Serotonin Inhibits Voltage-Gated K⁺ Currents in Pulmonary Artery Smooth Muscle Cells
Role of 5-HT₂A Receptors, Caveolin-1, and Kv1.5 Channel Internalization

Angel Cogolludo, Laura Moreno, Federica Lodi, Giovanna Frazziano, Laura Cobeño, Juan Tamargo, Francisco Perez-Vizcaino

Abstract—Multiple lines of evidence indicate that serotonin (5-hydroxytryptamine [5-HT]) and voltage-gated K⁺ (Kv) channels play a central role in the pathogenesis of pulmonary hypertension (PH). We hypothesized that 5-HT might modulate the activity of Kv channels, therefore establishing a link between these pathogenetic factors in PH. Here, we studied the effects of 5-HT on Kv channels present in rat pulmonary artery smooth muscle cells (PASMC) and on hKv1.5 channels stably expressed in Ltk⁻ cells. 5-HT reduced native Kv and hKv1.5 currents, depolarized cell membrane, and caused a contraction of isolated pulmonary arteries. The effects of 5-HT on Kv currents and contraction were markedly prevented by the 5-HT₂A receptor antagonist ketanserin. Incubation with inhibitors of phospholipase C (U73122), classic protein kinase Cs (G66976), or tyrosine kinases (genistein and tyrphostin 23), the cholesterol depletion agent β-cyclodextrin or concanavalin A, an inhibitor of endocytotic processes, also prevented the effects of 5-HT. In homogenates from pulmonary arteries, 5-HT₂A receptors and caveolin-1 coimmunoprecipitated with Kv1.5 channels, and this was increased on stimulation with 5-HT. Moreover, Kv1.5 channels were internalized when cells were stimulated with 5-HT, and this was prevented by concanavalin A. These findings indicate that activation of 5-HT₂A receptors inhibits native Kv and hKv1.5 currents via phospholipase C, protein kinase C, tyrosine kinase, and a caveola pathway. Kv channel inhibition accounts, at least partly, for 5-HT–induced pulmonary vasoconstriction and might play a role in PH. (Circ Res. 2006;98:931-938.)

Key Words: potassium ion channels hypertension ▪ pulmonary arteries ▪ receptors

Pulmonary hypertension (PH) is a heterogeneous group of disorders characterized by a sustained increase in pulmonary artery (PA) pressure leading to progressive right ventricular failure and death. Several lines of evidence indicate that serotonin (5-hydroxytryptamine [5-HT]) plays a central role in the pathogenesis of PH. First, 5-HT is an effective pulmonary vasoconstrictor and induces vascular smooth muscle hyperplasia. Moreover, plasma levels of 5-HT are increased in patients with primary PH. Conversely, mild pulmonary hypertension has been reported in some series of patients with carcinoid syndrome, a tumor of enterochromaffin cells releasing large amounts of 5-HT. Patients treated with fenfluramine or dexfenfluramine, anorectic drugs that induce platelet 5-HT release, inhibit the 5-HT transporter (5-HTT) and stimulate 5-HT receptors, also have a 23-fold increased risk of PH. In addition, 5-HTT overexpression or polymorphisms in the gene encoding the 5-HTT are associated with PH. Furthermore, mice lacking 5-HTT or 5-HT receptors (eg, 5-HT₁b or 5-HT₂a) show attenuated PH induced by hypoxia. Finally, specific pharmacological inhibition of 5-HT₁b or 5-HT₂a receptors or 5-HTT attenuates and/or reverses the development of PH and prolongs survival in animal models of PH.

K⁺ channels play an essential role in regulating resting membrane potential, intracellular calcium concentration ([Ca²⁺]i), and contraction of vascular smooth muscle. Activation of K⁺ channels leads to hyperpolarization, whereas their inhibition causes membrane depolarization, activation of voltage-gated L-type Ca²⁺ channels, increase in [Ca²⁺]i, and vasoconstriction. Voltage-gated K⁺ (Kv) channels present in pulmonary artery smooth muscle cells (PASMC) are inhibited by hypoxia, endothelin-1, thromboxane A₂, and anorectic drugs. Moreover, decreased expression or function of Kv channels in PASMC has been involved in the pathogenesis of primary and anorexigen-induced PH. From the variety of Kv channels expressed in PASMC, special interest has been paid to Kv1.5, because decreased expression or activity and mutations of Kv1.5 occurs in primary PH and in vivo gene transfer of Kv1.5 reduces PH and restores hypoxic pulmonary vasoconstriction. Furthermore, decreased expression and function of Kv channels in PASMC leads to inhibition of apoptosis and

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931
hypothesized that 5-HT might inhibit Kv channels, thereby inhibiting the inhibition of K(V) channels in PASMC and through cloned hKv1.5 channels (I(_Kv1.5)) stably expressed in Ltk(−) cells. The role of 5-HT receptors and 5-HTT in 5-HT-induced effects has also been studied.

