Ritanserin blocks CaV1.2 channels in rat artery smooth muscles: electrophysiological, functional, and computational studies

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INTRODUCTION

Ritanserin (6-[2-[4-bis(4-fluorophenyl)methylidene]piperidin-1-yl]ethyl)-7-methyl-[1,3]thiazolo[3,2-al]pyrimidin-5-one) is a potent, long-acting, nonselective 5-HT2 receptor antagonist [1, 2] and is classified as an antidepressant agent [3], although it has never been approved for clinical use. It has an intrinsic antidopaminergic effect, possibly underlying the reported improvement of negative symptoms in patients who have schizophrenia [4]. Moreover, a number of preclinical and clinical studies have examined the effectiveness of ritanserin at reducing cocaine cravings and/or cocaine use [5].

The direct action of ritanserin on Na+ and Ca2+ channels has been shown in canine Purkinje fibers, where it produces significant depressant effects on transmembrane action potentials [6], which are credited for its antiarrhythmic activity.

Recently, evidence that ritanserin inhibits several lipids (e.g., diacylglycerol kinase-α, DGKα, a novel, potential therapeutic target in various cancers as well as in immunotherapy) [7-9] and protein kinases (e.g., the feline encephalitis virus-related kinase FER) [10], as well as the rapidly accelerated fibrosarcoma kinase c-RAF [11] has been provided. The capacity of perturbing cellular signaling pathways important for cell survival and proliferation, through serotonin-independent mechanisms suggests that ritanserin may be a viable option for in vivo translation and a novel therapeutic tool with potential applications in various tumors.

The serotoninergic antagonism of ritanserin, characterized by a long duration of action, has been shown in a variety of peripheral cardiovascular, gastrointestinal, and respiratory vascular tissues [12]. In the rat tail artery, the interaction of ritanserin with an allosteric site near the 5-HT2 receptor was presumed, owing to ritanserin antagonism of the effects of 5-HT that are different from that of another 5-HT2 receptor antagonist, ketanserin (3-[2-[4-(4-fluorobenzoyl)piperidin-1-yl]ethyl]-1H-quinazoline-2,4-dione) [13]. Furthermore, ritanserin also antagonizes histaminergic (H1) and adrenergic (α1) responses in the rabbit femoral artery, though at concentrations one to three orders of magnitude higher than those needed to block 5-HT2 receptors.

Several studies have demonstrated the activity of this antagonist on vascular function. In vitro as well as in vivo, ritanserin potently inhibits the 5-HT-induced vasoconstriction and pressure responses of an isolated, perfused mesenteric artery preparation [14]. Furthermore, ritanserin is also an in vitro CaV1.2 channel blockers or 5-HT2 receptor antagonists constitute effective therapy for Raynaud’s syndrome. A functional link between the inhibition of 5-HT2 receptors and CaV1.2 channel blockade in arterial smooth muscles has been hypothesized. Therefore, the effects of ritanserin, a nonselective 5-HT2 receptor antagonist, on vascular CaV1.2 channels were investigated through electrophysiological, functional, and computational studies. Ritanserin blocked CaV1.2 channel currents (ICaV1.2) in a concentration-dependent manner (Ki = 3.61 µM); ICaV1.2 inhibition was antagonized by Bay K 8644 and partially reverted upon washout. Conversely, the ritanserin analog ketanserin (100 µM) inhibited ICaV1.2 by ~50%. Ritanserin concentration-dependently shifted the voltage dependence of the steady-state inactivation curve to more negative potentials (Ki = 1.58 µM) without affecting the slope of inactivation and the activation curve, and decreased ICaV1.2 progressively during repetitive (1 Hz) step depolarizations (use-dependent block). The addition of ritanserin caused the contraction of single myocytes not yet dialyzed with the conventional method. Furthermore, in depolarized rings, ritanserin, and to a lesser extent, ketanserin, caused a concentration-dependent relaxation, which was antagonized by Bay K 8644. Ritanserin and ketanserin were docked at a region of the CaV1.2 α1C subunit nearby that of Bay K 8644; however, only ritanserin and Bay K 8644 formed a hydrogen bond with key residue Tyr-1489. In conclusion, ritanserin caused in vitro vasodilation, accomplished through the blockade of CaV1.2 channels, which was achieved preferentially in the inactivated and/or resting state of the channel. This novel activity encourages the development of ritanserin derivatives for their potential use in the treatment of Raynaud’s syndrome.

Keywords: ritanserin; CaV1.2 channel; Raynaud’s syndrome; 5-HT2 receptor; docking simulation; homology modeling

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competitive antagonist of noradrenaline and decreases the mean arterial blood pressure of anaesthetized rats.

