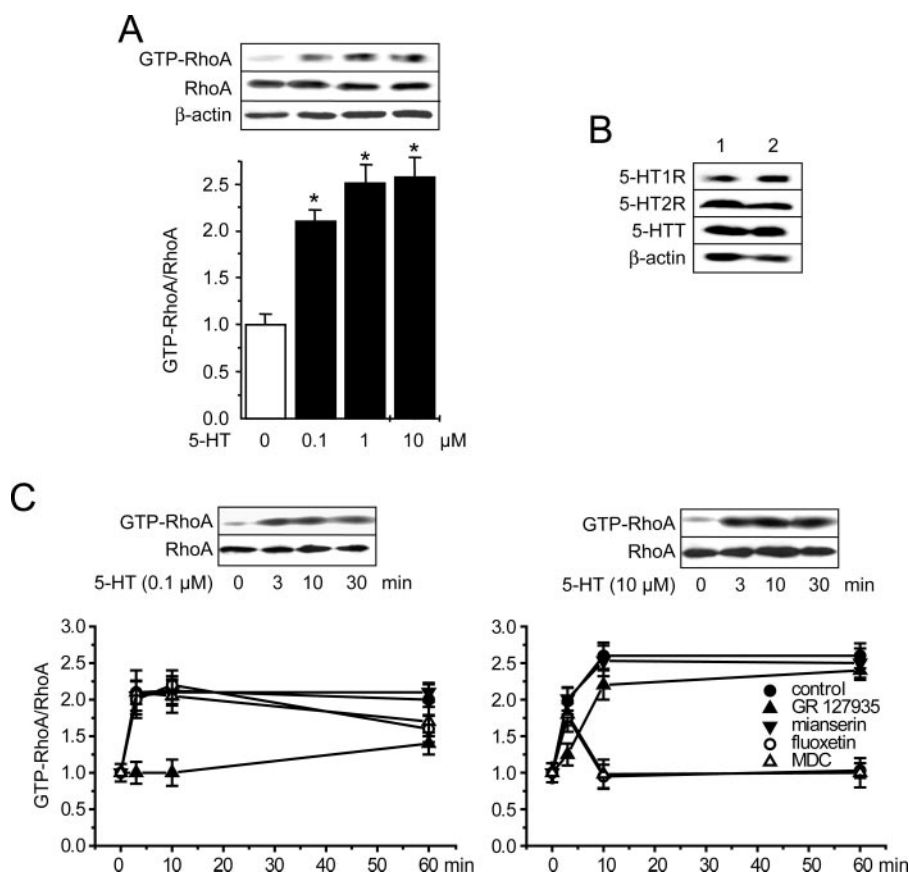
FIGURE 1. **5-HT activates RhoA.** *A,* Western blot and corresponding densitometric analyses for RhoA activity (GTP-RhoA) and RhoA levels in cells stimulated for 10 min by 0–10 μM 5-HT. *B,* Western blot for 5-HT1B/1D receptor, 5-HT2B receptor, and 5-HTT expression in two different batches of aortic smooth muscle cells in culture (third passage). *C,* Western blot and corresponding densitometric analyses for RhoA activity (GTP-RhoA) and RhoA levels in cells stimulated for 3–60 min by 0.1 μM 5-HT (*left*) and 10 μM 5-HT in the absence or in the presence of GR 127935 (1 μM), mianserin (10 μM), fluoxetine (10 μM), or MDC (200 μM). Equal loading was confirmed by examination of β-actin expression. RhoA activity is normalized to RhoA levels and expressed relative to the control in the absence of 5-HT taken as 1. The data presented are representative of at least three independent experiments. *, $p < 0.001$ *versus* control.

sometric conditions and connected to a force transducer (Pioden Controls Ltd., Canterbury, UK) in organ baths filled with Krebs-Henseleit solution (118.4 mM NaCl, 4.7 mM KCl, 2 mM CaCl$_2$, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, 11 mM glucose) maintained at 37 °C and equilibrated with 95% O$_2$, 5% CO$_2$. Calcium-dependent contractions were induced by stimulation with 60 mM KCl. Cumulative concentration-response curves were constructed in response to U46619. Amplitude of the contraction was expressed as a percentage of the maximal 60 mM KCl-induced contraction.

Chemicals and Drugs—Mouse monoclonal anti-RhoA antibody (26C4) and goat polyclonal anti-Smurf1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Akt, anti-phosphorylated-Akt, anti-PTEN, and anti-P-PTEN were purchased from Cell Signaling Technology (Ozyme, Saint Quentin, France). Anti-5HT1B/1D receptor antibody was from Novus Biological (Interchim, Montluçon, France), and the anti-5-HTT antibody was from Chemicon (Hampshire, UK) The permeant RhoA inhibitor Tat-C3 protein was produced in *Escherichia coli* and purified as already described (21, 22). All other reagents, including 5-HT (hydrochloride), rabbit polyclonal anti-5-HT, and anti-5-HT2B

receptor antibodies, were purchased from Sigma (Saint-Quentin Fallavier, France).

Statistics—All results are expressed as the means $\pm$ S.D. of sample size *n.* Significance was tested by analysis of variance or Student's *t* test.