Results

Effects of 5-HT on I(_Kv) and Membrane Potential in PASMC

Addition of 5-HT (10 μmol/L) markedly inhibited I(_Kv) at all potentials tested (Figure 1A and 1B) and depolarized PASMC (Figure 1D) (P<0.01). The concentration-response curve for the inhibition of I(_Kv) by 5-HT at test potentials of −30 and +60 mV was fitted to a Hill equation leading to E_max values of 41.2±3.4% and 50.2±0.5% and EC_{50} of 1.23±0.42 and 2.33±0.09 μmol/L, respectively (Figure 1C), indicating a voltage-independent inhibition. Kv currents recorded before and after treatment with 5-HT showed similar activation kinetics and voltage-activation curves (supplemental Figure I). After washing with 5-HT-free solution for 10 to 15 minutes, the effects of 5-HT on Kv currents (n=5; not shown) or on membrane potential (Figure 1D; n=3) were only weakly or not reversed. In contrast, the effects of the Kv channel blocker 4-aminopyridine (4-AP) (3 mmol/L) on Kv currents (not shown; n=3) or on membrane potential were reversed by ≈80% following a 5 minutes washing period (Figure 1E; n=3).

To test the involvement of 5-HT(3A) receptor on the I(_Kv) blocking properties of 5-HT, we performed experiments in the presence of the selective 5-HT(3A) receptor antagonist ketanserin (0.1 μmol/L). This drug had negligible effects on I(_Kv) but inhibited the effects of 5-HT on I(_Kv), (Figure 2) and on membrane potential (−46.3±2.1 and −45.2±2.1 before and after 5-HT, respectively; n=4). Similarly, the inhibitory effect of 5-HT on I(_Kv) was markedly attenuated in the presence of the 5-HTT inhibitor fluoxetine (0.1 μmol/L, Figure 2C; current–voltage [I–V] relationship in supplemental Figure II). However, other specific 5-HTT inhibitors such as fluvoxamine or citalopram or the 5-HT1B antagonist SB224289 (3 μmol/L) had no effects on the I(_Kv) blocking properties of 5-HT (Figure 2C; I–V relationships in supplemental Figure II).

Inhibition of hKv1.5 Currents by 5-HT

Figure 3 shows hKv1.5 current traces recorded in Ltk(−) cells stably expressing hKv1.5 channels. 5-HT (10 μmol/L) markedly inhibited hKv1.5 currents to a similar extent at all potentials tested, this effect being essentially prevented by ketanserin (0.1 μmol/L).

Role of Phospholipase C and Protein Kinases

To further assess the mechanisms involved in 5-HT–induced inhibition of I(_Kv), PASMC were perfused with different

Materials and Methods

All experiments were performed in accordance with the European Animals Act 1986 (Scientific Procedures) and approved by our institutional review board. A detailed description of the experimental methods is available in the online data supplement at http://circres.ahajournals.org.