Ketanserin fails to antagonize 5-HT-induced contractions in rat aorta rings pretreated with verapamil, a CaV1.2 channel blocker [15]. This phenomenon was explained by the direct interaction of 5-HT2 receptor antagonists with the CaV1.2 channel protein [16]. Finally, similarity in the pharmacology of CaV1.2 channel blockers and 5-HT2 receptor antagonists has been suggested [17]. In fact, both classes of compounds are capable of antagonizing 5-HT-induced and high K+-induced contractions in rat aorta rings.

Sarpogrelate, a 5-HT3 receptor antagonist registered in Japan, China, and South Korea, is used to improve vascular function in patients with peripheral artery disease and symptoms related to Raynaud’s syndrome, which is characterized by reduced blood flow to the fingers and toes caused by vessel tightening or spasms in the cold [18–20]. As CaV1.2 channel blockers, such as nifedipine, have recently been confirmed as useful agents to reduce the frequency, duration, severity of attacks, pain, and disability associated with Raynaud’s syndrome [21], the aim of the present study was to analyze the effect of ritanserin on the vascular CaV1.2 channel. Although the functional effects of ritanserin on blood vessels have been extensively investigated, its possible interaction with CaV1.2 channels, which are fundamental regulators of vascular muscle tone and function [22], has never been investigated. Therefore, patch-clamp, functional, and molecular-docking analyses of ritanserin effects were performed on rat tail arteries.

**MATERIALS AND METHODS**

**Animals**

All animal care and experimental protocols conformed to the European Union Guidelines for the Care and Use of Laboratory Animals (European Union Directive 2010/63/EU) and were approved by the Italian Department of Health (666/2015-PR). Male Wistar rats (250–350 g, Charles River Italia, Calco, Italy) were anaesthetized (i.p.) with a mixture of Zoletil 100® (7.5 mg·kg⁻¹ tiletamine and 7.5 mg·kg⁻¹ zolazepam; Virbac Srl, Milan, Italy) and Rompun® (4 mg·kg⁻¹ xylazine; Bayer, Milan, Italy), decapitated and exsanguinated. The tail was isolated immediately, cleaned of skin and placed in physiological solution (namely an external solution or modified Krebs–Henseleit solution; see sections “Cell isolation procedure” and “Functional experiments”, respectively). The tail main artery was dissected free of its connective tissue and cells or rings prepared as detailed in sections “Cell isolation procedure” and “Functional experiments”, respectively.

**Cell isolation procedure**

Smooth muscle cells were freshly isolated from the tail main artery under the following conditions. A 5-mm long piece of artery was incubated at 37 °C for 40–45 min in 2 mL of 0.1 mM Ca²⁺ external solution (in mM: 130 NaCl, 5.6 KCl, 10 HEPES, 20 glucose, 1.2 MgCl₂, and 5 Na-pyruvate; pH 7.4) containing 20 mM taurine, which replaced an equimolar amount of NaCl, 1.35 mg·mL⁻¹ bovine serum albumin at 4 °C under a normal atmosphere were used for experiments within 2 days after isolation [24].

Whole-cell patch clamp recordings

Cells were continuously superfused with external solution containing 0.1 mM Ca²⁺ and 30 mM tetraethylammonium (TEA) using a peristaltic pump (LKB 2132, Bromma, Sweden) at a flow rate of 400 μL·min⁻¹. The conventional whole-cell patch-clamp method was employed to voltage clamp smooth muscle cells. Recording electrodes were pulled from borosilicate glass capillaries (WPI, Berlin, Germany) and fire-polished to obtain a pipette resistance of 2–5 MΩ when filled with internal solution. The internal solution (pCa 8.4) consisted of (in mM): 100 CsCl, 10 HEPES, 11 EGTA, 2 MgCl₂, 1 CaCl₂, 5 Na-pyruvate, 5 succinic acid, 5 oxaloacetic acid, 3 Na₂ATP, and 5 phosphocreatine; the pH was adjusted to 7.4 with CsOH.

An Axopatch 200B patch-clamp amplifier (Molecular Devices Corporation, Sunnyvale, CA, USA) was used to generate and apply voltage pulses to the clamped cells and record the corresponding membrane currents. At the beginning of each experiment, the junction potential between the pipette and bath solution was electronically adjusted to zero. Current signals, after compensation for whole-cell capacitance and series resistance (between 70% and 75%), were low-pass filtered at 1 kHz and digitized at 3 kHz prior to being stored on the computer hard disk. Electrophysiological responses were tested at room temperature (20–22 °C).

The I_{Ca,1.2} was recorded in an external solution containing 30 mM TEA and 5 mM Ca²⁺. The current was elicited with 250 ms clamp pulses (0.067 Hz) to 10 mV from a V_h of either −50 or −80 mV. Data were collected once the current amplitude had been stabilized (usually 7–10 min after the whole-cell configuration had been obtained). Then the various experimental protocols were performed as detailed below. Under these conditions, the current, which did not run down during the following 40 min [25], was carried almost entirely by CaV1.2 channels [24, 26].