RESULTS

Long-term 5-HT Stimulation Induces 5-HTT- and TG-dependent RhoA Activation—RhoA activity has been determined by pulldown assay in aortic smooth muscle cells stimulated by 5-HT. Results shown in Fig. 1*A* indicate that 5-HT applied for 10 min dose-dependently (0.1–10 μM) activated RhoA. This stimulation of RhoA activity could result either from 5HT1, 5HT2 receptor, or 5-HTT activation, which were all expressed in cultured aortic smooth muscle cells (Fig. 1*B*). The use of selective inhibitors revealed that the pharmacological profile of 5-HT-induced RhoA activation depended on the concentration and the duration of 5-HT stimulation. Time-course analysis showed that 5-HT-induced RhoA activation was maintained (Fig. 1*C*). Maximal RhoA activation induced by 0.1 μM 5-HT (2.10 $\pm$ 0.16-fold over control, $n = 5$) was observed after 3 min of stimulation, whereas 10 min were required to reach maximal RhoA activation in the presence of 10 μM 5-HT (2.60 $\pm$ 0.18-fold over control, $n = 5$) (Fig. 1*C*). Activation of RhoA induced by short-term stimulation (3–10 min) with 0.1 μM 5-HT was inhibited by the pharmacological inhibition of 5-HT1B/1D receptor by the GR 127935 (1 μM) but was unaffected by the inhibition of 5-HT2A receptor by mianserin (10 μM) or 5-HTT by fluoxetine (10 μM), suggesting that the first phase of RhoA activation only depended on 5-HT1B/1D receptor activation (Fig. 1*C*). In contrast, at 60 min RhoA activation induced by 0.1 μM 5-HT was partially inhibited by GR 127935 and fluoxetine, indicating that both 5-HT1B/1D and 5-HTT were involved in the delayed activation of RhoA (Fig. 1*C*). In the presence of 10 μM 5-HT, activation of RhoA at 3 min essentially depended on 5-HT1B/1D receptor (Fig. 1*C*). However, the involvement of 5-HTT occurred earlier than that observed with 0.1 μM as fluoxetine inhibited RhoA activation induced by 10 min of stimulation with 10 μM 5-HT (Fig. 1*C*). These results, thus, indicate that 5-HT1B/1D receptor was responsible for early 5-HT-induced RhoA activation, whereas delayed RhoA activation induced by longer 5-HT stimulation was due to 5-HTT, the involvement of which was favored at high 5-HT concentration. The delayed participation of 5-HTT to RhoA activation by 0.1 μM 5-HT
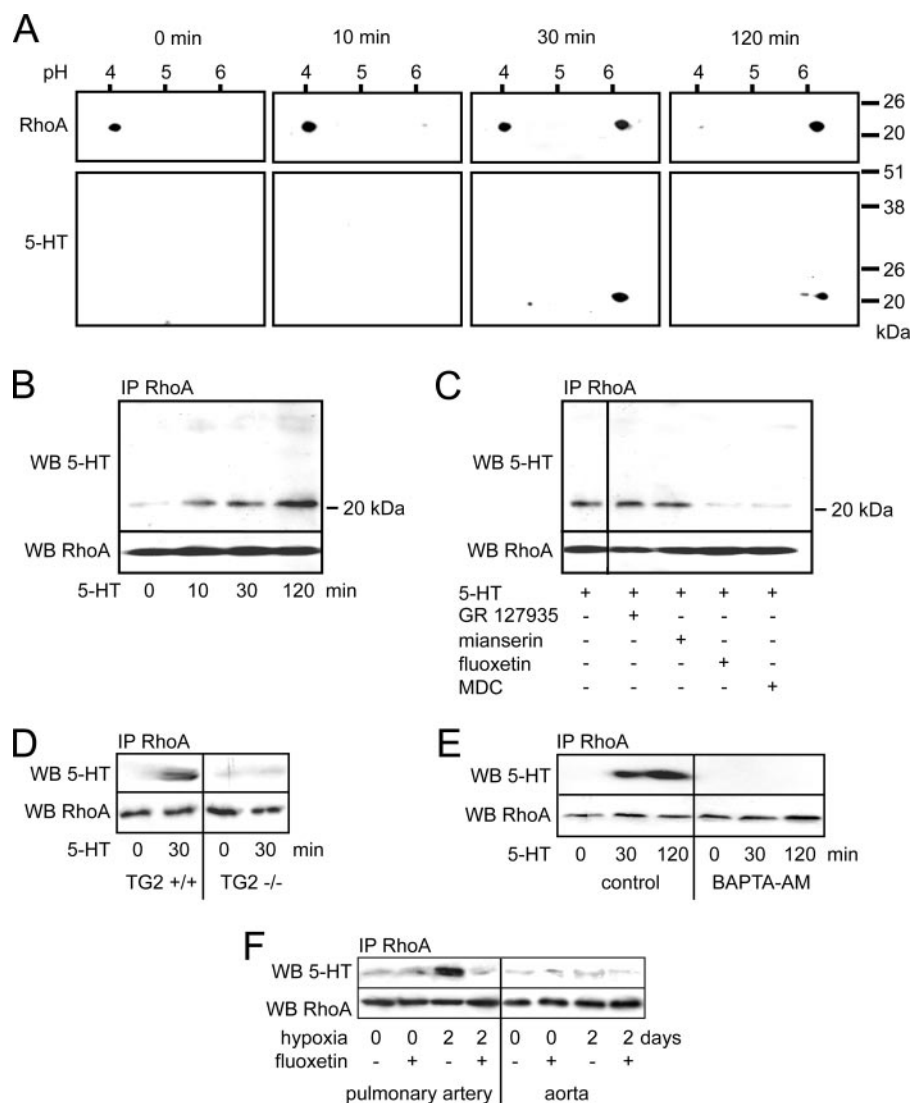
FIGURE 2. **5-HT binds RhoA.** *A*, protein extracts of aortic smooth muscle cells stimulated with 10 μM 5-HT for 0–120 min were separated by two-dimension gel electrophoresis followed by Western blot (*WB*) analysis using anti-RhoA and anti-5-HT antibodies. *B* and *C*, RhoA was immunoprecipitated (*IP*) with anti-RhoA antibody in lysates from cells stimulated for 10 μM 5-HT for 0–120 min (*B*) and for 30 min in the absence or in the presence of GR 127935 (1 μM), mianserin (10 μM), fluoxetine (10 μM), or MDC (200 μM) (*C*). 5-HT binding was assessed using anti-5-HT antibody. *D*, Western blotting with anti-5-HT antibody of immunoprecipitated RhoA from aortic smooth muscle cells of control (TG2$^{+/+}$) and TG2$^{-/-}$ mice in the absence and presence of 10 μM 5-HT for 30 min. *E*, Western blotting with anti-5-HT antibody of immunoprecipitated RhoA from rat aortic smooth muscle cells stimulated with 10 μM 5-HT for 30 and 120 min in the absence and the presence of BAPTA-AM (30 μM), added 30 min before 5-HT stimulation. *F*, Western blotting with anti-5-HT antibody of immunoprecipitated RhoA from rat pulmonary artery and aorta of normoxic (*0 days*) or hypoxic rats (*2 days* hypoxia) treated or not with fluoxetine. Equal amounts of RhoA were confirmed by Western blot with anti-RhoA antibody (*B–F*). The data presented are representative of at least three independent experiments.

could be related to reduced intracellular 5-HT accumulation (Fig. 1*C*).

The 5-HTT allows internalization of 5-HT that can then be used as substrate for TG, and it has been recently demonstrated that TG can transamidate Glu-63 of RhoA, leading to its constitutive activation (23). We, therefore, analyzed the potential involvement of TG in the activation of RhoA induced by 5-HT by using the pharmacological TG inhibitor monodansylcadaverin (MDC, 200 μM). Inhibition of TG mimicked the effect of fluoxetine because it prevented 5-HT-induced delayed RhoA activation (Fig. 1*C*), suggesting that it was mediated by transamidation.

5-HT Binds to RhoA—If 5-HT-induced RhoA activation was due to TG-mediated transamidation of RhoA, 5-HT should be covalently linked to RhoA (24, 25). To address this hypothesis, we first used two-dimension gel electrophoresis and immunoblotting (Fig. 2*A*). RhoA immunoblotting after two-dimension gel electrophoresis of total protein extracts derived from aortic smooth muscle cells revealed that 5-HT induced a shift of the RhoA spot to alkaline pH without a change in the molecular mass, ~21 kDa (Fig. 2*A*). Under control conditions, a single RhoA positive spot was detected at pH 4. In the presence of 5-HT, another RhoA spot appeared at a pH close to 6. The density of this second spot increased with the duration of 5-HT application becoming the major spot after 120 min. Western blot with an anti-5-HT antibody showed that in the absence of 5-HT and after 10 min of 5-HT stimulation, there was no 5-HT positive spot in the range of 18–50 kDa (Fig. 2*A*). However, a positive spot, corresponding to the more alkaline RhoA spot appeared at 30 and 120 min of 5-HT stimulation. To directly assess the binding of 5-HT to RhoA, we next performed coimmunoprecipitation experiments (Fig. 2*B*). In RhoA precipitated from cells stimulated for 0–120 min with 5-HT, a Western blot with a specific anti-5-HT antibody revealed an immunoreactive band at ~21 kDa, which corresponded to the molecular mass of RhoA. The amount of 5-HT that coimmunoprecipitated with RhoA increased with the time of 5-HT application. Inhibition of 5-HT1 receptor by GR 127935 or 5-HT2 receptor by mianserin did not alter 5-HT/RhoA interaction (Fig. 2*C*). In contrast, in the presence of fluoxetine or MDC, the amount of 5-HT that coimmunoprecipitated with RhoA was extremely low, indicating that inhibition of the 5-HTT or TG prevented 5-HT binding to RhoA (Fig. 2*C*).