Second- to third-order branches of the PA isolated from male Wistar rats were dissected and denuded of endothelium and PASMC were enzymatically isolated. Membrane currents were recorded in PASMC or in Ltk(−) cells stably expressing hKv1.5 channels using the whole-cell configuration of the patch-clamp technique and membrane potential was measured under current-clamp configuration.19,27 Coimmunoprecipitation, confocal immunostaining, and contractile tension studies are described in the online data supplement.
inhibitors before and during the addition of the agonist (summarized in Figure 4; I-V relationships in supplemental Figure III). The phospholipase C (PLC) inhibitor U73122 (3 μmol/L) and the classic protein kinase C (PKC) inhibitor Go6976 (0.1 μmol/L) prevented the inhibitory effects of 5-HT on Kv, currents, whereas the PKCζ pseudosubstrate inhibitor (PKCζ-PI) (0.1 μmol/L, added in the internal solution) had no effect. The tyrosine kinases inhibitor genistein (10 μmol/L) per se caused a ~25% inhibition of \( I_{K(V)} \) and prevented the effects of 5-HT on the current. The inhibitory effect of genistein on \( I_{K(V)} \), has been previously reported and seems to be independent of its tyrosine kinase inhibition activity.\(^{28}\) In the presence of the more selective tyrosine kinase inhibitor tyrphostin 23 (30 μmol/L), which had no effect per se, the effects of 5-HT on \( I_{K(V)} \) were inhibited (Figure 4B and 4C). Likewise, tyrphostin 23 prevented the inhibitory effects of 5-HT on hKV1.5 recorded in Ltk\(^{-}\) cells (Figure 4D). Furthermore, the incubation with the tyrosine phosphatase inhibitor vanadate (100 μmol/L) increased the inhibitory effect of a low concentration of 5-HT (0.1 μmol/L) on \( I_{K(V)} \) in PASMC (Figure 4E). Taken together, these results indicated the involvement of tyrosine kinases in the electrophysiological effects of 5-HT. However, we did not find changes in the Kv1.5 protein phosphorylation in tyrosine or serine residues, whereas at least 2 bands with a molecular mass of ~20 to 24 kDa that coimmunoprecipitated with Kv1.5 proteins showed increased tyrosine phosphorylation after 5 minutes of treatment with 5-HT (Figure 4F). Their nature is presently unknown.

Kv1.5 Association With 5-HT2A Receptors and Caveolin-1
Homogenates from PA were immunoprecipitated with anti-Kv1.5 antibodies and analyzed for 5-HT2A receptors and caveolin-1 content via Western blot analysis. Figure 5A shows that both proteins commmunoprecipitated with Kv1.5. Furthermore, the interaction among Kv1.5, caveolin-1, and 5-HT2A augmented after stimulation with 5-HT. To analyze the potential functional role of caveolae, PASMC were incubated with β-cyclodextrin (2% for 2 hours), a cholesterol-modifying agent that disrupts membrane lipid rafts. Interestingly, 5-HT failed to inhibit \( I_{K(V)} \) in these lipid raft-disrupted myocytes (Figure 5B and 5C). In addition, concanavalin A, a widely used inhibitor of endocytotic processes, had no effect on \( I_{K(V)} \) but prevented the effects of 5-HT on \( I_{K(V)} \) (Figure 5D). Finally, confocal microscopy revealed that Kv1.5 channels were localized preferentially in the plasma membrane of PASMC but were partly internalized on 5-HT stimulation, and this effect was prevented with concanavalin A (Figure 5E).

Contractile Responses to 5-HT
Stimulation of endothelium-denuded PA rings with 5-HT (10 μmol/L) induced a sustained contractile response of 113±6 mg (n=47). Under control conditions, the contraction elicited by 5-HT was suitably reproduced after a 30 minute washout (99±6% of the first contraction, \( P > 0.05 \)). Pretreat-

![Figure 2](https://circres.ahajournals.org/content/93/11/933/F2)

**Figure 2.** A, Current traces comparing the effects of 5-HT (10 μmol/L) in the absence and in the presence of ketanserin (0.1 μmol/L) when stepping to +60 mV. B, I-V relationships of \( I_{K(V)} \) under control conditions and after perfusing with ketanserin and ketanserin plus 5-HT in PASMC. C, Percentage of \( I_{K(V)} \) blockade induced by 5-HT at the end of the pulse at +60 mV in the absence or the presence of ketanserin, SB224289 (3 μmol/L), fluoxetine, fluvoxamine, or citalopram (all at 0.1 μmol/L). I-V relationships are shown in supplemental Figure II. Data show mean±SEM (n in parenthesis). *P<0.05 and **P<0.01 vs control.

![Figure 3](https://circres.ahajournals.org/content/93/11/933/F3)

**Figure 3.** 5-HT inhibits hKv1.5 stably expressed in Ltk\(^{-}\) cells. A, Current traces are shown for depolarization pulses from −80 mV to +60 mV (in 10-mV increments) from a holding potential of −60 mV. B, I-V relationships of \( I_{K(V1.5)} \) in the absence and the presence of 5-HT (10 μmol/L, n=5). C, Blockade of \( I_{K(V1.5)} \) induced by 5-HT at +60 mV in the absence and in the presence of ketanserin (0.1 μmol/L). Data show mean±SEM (n in parenthesis). *P<0.05 and **P<0.01 vs control, respectively.
ment with 0.1 μmol/L ketanserin or 3 μmol/L SB224289 before the second addition of 5-HT inhibited the vasoconstriction (Figure 6A). Fluoxetine, but not the other 5-HTT inhibitors fluvoxamine and citalopram, also caused a marked inhibition of the vasoconstriction induced by 5-HT. Furthermore, the contraction induced by 5-HT was markedly inhibited by the L-type Ca\(^{2+}\) channel blocker nifedipine (0.1 μmol/L), the classic PKC inhibitor Go6976 (0.1 μmol/L), the tyrosine kinases inhibitors genistein (10 μmol/L) or tyrphostin 23 (30 μmol/L). B, Current traces comparing the effects of 5-HT in the absence and in the presence of tyrophostin when stepping to +60 mV. I-V relationships for the effects of tyrophostin and tyrophostin plus 5-HT in PASMC and Ltk\(^-\) are shown in C and D, respectively, and for other drugs in PASMC in supplemental Figure III. E, Vanadate (100 μmol/L) potentiates the \(I_{K(V)}\) blockade induced by 5-HT (0.1 μmol/L) in PASMC. F, \(K\textsubscript{V}1.5\) immunoprecipitates (IP) from Ltk\(^-\) cells expressing \(K\textsubscript{V}1.5\) treated with or without 5-HT (100 μmol/L) for 5 minutes were immunoblotted (IB) with anti-phosphotyrosine, anti-phosphoserine, and anti-\(K\textsubscript{V}1.5\) antibodies (the arrows indicate the expected \(K\textsubscript{V}1.5\) protein, representative of 3 Western blots). Data show mean±SEM (n in parenthesis). *P<0.05 and **P<0.01 vs control.

**Figure 4.** Inhibition of PLC, classic PKCs, and tyrosine kinases reduces \(I_{K(V)}\) blockade induced by 5-HT. A, Percentage of \(I_{K(V)}\) blockade at +60 mV by 5-HT (10 μmol/L) in the absence or presence of the PLC inhibitor U73122 (3 μmol/L) and the classic PKCs inhibitor Go6976 (0.1 μmol/L), the PKC\(_C\) pseudosubstrate inhibitor (PKC\(_C\)-PI, 0.1 μmol/L), or the tyrosine kinase inhibitors genistein (10 μmol/L) and tyrophostin 23 (30 μmol/L). B, Current traces comparing the effects of 5-HT in the absence and in the presence of tyrophostin when stepping to +60 mV. I-V relationships for the effects of tyrophostin and tyrophostin plus 5-HT in PASMC and Ltk\(^-\) are shown in C and D, respectively, and for other drugs in PASMC in supplemental Figure III. E, Vanadate (100 μmol/L) potentiates the \(I_{K(V)}\) blockade induced by 5-HT (0.1 μmol/L) in PASMC. F, \(K\textsubscript{V}1.5\) immunoprecipitates (IP) from Ltk\(^-\) cells expressing \(K\textsubscript{V}1.5\) treated with or without 5-HT (100 μmol/L) for 5 minutes were immunoblotted (IB) with anti-phosphotyrosine, anti-phosphoserine, and anti-\(K\textsubscript{V}1.5\) antibodies (the arrows indicate the expected \(K\textsubscript{V}1.5\) protein, representative of 3 Western blots). Data show mean±SEM (n in parenthesis). *P<0.05 and **P<0.01 vs control.

**Discussion**

This is the first study demonstrating regulation of the native PA \(K\textsubscript{v}\) channels and cloned human \(K\textsubscript{V}1.5\) channels by 5-HT. The main findings of the study can be summarized as follows. 5-HT depolarized PASMC and inhibited the \(K\textsubscript{v}\) current in PASMC and in Ltk\(^-\) cells expressing cloned \(K\textsubscript{V}1.5\) but did not modify the activation curve and the kinetics of the currents. These effects were inhibited by antagonists/inhibitors of 5-HT\(_{2A}\) receptors, PLC, classic PKCs, and tyrosine kinases and by cyclodextrin and concanavalin A. In addition, 5-HT\(_{2A}\) receptors and caveolin-1 coimmunoprecipitated with \(K\textsubscript{V}1.5\) channels, and \(K\textsubscript{V}1.5\) channels internalized when cells were stimulated with 5-HT. Finally, the contraction induced by 5-HT in isolated rat PA was inhibited by the same drugs preventing \(K\textsubscript{V}\) channels inhibition.