The TG2, identified as the most interesting member of the TG family (26), has been shown to be expressed in smooth muscle cells (27). To further analyze TG-mediated 5-HT binding to RhoA, 5-HT stimulation was applied to aortic smooth muscle cells isolated from TG2$^{-/-}$ mice (18). Coimmunoprecipitation and immunoblot experiments indicated that stimu-
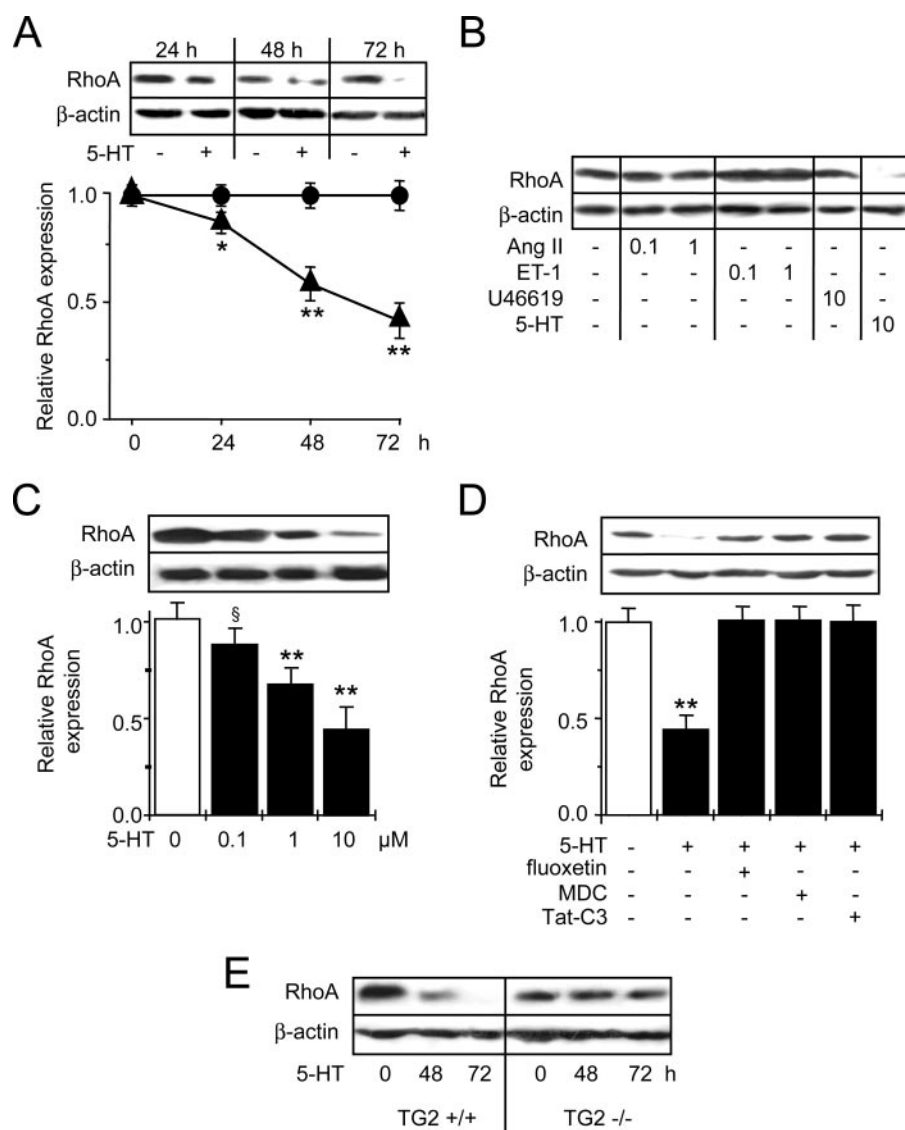
FIGURE 3. **5-HT induces depletion of RhoA.** *A*, Western blot and corresponding quantitative analyses for RhoA expression in control and in cells stimulated by 10 μM 5-HT for 0–72 h. *B*, Western blot analyses for RhoA expression in control and in cells stimulated by 0.1 and 1 μM angiotensin II (*Ang II*), 0.1 and 1 μM endothelin 1 (*ET-1*), 10 μM U46619, and 10 μM 5-HT for 72 h. *C*, Western blot and corresponding quantitative analyses for RhoA expression in control and in cells stimulated by increasing concentrations of 5-HT (0.1–10 μM) for 72 h. *D*, Western blot and corresponding quantitative analyses for RhoA expression in control and in cells stimulated by 10 μM 5-HT for 72 h in the absence or in the presence of fluoxetine (10 μM), MDC (200 μM), or Tat-C3 (20 μg/ml). *E*, Western blot analyses for RhoA expression in aortic smooth muscle cells of control (TG2$^{+/+}$) and TG2$^{-/-}$ mice in the absence and presence of 10 μM 5-HT for 48 and 72 h. RhoA expression is normalized to β-actin and expressed relative to the control in the absence of 5-HT taken as 1. The data presented are representative of at least three independent experiments. §, $p < 0.05$; *, $p < 0.01$; **, $p < 0.001$.

lation with 5-HT for 30 min induced association of 5-HT to RhoA in cells from control mice but not in cells isolated from TG2$^{-/-}$ mice, suggesting that transamidation of RhoA was mediated by TG2 (Fig. 2*D*). TG2 is predominantly a cytosolic protein catalyzing Ca^{2+}-dependent protein modifications (28). To address a potential role of Ca^{2+} in 5-HT-induced TG2-mediated 5-HT/RhoA association, we used the cell-permeable Ca^{2+} chelator BAPTA-AM. As shown in Fig. 2*E*, treatment of rat aortic smooth muscle cells with BAPTA-AM completely prevented 5-HT attachment to RhoA. These results, thus, provide direct evidence for Ca^{2+}-dependent transamidation of intracellular 5-HT to RhoA by TG2 in vascular smooth muscle cells.

5-HT Binds to RhoA in Vivo—We next assessed whether transamidation of intracellular 5-HT to RhoA occurred in arteries *in vivo*. 5-HT and 5-HTT have been shown to participate in the development of hypoxia-induced pulmonary remodeling and hypertension (29–31), which also involved RhoA activation (32). Furthermore, hypoxia up-regulated 5-HTT expression and enhanced 5-HT uptake in pulmonary artery smooth muscle cells (29, 33). We, therefore, analyzed 5-HT/RhoA association in aorta and pulmonary arteries of rat maintained in hypoxic condition for 2 days. Hypoxia induced binding of 5-HT to RhoA in pulmonary artery but not in aorta (Fig. 2*F*). This effect was blocked by fluoxetine, indicating the role of 5-HTT (Fig. 2*F*). This result, thus, provides evidence that 5-HTT-dependent transamidation of 5-HT to RhoA exists in arteries *in vivo*.

5-HT Induces RhoA Depletion by Stimulation of Its Proteasomal Degradation—Transamidated RhoA is constitutively active (23), and constitutively active RhoA has been shown to be subject to rapid proteasomal degradation (7). It could, thus, be expected that maintained stimulation with 5-HT should promote decrease of RhoA expression in vascular smooth muscle cells. To address this hypothesis, we performed time-course analysis of RhoA expression by Western blot in the absence and presence of 5-HT for up to 72 h. As shown in Fig. 3*A*, 5-HT induced a time-dependent depletion of RhoA. Analysis of RhoA mRNA by real-time PCR indicated that there was no change in the amount of RhoA mRNA in

the presence of 5-HT (not shown). This decrease in RhoA expression is not a general phenomenon in response to long-term stimulation of vascular smooth muscle cells with vasoconstrictors as application of other RhoA-activating vasoconstricting agents such as angiotensin II, endothelin 1, or the thromboxane A2 analog U46619, at concentrations producing maximal contraction for 72 h, had no effect on RhoA expression (Fig. 3*B*). RhoA depletion was observed in response to 5-HT concentration ranging from 0.1 to 10 μM, similar to those inducing RhoA activation (Fig. 3*C*). The inhibitory effect of 5-HT on RhoA expression was blocked by fluoxetine and by MDC, indicating that it depended on transamidation of intra-
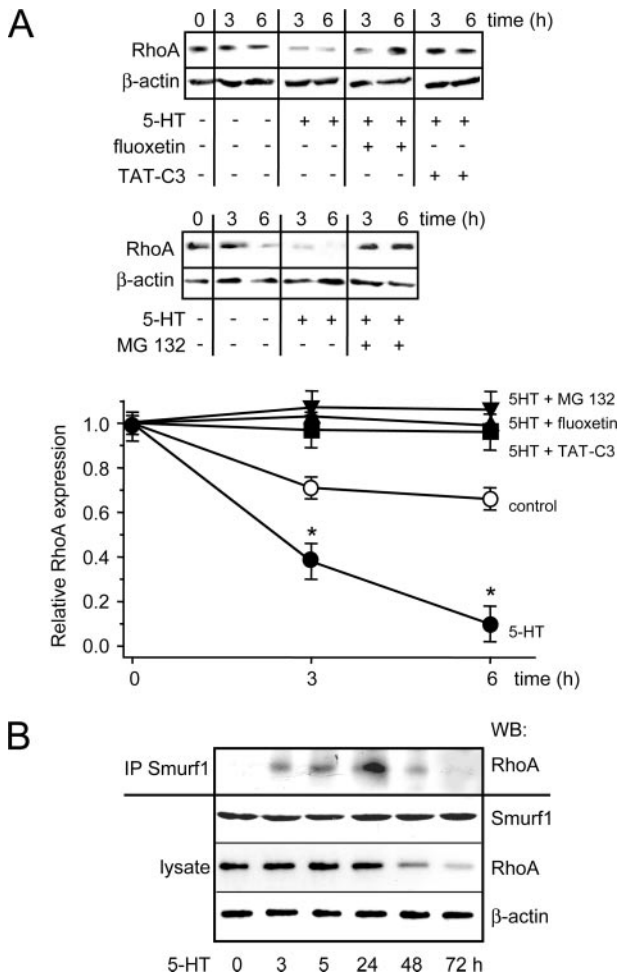
FIGURE 4. **5-HT induces accelerated proteasomal degradation of RhoA.** *A*, cells were preincubated with 15 μg/ml cycloheximide for 30 min, then stimulated for 3 and 6 h by 10 μM 5-HT in the absence and presence of fluoxetine (10 μM), Tat-C3 (20 μg/ml), or MG132 (100 μM). RhoA protein levels detected by Western blot analysis at various time are expressed relative to that of control (0 h) taken as 1. *B*, Smurf1 was immunoprecipitated (*IP*) with anti-Smurf1 antibody, and RhoA binding was assessed using anti-RhoA antibody. Western blotting (*WB*) with anti-Smurf1, RhoA, and β-actin antibody shows the presence of similar amounts of proteins in the samples. Data shown are the mean of 3–4 independent experiments. *, $p < 0.001$.

cellular 5-HT (Fig. 3*D*). In addition, 5-HT-induced depletion of RhoA was also prevented by the inhibition of RhoA activity by Tat-C3 (20 μg/ml) (Fig. 3*D*). In mouse aortic smooth muscle cells, 5-HT stimulation for 48 or 72 h induced RhoA depletion similar to that described in rat cells. However, this effect of 5-HT was completely lost in smooth muscle cells from TG2$^{-/-}$ mice (Fig. 3*E*). Together, these results, thus, suggest that 5-HT-induced RhoA depletion was the consequence of RhoA activation by transamidation of intracellular 5-HT by TG2.

To assess whether 5-HT-induced RhoA depletion resulted from its accelerated proteasomal degradation, we examined the fate of RhoA by Western blotting in the presence cycloheximide. Under these conditions, 5-HT increased the rate of RhoA degradation (Fig. 4*A*). This effect was inhibited by the blockade of 5-HTT by fluoxetine and inhibition of RhoA activation by Tat-C3 (Fig. 4*A*). In fact, in the presence of fluoxetine or Tat-C3, RhoA degradation was even slower than that occurring in

control, suggesting that basal 5-HTT and RhoA activities were involved in the slow RhoA degradation under control conditions. Inhibition of the proteasome activity by MG132 (100 μM) also prevented the acceleration of RhoA degradation induced by 5-HT (Fig. 4*A*), indicating that the loss of RhoA expression induced by 5-HT was due to its increased proteasomal degradation.

The E3 ubiquitin ligase Smurf1 has been recently shown to target active RhoA for ubiquitination and subsequent proteasomal degradation (34). If Smurf1 is involved in the accelerated degradation of RhoA induced by 5-HT, it could be expected that 5-HT stimulation might increase the association of RhoA to Smurf1. To address this hypothesis, lysates of vascular smooth muscle cells were immunoprecipitated with an anti-Smurf1 antibody, and a Western blot was performed using an anti-RhoA antibody. Under control conditions, RhoA was not associated to Smurf1 (Fig. 4*B*). 5-HT (3–72 h) increased the amount of RhoA that coprecipitated with Smurf1, with a maximal effect at 24 h (Fig. 3*B*). For longer times of 5-HT stimulation, the amount of RhoA coprecipitated with Smurf1 decreased, in parallel to RhoA expression (Fig. 3*B*). These results, thus, show that, in agreement with the increased proteasomal degradation of RhoA, 5-HT induced association of RhoA to Smurf1.

Long-term 5-HT Stimulation Depressed Arterial Contractility—The next question to address was to determine the functional consequences of 5-HT-induced RhoA depletion. We first examined whether long-term stimulation with 5-HT altered the contractile properties of arterial smooth muscle cells, known to be largely dependent on the RhoA/Rho kinase activity (1). Endothelium-denuded aorta rings were maintained for 48 h in the absence or presence of 10 μM 5-HT before measurements of contractile activity. As shown in Fig. 5*A*, in agreement with the results obtained in cell cultures, this treatment induced RhoA depletion in aorta rings. The long-term pretreatment with 5-HT did not modify the amplitude of the calcium-dependent contraction induced by 60 mM KCl (Fig. 5*B*). In contrast, 5-HT pretreatment inhibited by 75% the contractile response to U46619 (Fig. 5*C*). This inhibitory effect was completely lost by co-treatment with fluoxetine (not shown). In the presence of the Rho kinase inhibitor Y-27632 (10 μM), the amplitude of the contraction induced by 10 μM U46619 was similar in control and in 5-HT-pretreated rings and corresponded to ~20% that of the control response (Fig. 5*C*). This observation, thus, indicates that long-term treatment with 5-HT almost completely inhibited the RhoA/Rho kinase-dependent component of the U46619-induced contraction, which is in agreement with the observed 5-HT-induced RhoA depletion.