Decreased \(K\textsubscript{v}\) channel activity leads to depolarization, opening of L-type voltage-dependent Ca\(^{2+}\) channels, increased \([Ca^{2+}]_i\), and vasoconstriction.\(^{16–18}\) Thus, \(K\textsubscript{v}\) channel blockers such as 4-AP induce depolarization and pulmonary vasoconstriction. In the present report, 5-HT caused \(K\textsubscript{v}\) current inhibition with a similar potency (EC\(_{50}\) values of ~2 μmol/L) than that previously reported for pulmonary vasoconstriction (EC\(_{50}\) values of ~1 and 3 μmol/L in fawn-hooded and Sprague–Dawley rats, respectively\(^{29}\)) and with a similar or higher efficacy (50% inhibition) than other vasoactive factors known to inhibit \(K\textsubscript{v}\) channels such as endothelin-1 (29%\(^{29}\)), the thromboxane A\(_2\) analog U46619 (56%\(^{19}\)), or hypoxia (~49%\(^{30}\)). In addition, 5-HT caused membrane depolarization; its vasoconstrictor response was inhibited by the L-type Ca\(^{2+}\) channel blocker nifedipine and by the same drugs preventing \(K\textsubscript{v}\) channel inhibition. Further-
more, in 5-HT–contracted PA, the relaxation induced by the tyrosine kinase inhibitor tyrphostin 23 was abolished by 4-AP. All of these results are consistent with the view that 5-HT-induced vasoconstriction is, at least in part, mediated by KV channel inhibition. Accordingly, 5-HT has been previously reported to block KV channels in carotid body type I cells and in choroid plexus epithelial cells. 

As outlined in the introduction, multiple evidences indicate that 5-HT plays a key role in the pathogenesis of PH. However, the molecular target of 5-HT is not clearly defined, and it seems likely that several G protein–coupled receptors, as well as the 5-HTT, are involved in PH. 5-HT-induced pulmonary vasoconstriction seems to be mainly attributable to the activation of 5-HT2A and 5-HT1B/1D receptors. In the present study, 5-HT–induced vasoconstriction was inhibited by the selective antagonists of 5-HT2A and 5-HT1B receptors ketanserin and SB224289, respectively, whereas the inhibition of KV currents was prevented only by ketanserin.

Figure 5. KV1.5 coimmunoprecipitation with 5-HT2A receptors and caveolin-1 and functional consequences of lipid raft depletion. A, PA were treated with or without 5-HT (100 μmol/L) for 5 minutes. Homogenates were immunoprecipitated with anti-KV1.5 antibody submitted to SDS-PAGE, and membranes were probed for 5-HT2A receptors (molecular mass of ~52 kDa), caveolin-1 (~25 kDa), and KV1.5. B, Current traces are shown for depolarization pulses from −60 mV to +60 mV from a holding potential of −60 mV obtained from PASMC incubated with β-cyclodextrin (2%) for 2 hours. C, I-V relationships of I(V) measured at the end of the pulse in the absence and the presence of 5-HT (10 μmol/L) in β-cyclodextrin–treated cells (n=4). D, I-V relationships of I(V) under control conditions and after perfusing with concanavalin A (250 μg/mL) and concanavalin A plus 5-HT (n=5). E, Confocal images of PASMC stained with anti-KV1.5 antibody. PASMC were incubated in the absence or presence of 5-HT for 5 minutes; some cells were pretreated with concanavalin A for 15 minutes. Data show mean±SEM.