RhoA Depletion Induces Akt Activation—In endothelial cells, inhibition of RhoA leads to Akt activation (14). We, therefore, hypothesized that in addition to the inhibition of vascular contractility, 5-HT-induced RhoA depletion could also induce Akt activation in vascular smooth muscle cells. To address this hypothesis, we first examined whether inhibition of RhoA was able to activate Akt in vascular smooth muscle cells. Pharmacological inhibition of RhoA by the non-selective Rho protein inhibitor simvastatin (10 μM, 24 h) induced a 3–4-fold increase
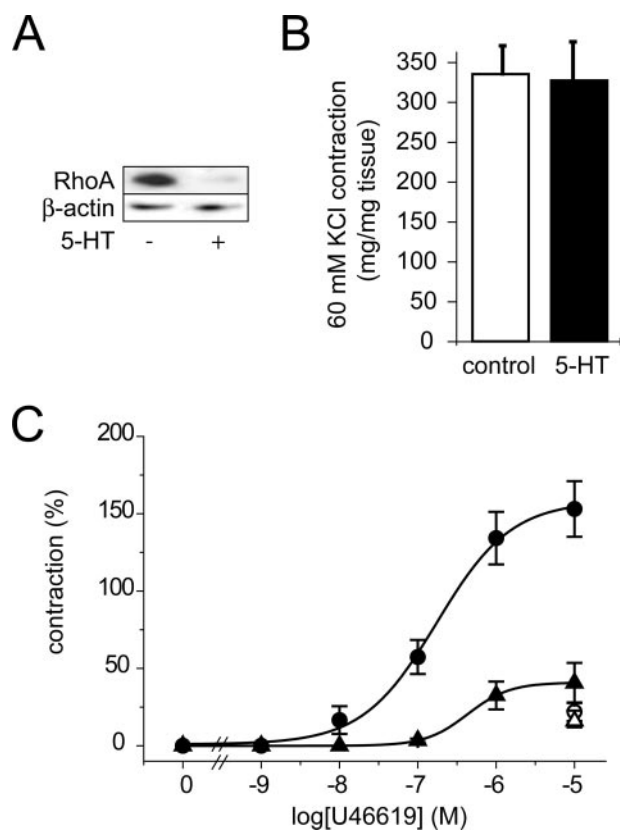
FIGURE 5. **Effect of long-term 5-HT stimulation on contractile properties of the aorta.** *A*, amplitude of the calcium-dependent contraction induced by 60 mM KCl in aorta rings incubated for 48 h in the absence (control) and presence of 10 μM 5-HT. *B*, cumulative concentration-response curves for the action of U46619 in aorta rings incubated for 48 h in the absence (*circles*) and presence of 10 μM 5-HT (*triangles*). *C*, *open symbols* show the amplitude of the contraction of control (*circles*) and 5-HT treated rings (*triangles*) in response to 10 μM U46619 in the presence of 10 μM Y-27632. Contraction is expressed as a percentage of the 60 mM KCl-induced contraction. Data shown are the mean of four independent experiments.

FIGURE 6. **Inhibition or depletion of RhoA induces Akt activation.** Western blot and corresponding densitometric analyses for Akt activity monitored by serine 473 phosphorylated (*P-Akt*), Akt, and RhoA (*B*) in control (*0*) and in cells treated with simvastatin (10 μM) or Y-27632 (10 μM) for 24 h (*A*) and with scrambled- or RhoA-siRNA for 24 and 48 h (*B*). Akt phosphorylation is normalized to Akt levels and expressed relative to the control taken as 1. The data presented are representative of at least three independent experiments in *A* and 5 in *B*. *, $p < 0.001$.

in Akt activity, monitored by the measurement of serine 473 phosphorylation by Western blot (Fig. 6*A*). Similarly, inhibition of the RhoA effector Rho kinase by Y-27632 (10 μM) also activated Akt (Fig. 6*A*). To mimic 5-HT-induced RhoA depletion, we next used siRNA targeting RhoA. Treatment of vascular smooth muscle cells with RhoA-siRNA for 24 or 48 h nearly abolished RhoA expression (Fig. 6*B*). siRNA-mediated RhoA knockdown was associated with a 3–4-fold increase in Akt activation (Fig. 6*B*). These results, thus, indicate that basal expression and activity of RhoA exert a Rho kinase-dependent negative control on Akt activity in smooth muscle cells. Consequently, inhibition or depletion of RhoA activates Akt. It is, therefore, expected that by inducing RhoA depletion, maintained 5-HT stimulation should activate Akt in vascular smooth muscle cells.

Long-term 5-HT Stimulation Induces 5-HTT- and TG-dependent Akt Activation—Stimulation of rat aortic smooth muscle cells with 5-HT induced an early and transient Akt activation followed by a delayed time-dependent activation of Akt (Fig. 7*A*). The use of pharmacological inhibitors of 5-HT receptors and 5-HTT indicated that the transient Akt activation (3 min), inhibited by mianserin but not by GR 127935 or fluoxetine, was mediated by 5-HT2A receptor activation (Fig. 7*B*). In
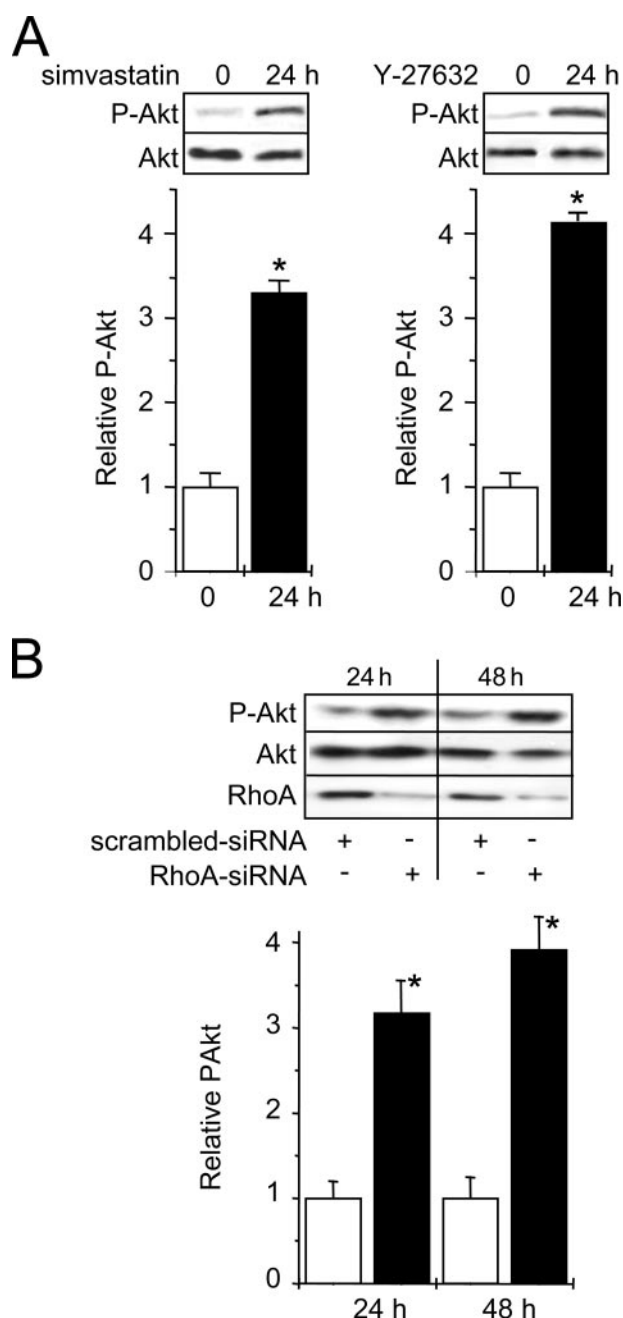
contrast, the pharmacological profile of the delayed 5-HT-induced Akt activation (72 h) was similar to that of RhoA activation and depletion; it was not modified by GR 127935 or mianserin but was abolished in the presence of fluoxetine or MDC (Fig. 7*B*). These results provide evidence that, like 5-HT-induced RhoA activation and depletion, 5-HT-induced delayed Akt activation depends on 5-HTT and TG activity. To further analyze the role of proteasomal degradation of RhoA in 5-HT-induced Akt activation, we next used the proteasome inhibitor
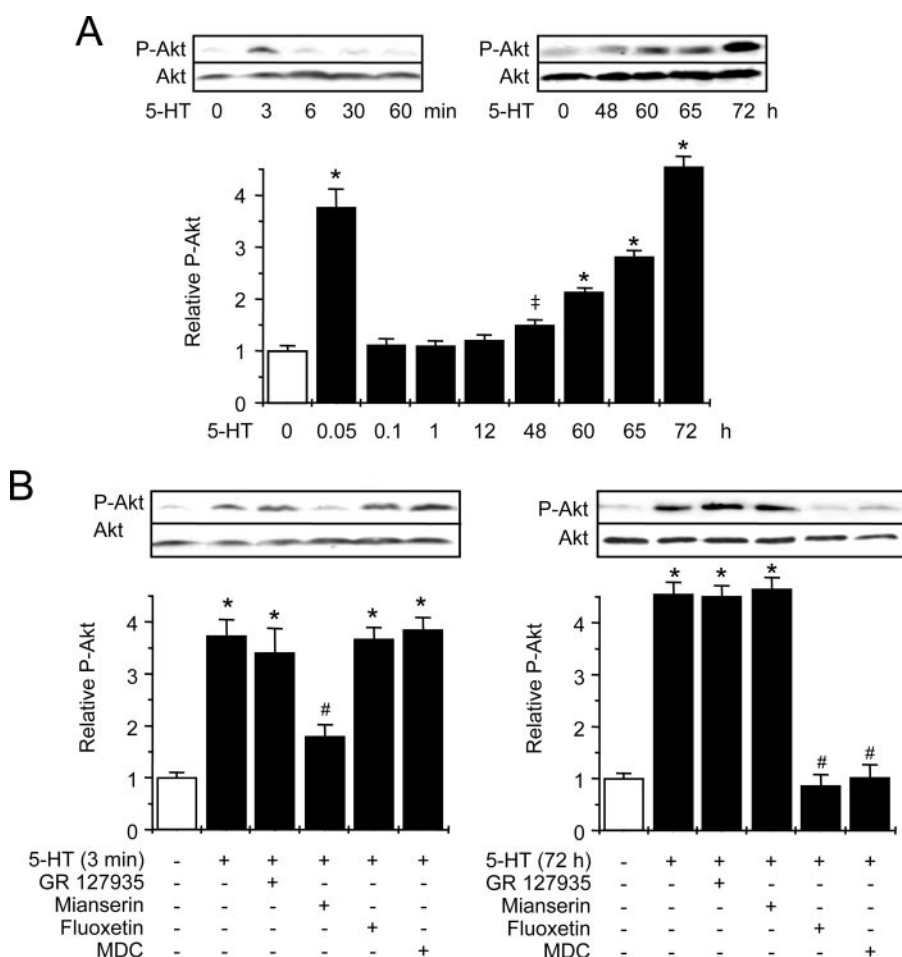
FIGURE 7. **5-HT activates Akt in aortic smooth muscle cells.** Western blots and corresponding densitometric analyses for phosphorylated Akt (*P-Akt*) and Akt levels in cells stimulated by 5-HT (10 μM) for 0–72 h (*A*) and for 3 min (*left*) or 72 h (*right*) in the absence or in the presence of GR 127935 (1 μM), mianserin (10 μM), fluoxetine (10 μM), or MDC (200 μM) (*B*). Akt phosphorylation is normalized to Akt levels and expressed relative to the control in the absence of 5-HT taken as 1. The data presented are representative of at least three independent experiments. ‡, $p < 0.05$ *versus* control; *, $p < 0.001$ *versus* control; #, $p < 0.001$ *versus* 5-HT.

MG132. As shown in Fig. 8*A*, in the presence of MG132, stimulation with 5-HT did not affect RhoA expression and did not activate Akt. These results, thus, suggest that depletion of RhoA by stimulation of its proteasomal degradation is responsible for 5-HT-induced Akt activation.

5-HT Induces Inhibition of PTEN Phosphorylation—Activation of Akt is generally ascribed to the increased generation of phosphatidylinositol 3,4,5-trisphosphate, which recruits Akt to the plasma membrane and allows its phosphorylation by the phosphoinositide-dependent kinase 1 (35). The recently described RhoA/Rho kinase-dependent phosphorylation of PTEN provides a molecular mechanism for the inhibitory action of RhoA/Rho kinase on Akt signaling (36). Rho kinase-mediated PTEN phosphorylation stimulates its lipid phosphatase activity, which decreases the amount of phosphatidylinositol 3,4,5-trisphosphate. Basal RhoA/Rho kinase activity, thus, contributes to maintain low levels of Akt phosphorylation. It is, thus, expected that, by promoting RhoA depletion, 5-HT inhibits basal Rho kinase activity and, consequently, PTEN phosphorylation, which in turn should result in an increased Akt phosphorylation. To address this hypothesis, we examined the effect

of Rho kinase and RhoA inhibition on PTEN phosphorylation by Western blot (Fig. 8*B*). Under basal conditions, PTEN was phosphorylated, and both inhibition of Rho kinase by Y-27632 (10 μM; 6 or 12 h) and depletion of RhoA by RhoA-siRNA reduced PTEN phosphorylation. Similarly, long-term stimulation with 5-HT (48 h) inhibited PTEN phosphorylation (Fig. 8*C*). Shorter applications of 5-HT (24 h) had only a slight effect. The inhibitory effect of 5-HT on PTEN phosphorylation was blocked by fluoxetine (10 μM) (Fig. 8*C*). These results, thus, suggest that the decreased Rho kinase activity and PTEN phosphorylation are the mediators of Akt activation induced by 5-HT-mediated RhoA depletion.