Figure 6. Blockade of 5-HT2A receptors and inhibition of tyrosine kinases decreases 5-HT–induced (10 μmol/L) contraction. A, Effects of ketanserin, SB224289, fluoxetine, fluvoxamine, and citalopram (all at 1 μmol/L, except SB224289 at 3 μmol/L) on 5-HT—induced (10 μmol/L) contractions in rat PA. B, Effects of nifedipine (0.1 μmol/L), G66976 (0.1 μmol/L), genistein (10 μmol/L), tyrphostin 23 (100 μmol/L), and concanavalin A (250 μg/mL) on 5-HT—induced contractions in rat PA. C, Recordings of the contractile effect of 5-HT and the relaxant effect of tyrphostin 23 in the absence and presence of the KV channel blocker 4-AP (10 mmol/L). Data show mean±SEM (n in parenthesis). *P<0.05 and **P<0.01 vs control.
Therefore, 5-HT–induced vasoconstriction was mediated by at least 2 types of receptors, 5-HT₁A and 5-HT₁B, but only the former signaled via Kᵥ channels. The 5-HT₁A inhibitor fluoxetine also inhibited the effects of 5-HT on Kᵥ currents and vasoconstriction. In contrast, other selective 5-HT₁ inhibitors such as fluvoxamine and citalopram at concentrations sufficient to fully inhibit the transporter had no effect. The different behavior of fluoxetine suggests that this drug is acting via a 5-HTT–independent mechanism. In fact, fluoxetine inhibited (+)-norfenfluramine- and 5-HT–induced vasoconstriction in mice lacking 5-HTT. One possibility is that fluoxetine antagonized 5-HT₂A receptors, because this drug, in contrast to the other 5-HTT inhibitors tested, has a relatively high affinity against the 5-HT₂A receptor (Kᵢ ≈ 140 nmol/L). This drug, 1 of the most widely prescribed antidepressant, has been recently proposed as a novel treatment for PH. Its inhibitory effect on 5-HT–induced Kᵥ current attenuation described herein might contribute to its ability to prevent and reverse PH.

Kᵥ channels exist as tetramers formed by 4 transmembrane Kᵥα subunits combined with modulatory cytosolic Kᵥβ subunits. In human PA, 22 transcripts of Kᵥα (Kᵥ1.1 to -1.7, Kᵥ1.10, Kᵥ2.1, Kᵥ3.1, Kᵥ3.3, Kᵥ3.4, Kᵥ4.1, Kᵥ4.2, Kᵥ5.1, Kᵥ6.1 to -6.3, Kᵥ9.1, Kᵥ9.3, Kᵥ10.1, and Kᵥ11.1), and 3 of Kᵥβ subunits (Kᵥβ1 to -3) have been identified by RT-PCR. Further diversity can be found in native channels because heterotetramers can be formed by the combination of distinct Kᵥα subunits. Because Kᵥ1.5 subunits are believed to be major contributors of the native Kᵥ currents in PA, we analyzed the effects of 5-HT on the Kᵥ current carried by human cloned Kᵥ1.5 channels expressed in Ltk⁻⁻ cells. 5-HT induced an inhibitory effect on this current of similar characteristics of that in native PA myocytes. This effect was also prevented by ketanserin and tyrphostin 23, suggesting a similar signaling pathway in both types of cells. In addition, we found a strong expression of 5-HT₂A receptors by Western blot in Ltk⁻⁻ cells (not shown). Thus, Kᵥ1.5 channels are likely candidates to underlie 5-HT–sensitive currents in native cells, even when we cannot rule out that other Kᵥ channel subunits might also contribute to the effects of 5-HT.

In previous studies, we reported that Kᵥ channel inhibition induced by the thromboxane A₂ analog U46619 in both rat and porcine PA, involved the activation of PKCγ, an atypical PKC that is insensitive to diacylglycerol. However, 5-HT–induced inhibition of Kᵥ current was not significantly affected by the specific PKCγ inhibitor. 5-HT₂A receptors signal primarily through heterotrimERIC proteins of the Gₛ₁₁ subfamily, activation of PLC, the subsequent formation of diacylglycerol, and activation of classic diacylglycerol-sensitive PKC. Likewise, the effects of 5-HT on Kᵥ currents were prevented by U73122, a PLC inhibitor, and by Gö6976, an inhibitor of classic diacylglycerol-sensitive PKCs, that does not inhibit PKCγ and did not modify the effects of U46619 in rat PA. Accordingly, Kᵥ channel inhibition by 5-HT in carotid body type I cells and rat choroid plexus epithelial cells was sensitive to PKC inhibitors. In addition, 5-HT–induced (but not U46619–induced) inhibition was prevented by genistein, a widely used tyrosine kinase inhibitor, and by tyrphostin 23, a selective tyrosine kinase inhib-
suggest that the 5-HT–induced inhibition of Kv currents and the subsequent vasoconstriction of PA result from the interaction of 5-HT₃A receptors with Kv channels within caveolar membrane microdomains and involve tyrosine phosphorylation and endocytotic processes. This is consistent with a reduction in Kv current amplitude without modification of its biophysical features.

PH has a multifactorial origin characterized by sustained elevation of pulmonary arterial pressure associated with vasoconstriction and PA smooth muscle proliferation. Both 5-HT and Kv channels have been implicated in the pathogenesis of PH. In the present study, we demonstrate that a reduction of Kv channel activity not only causes pulmonary vasoconstriction but also contributes to pulmonary vascular medial hypertrophy by inhibiting apoptotic cell shrinkage and apoptosis, whereas overexpression of the Kv1.5 gene (KCNA5) induces apoptosis in PASMC. Thus, Kv channel inhibition might also be involved in the medial hypertrophy of PA induced by 5-HT. Additionally, it must be noted that Kv channel blockade has also been involved in the vasoconstriction and pulmonary hypertension induced by anorexigenic drugs and the antiparkinsonian drug pergolide. Interestingly, dhexfenfluramine and pergolide activate 5-HT₃A receptors, which suggests that Kv channel blockade induced by these drugs might be secondary to 5-HT₃A receptor activation.

In conclusion, 5-HT inhibits Kv currents in PA myocytes and in Ltk– cells stably transfected with human Kv1.5 channels through the activation of 5-HT₃A receptors, PLC, classic PKCs, tyrosine kinases, and endocytotic processes. This effect is involved, at least partly, in PA vasoconstriction and might contribute to the development of human PH.

Acknowledgments

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References


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Methods

All experiments were carried out in accordance with the European Animals Act 1986 (Scientific Procedures) and approved by our institutional review board.

Reagents

Drugs and reagents were obtained from Sigma except nifedipine (Bayer), fluoxetine (Lilly), Gö6976, PKCζ pseudosubstrate inhibitor and secondary horseradish peroxidase conjugated antibodies (Calbiochem), mouse anti-Caveolin-1 (Transduction Laboratories), mouse anti-5-HT2A receptor (Pharmingen), rabbit anti-KV1.5 (Alomone), rabbit anti-phosphotyrosine, anti-phosphoserine (Santa Cruz Biotechnology) and secondary Cy3 conjugated antibodies (Jackson Immunoresearch).

Tissue preparation and cell isolation

Second to third-order branches of PA isolated from male Wistar rats (250-300 g) were dissected into a nominally calcium-free physiological salt solution (Ca2+-free PSS) of composition (in mmol/L): NaCl 130, KCl 5, MgCl2 1.2, glucose 10, HEPES 10 (pH 7.3 with NaOH). Endothelium-denuded PA were cut into small segments (2x2 mm) and cells were isolated in Ca2+-free PSS containing (in mg/mL) papain 1, dithiothreitol 0.8 and albumin 0.7. Cells were stored in Ca2+-free PSS (4°C).

Electrophysiological studies

Membrane currents were recorded using the whole-cell configuration of the patch clamp technique, normalized for cell capacitance and expressed in pA/pF as previously described (19,27). Membrane potential was measured under current-clamp configuration (I=0, sampling rate 500 Hz) and the electric noise was reduced by data
reduction (substitute average, reduction factor 1000 using Clampfit 9.0 software). \( I_{K(V)} \) were recorded under essentially \( Ca^{2+} \)-free conditions using an external \( Ca^{2+} \)-free PSS (see above) and a \( Ca^{2+} \)-free pipette (internal) solution containing (mmol/L): KCl 110, MgCl2 1.2, Na2ATP 5, HEPES 10, EGTA 10, pH adjusted to 7.3 with KOH. Ltk\(^-\) cells stably expressing hKV1.5 channels (online reference 1) were superfused with PSS containing 1 mmol/L CaCl2. The internal solution contained (mmol/L): K-aspartate 80, KCl 42, KH2PO4 10, MgATP 5, phosphocreatine 3, HEPES 5 and EGTA 5 (pH=7.2 with KOH). Currents were evoked following the application of 200 ms depolarizing pulses from -60 mV to test potentials from -60 mV to +60 mV in 10 mV increments. All experiments were performed at room temperature (22-24°C). All drugs were applied in the external solution, except the PKC\( \zeta \) pseudosubstrate inhibitor which was included in the patch pipette solution. Antagonists were applied for 15 min before the addition of 5-HT.