DISCUSSION

Our data provide evidence for a receptor-independent signaling pathway, which leads to RhoA activation in vascular smooth muscle cells. We show that the delayed RhoA activation induced by maintained 5-HT stimulation and the resulting accelerated RhoA degradation depend on 5-HTT and Ca^{2+}-dependent TG2 activity, allowing internalization of 5-HT and its attachment to RhoA, respectively. This is the first demonstration that (i) transamidation of intracellular 5-HT to RhoA exists in arterial smooth muscle cells *in vivo* and *in vitro*, and (ii) depletion of RhoA, resulting from the accelerated degradation of serotonylated RhoA in cultured smooth muscle cells, is a physiological signal mediating Akt activation.

5-HT is a vasoconstrictor and a vascular smooth muscle cell mitogen. Although some studies have shown that 5-HT receptors could be involved in proliferative process (37, 38), most reports support an essential role of the 5-HTT in the mitogenic action of 5-HT (29, 39–41). In addition to its effect on 5-HT uptake in neurons, 5-HTT mediates uptake of indolamine by platelets and endothelial and vascular smooth muscle cells. Therefore, although the role of 5-HTT in the mitogenic effect of 5-HT on vascular smooth muscle cells has been ascribed to 5-HT internalization, the mechanisms linking intracellular 5-HT to the activation of downstream cell growth signal transduction pathways remained unknown. It has recently been shown that 5-HT could be used as a substrate by the platelet TG (FXIIIa) to transamidate the von Willebrand factor and other proaggregatory proteins (42, 43). The observation that delayed 5-HT-mediated RhoA activation in vascular smooth muscle cells was only dependent on 5-HTT (Fig. 1*D*) prompted us to
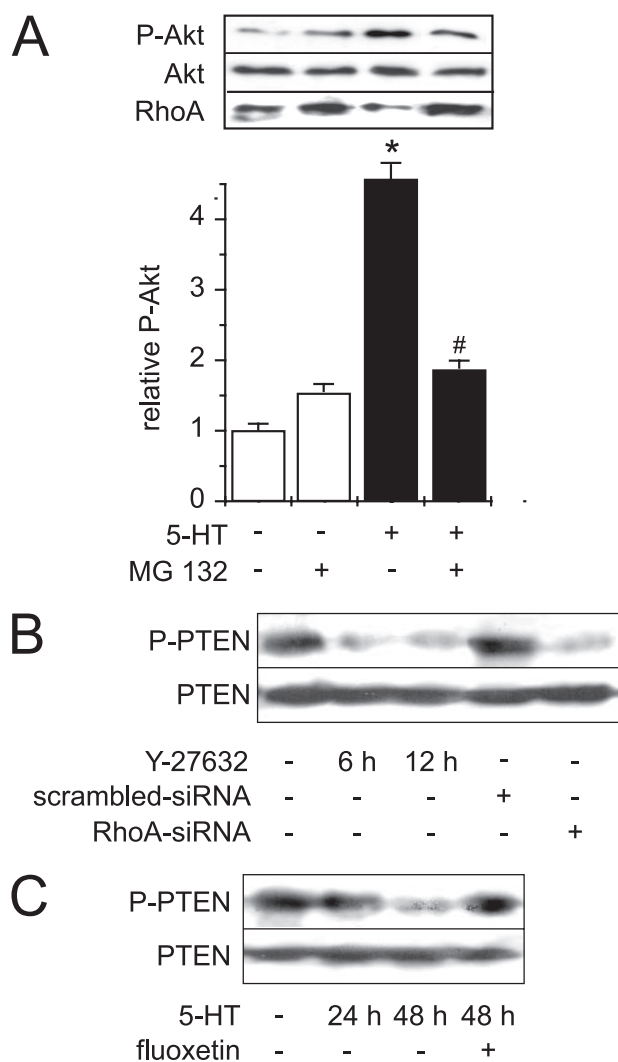
FIGURE 8. **Role of RhoA depletion and PTEN dephosphorylation.** *A*, Western blot and corresponding densitometric analyses for phosphorylated Akt (*P-Akt*), Akt, and RhoA in the absence or presence of 10 μM 5-HT for 72 h with or without 100 μM MG132. Akt phosphorylation is normalized to Akt level and expressed relative to the control in the absence of 5-HT taken as 1. *B* and *C*, Western blot analyses for PTEN phosphorylation (*P-PTEN*) and PTEN in cells treated with Y-27632 (10 μM) for 6 and 12 h and with scrambled- or RhoA-siRNA for 48 h (*B*) with 5-HT (10 μM) for 24 h and 48 h in the absence and presence of fluoxetine (10 μM) (*C*). The data presented are representative of at least three independent experiments. *, $p < 0.001$ *versus* control; #, $p < 0.001$ *versus* 5-HT.

analyze the potential role of transamidation of intracellular 5-HT by TG to RhoA.

TGs constitute a superfamily of cross-linking enzymes that are able to covalently link simple primary amines such as 5-HT to Gln residues (44). Among the TG family, the ubiquitous TG2 has been shown to post-translationally modify intra- and extracellular proteins, and RhoA was identified as an intracellular substrate for TG2 (24, 25). RhoA is transamidated on Gln at position 52, 63, and 136 by TG2. Because Gln-63 is essential for the intrinsic and GTPase-activating protein-stimulated GTPase activity of RhoA, transamidated RhoA is constitutively active (24, 45). The delayed activation of RhoA induced by long-term 5-HT stimulation in aortic smooth muscle cells depended on 5-HTT and was abolished by inhibition of TG (Fig. 1*C*). In addition, our results directly demonstrate a physi-

cal interaction between RhoA and 5-HT that was prevented by 5-HTT inhibition, TG2 inhibition/deletion, and intracellular Ca^{2+} buffering, indicating that this process was Ca^{2+}-dependent (Fig. 2). In two-dimension gel experiments, "serotonylation" shifted the pI of RhoA to more basic value, similar to that previously described for RhoA methylation (46). These data, thus, provide evidence that, as described in platelets, 5-HT can induce RhoA activation by serotonylation of RhoA in vascular smooth muscle cells. Although activation of RhoA by post-translational modification at Gln63 leads to permanent RhoA activation *in vitro* in cell-free system, it only induces a transient activation of RhoA in cells due to the increased susceptibility to proteasomal degradation of constitutively activated RhoA (7). In agreement with these data, 5-HT accelerated RhoA degradation in vascular smooth muscle cells, and this effect was dependent on 5-HTT, TG2, and RhoA activation (Fig. 4). The accelerated degradation of RhoA induced by 5-HT is, therefore, the direct consequence of RhoA activation by transamidation of intracellular 5-HT. The depletion of RhoA observed in smooth muscle cells in culture after maintained 5-HT stimulation resulted from the increased degradation of serotonylated RhoA that was not compensated by an increased RhoA synthesis. The induction of RhoA-Smurf1 association by 5-HT suggests that the increased proteasomal degradation of RhoA was due to its increased ubiquitylation by Smurf1, in agreement with previous observations (34). Although depletion of Rho protein has been shown to occur after Rho protein activation by bacterial toxin as the cytotoxic necrotizing factor-1 (7), this is the first demonstration that depletion of RhoA is also induced by physiological stimuli. Depletion of RhoA by 5-HT is a long-term effect, becoming potent after 48 h (Fig. 3*A*). This mechanism probably cannot be used by cells to rapidly down-regulate RhoA activation, for example by growth factors, and therefore, does not substitute for Rho GTPase-activating proteins to stop RhoA activation occurring in response to stimulation of exchange factor activity by upstream signals. In agreement with this, long-term stimulation with vasoconstrictors other than 5-HT, activating RhoA through exchange factor-mediated GDP/GTP exchange, do not lead to RhoA depletion (Fig. 3*B*). Therefore, the observed accelerated degradation of RhoA in vascular smooth muscle cells stimulated by 5-HT is, thus, directly related to the original mechanism of RhoA activation by transamidation of intracellular 5-HT. It is likely that this mechanism constitutes a vigilance system elaborated by cells to counteract harmful effects of a permanent activation of RhoA.