**Co-immunoprecipitation and phosphorylation**

After dissection, PA were placed in warm PSS solution for 60 minutes and then in the absence or presence of 5-HT (100 \( \mu \)mol/L) for 5 min. PA were rapidly frozen in liquid nitrogen and homogenized in a glass potter in 200 \( \mu \)L of a buffer of the following composition (mmol/L): HEPES 10 (pH 8), KCl 10, EDTA 1, EGTA 1, dithiothreitol 1, aprotinin 0.006, leupeptin 0.009, Na\( \alpha \)-p-tosyl-l-lysine chloromethyl ketone 0.011, NaF 5, Na2MoO4 10, NaVO4 1, phenylmethanesulfonyl fluoride 0.5 and okadaic acid 0.00001. Homogenates were centrifuged at 13,000g for 5 min at 4°C and the supernatant fraction was collected. Sixty \( \mu \)g of protein were incubated for 2 hours with anti-KV1.5 antibody at 4°C, followed by the addition of protein A/G beads and further incubation overnight. The immune complexes were collected, separated by SDS-PAGE and transferred to a
PVDF membrane for western blotting as described (19). Membranes were sequentially probed for 5-HT$_{2A}$-, caveolin-1- and Kv1.5-like immunoreactivity or for phosphotyrosine- phosphoserine- and Kv1.5-like immunoreactivity.

**Immunofluorescence staining**

The subcellular location of Kv1.5 protein was analyzed by confocal images of immunofluorescence-stained samples. PASMC were plated onto gelatine-coated cover slips and allowed to attach for 30 min at 4ºC. Then, the cells were washed with Ca$^{2+}$-free PSS and incubated in the presence or absence of concanavalin A (250 µg/mL) for 15 min and then in the absence or presence of 5-HT (10 µmol/l) for 5 min at 37ºC. Cells were immediately fixed for 20 min in 4% paraformaldehyde in PBS and then permeabilized and blocked (0.4% Triton-x100 and 3% BSA in PBS) for 1 h at room temperature. Finally, PASMC were incubated with rabbit anti-Kv1.5 antibody (1:70) overnight at 4ºC. Once excess primary antibody was removed, cells were incubated with donkey anti-rabbit Cy3 (1:200) for 1h. Images were captured using a Leica TCS SP2 inverted confocal microscope.

**Contractile tension recording**

Contractile responses in endothelium-denuded intralobar PA rings were recorded as previously reported (19,27). The procedure of endothelium denudation was checked by the lack of a relaxant effect of acetylcholine. Arteries were stimulated with 5-HT (10 µmol/L) and once a stable contraction was reached, were washed with Krebs solution for 30 minutes. A second stimulation with 5-HT was elicited in the absence (controls) or after a 30 minute incubation period with different drugs. The values of the second contraction were expressed as a percentage of the initial response to the agonist.
**Statistical analysis**

Data are expressed as means±SEM; n indicates the number of arteries or cells tested from different animals. Statistical analysis was performed using Student’s t-test for paired observations or one-way ANOVA followed by a Newman Keuls’ test. Differences were considered statistically significant when $P<0.05$.

**Reference**

Online figures

Online Figure 1. (A) Activation kinetics and (B) voltage-activation curve of Kv currents in PASMCs in the absence and presence of 5-HT. 5-HT did not significantly change these parameters. Data show mean±SEM (n=9).
Online Figure 2. Current-voltage relationships of \(I_{K(V)}\) in PASMCs measured at the end of the pulse in the absence and the presence of (A) SB224289 (3 µmol/L), (B) fluoxetine (0.1 µmol/L), (C) fluvoxamine (0.1 µmol/L) or (D) citalopram (0.1 µmol/L) and these drugs plus 5-HT (10 µmol/L). Data show mean±SEM. *,** \(P<0.05\) and ** \(P<0.01\) in the absence vs in the presence of 5-HT.
Online Figure 3. Current-voltage relationships of \( I_{K(V)} \) in PASMCs measured at the end of the pulse in the absence and the presence of (A) U73122 (3 \( \mu \)mol/L), (B) Gö6976 (0.1 \( \mu \)mol/L), (C) the PKC\( \zeta \) pseudosubstrate inhibitor (PKC\( \zeta \)-PI, 0.1 \( \mu \)mol/L) or (D) genistein (10 \( \mu \)mol/L) and these drugs plus 5-HT (10 \( \mu \)mol/L). Data show mean±SEM. 

\(*, ** P<0.05 \) and \( P<0.01 \) in the absence vs in the presence of 5-HT.