Although RhoA activation has not been directly analyzed, previous pharmacological studies have reported that Rho kinase activation by short-term stimulation of bovine pulmonary arterial smooth muscle cells by 5-HT involved activation of 5-HT1B/1D receptor (12, 38), which is in agreement with our results obtained for short-term 5-HT stimulation (Fig. 1*C*). They also described that 5-HTT activity was necessary for the mitogenic effect of 5-HT. In addition, transient Akt stimulation induced by short-term 5-HT stimulation has been ascribed to 5-HT2A receptor (12). We confirm the presence of a similar early and transient Akt activation by short-term 5-HT stimulation in rat arterial smooth muscle cells, which was dependent on 5-HT2A receptor (Fig. 7). However, we also observed
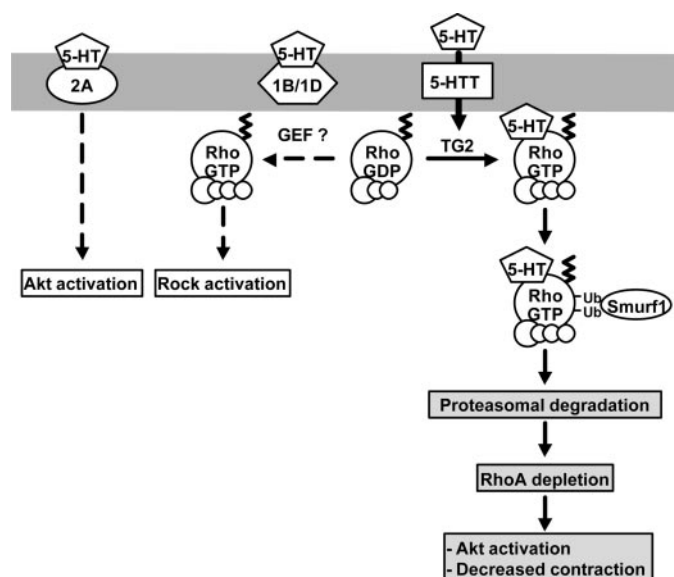
FIGURE 9. **Diagram of signaling pathways for 5-HT in smooth muscle cells.** *Broken lines* and *white boxes* indicate early events. *Black lines* and *gray boxes* show delayed events.

5-HT2A-independent delayed Akt activation for long-term stimulation of rat aortic smooth muscle cell with 5-HT (Fig. 7). Therefore, given the complexity of 5-HT signaling, it is possible that the respective roles of 5-HT receptors and 5-HTT are different depending on the cell types and relative expression levels of 5-HT receptors and 5-HTT (see Fig. 9). Nevertheless, 5-HT receptors seem rather implied in the activation of early and/or transient phenomena, whereas the new 5-HTT-dependent signaling described here, the activation of which is favored by stimulations of long duration and high 5-HT concentrations, is responsible for the activation of late events.

Pharmacological studies using Rho kinase inhibitors or statins have suggested that inhibition of the RhoA/Rho kinase pathway leads to the activation of Akt in endothelial cells (15, 16). However, inconsistent results have been reported in vascular smooth muscle cells as both inhibition and stimulation of Akt and apoptosis have been observed in the presence of statins or Rho kinase inhibitors (47–50). We observed that, as described in endothelial cells, RhoA inactivation by statin, Rho kinase inhibition, or siRNA-mediated RhoA depletion was sufficient to activate Akt in vascular smooth muscle cells and in this way mimicked the effect of long-term 5-HT stimulation (Fig. 6). The inhibition of 5-HT-induced delayed Akt activation by inhibition of proteasome by MG132 (Fig. 8) then provided evidence for a direct link between RhoA depletion and delayed Akt activation induced by maintained 5-HT stimulation. Time-course analyses indicated similar kinetics for 5-HT-induced RhoA depletion, delayed Akt phosphorylation, and PTEN dephosphorylation, with effects becoming significant after 48 h of 5-HT treatment (Figs. 3, 7, and 8). The association of the results of all these experiments strongly suggests that stimulation of Akt by long-term exposition to 5-HT was related to the decreased Rho kinase-mediated PTEN phosphorylation and activity resulting from RhoA depletion (36).

The kinetics of Akt activation by 5-HT were slower than those observed in response to statin treatment. Statins, by inhibit-

ing hydroxymethylglutaryl-CoA reductase induced depletion of both cholesterol and non-sterol isoprenoids. Inhibition of Rho proteins by statins is due to isoprenoid depletion, preventing Rho protein prenylation and leading to accumulation of Rho protein in the cytoplasm (51). In the presence of statin, relocalization of RhoA in the cytosol starts at 6 h and becomes significant at 12 h (52). Functional consequences of inhibition of Rho protein prenylation by statins are generally assessed after treatment with statin for 24 h or longer (47, 53). In endothelial cells Akt activation could be induced after 0.5–1 h of statin treatment (16, 54). However, the involvement of Rho protein inhibition in statin-mediated Akt activation has not been demonstrated. In contrast, it has been suggested that statin-induced Akt phosphorylation and translocation to membrane resulted from the rapid depletion of membrane cholesterol (54). Therefore, it seems likely that inhibition of Rho proteins could participate in activation of Akt induced by long-term statin treatment but not for early Akt activation.

In addition to Akt activation, long-term stimulation with 5-HT induced loss of contractility, in agreement with previous data showing that RhoA expression and activity were high in contractile smooth muscle cells, whereas the synthetic phenotype was associated with low RhoA expression (55). 5-HT could, thus, participate in vascular smooth muscle cell phenotype alterations associated with arterial disorders. Although not studied here, it is likely that Akt activation in vascular smooth muscle cells participates in migration, proliferation, and hypertrophy induced by 5-HT. The signaling pathway newly identified here could, thus, be involved in pathological accumulation of vascular smooth muscle cells observed in various type of vascular diseases. In fact, we show that 5-HTT-dependent serotonylation of RhoA occurred *in vivo* in pulmonary artery of rats in hypoxic conditions. In this model, the development of pulmonary hypertension is associated with RhoA activation (56, 57). As the contribution of the 5-HTT to pulmonary hypertension has been demonstrated (29–31), the involvement of serotonylation of RhoA and its downstream events in pulmonary artery remodeling and hypertension deserves to be analyzed.

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Transglutaminase-dependent RhoA Activation and Depletion by Serotonin in Vascular Smooth Muscle Cells

Christophe Guilluy, Malvyne Rolli-Derkinderen, Pierre-Louis Tharaux, Gerry Melino,
Pierre Pacaud and Gervaise Loirand

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