Steady-state activation curves were derived from the current–voltage relationships. Conductance (G) was calculated from the equation $G = I_{Ca,1.2}/(E_{rev} - E_{m})$, where $I_{Ca,1.2}$ is the peak current elicited by depolarizing test pulses between −50 and 20 mV from a $V_{h}$ of −80 mV; $E_{m}$ is the membrane potential; and $E_{rev}$ is the reversal potential (181 mV, as estimated by the Nernst equation). $G_{max}$ is the maximal Ca²⁺ conductance (calculated at potentials ≤20 mV). The $G/G_{max}$ ratio was plotted against the membrane potential and fitted to the Boltzmann equation [27].

Steady-state inactivation curves were obtained using a double-pulse protocol. Once various levels of the conditioning potential had been applied for 5 s, followed by a short (5 s) return to the $V_{h}$ of −80 mV, a test pulse (250 ms) to 10 mV was delivered to evoke the current. The delay between the conditioning potential and the test pulse allowed the full or near-complete deactivation of the channels, simultaneously avoiding partial recovery from inactivation...

$K^+$ currents were blocked with 30 mM TEA in the external solution and Cs⁺ in the internal solution. Current values were corrected for leakage and residual outward currents using 10 μM nifedipine, which completely blocked I_{Ca,1.2}.

The osmolality of the 30 mM TEA-containing and 5 mM Ca²⁺-containing external solution (320 mOsmol) and that of the internal solution (290 mOsmol) were measured with an osmometer (Osmostat OM 6020, Menarini Diagnostics, Florence, Italy).

**Functional experiments**

The HEPES buffer system present in the cell storage medium (i.e., external solution) may alter the response of vascular smooth muscle to vasoconstricting agents such as adrenaline and angiotensin II [28]. Therefore, in the functional experiments, the rings and cells were continuously superfused at 37 °C with a modified Krebs–Henseleit, HEPES-free solution containing (in mM): 118 NaCl, 4.75 KCl, 2.5 CaCl₂, 1.19 MgSO₄, 1.19 KH₂PO₄, 25 NaHCO₃, and 11.5 glucose bubbled with a 95% O₂–5% CO₂ gas mixture to create a pH of 7.4. Cells were randomly selected among those, phase-dense, presenting an elongated shape (i.e., relaxed).

Single-cell shortening was quantified using ImageJ by means of the Analyze/Measure plugin with the straight-line tool (ver 1.46r, NIH, http://imagej.nih.gov/ij/download.html). The length was measured in the same cell before and after drug challenge.
Two millimeter long rings, endothelium-denuded, were obtained from the tail main artery and mounted in a homemade plexiglass support for tension recording as previously described [29] with slight modifications. The endothelium was removed by gently rubbing the lumen of the rings with a very thin rough-surfaced tungsten wire. Rings were immersed in a double chambered organ bath at 37 °C filled with a modified Krebs–Henseleit solution. Contractile tension was recorded using an isometric force transducer (Ugo Basile, Comerio, Italy) connected to a digital PowerLab data acquisition system (PowerLab 8/30; ADInstruments, Castle Hill, Australia) and analyzed by LabChart Pro version 7.3.7 for Windows software (ADInstruments).

At the beginning of the experiment, a preload of 2 g (1 g/mm) was applied to each ring. After an equilibration period of 60 min, the rings were contracted with either 90 mM KCl or 10 µM phenylephrine until reproducible responses to each stimulus were obtained. The absence of a functional endothelium was confirmed by the lack of carbacol-induced relaxation of rings precontracted with phenylephrine.

Ritanserin vasoactivity was assessed on rings either depolarized with 90 mM KCl or stimulated with 100 nM Bay K 8644 in the presence of 20 mM KCl. A concentration-relaxation curve for ritanserin was subsequently constructed. Muscle tension was evaluated as a percent of the initial response to 90 mM KCl or Bay K 8644, which was taken as 100%. A high KCl concentration was achieved by directly adding KCl from a 2.4 M stock solution, to the organ bath solution, as neither efficacy nor potency of vasodilators is significantly affected by the resultant increase in the osmolarity compared to preparations in which the osmolarity is preserved [30].

Chemicals

The chemicals used included collagenase (type XI), trypsin inhibitor, bovine serum albumin, TEA chloride, HEPES, taurine, (S)-(−)-methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate (Bay K 8644), phenylephrine, ritanserin, and nifedipine (Sigma Chimica, Milan, Italy); ketanserin (Janssen, CEVA Logistics Italia Srl, Stradella (PV), Italy); Bay K 8644 and nifedipine, dissolved directly in DMSO, were diluted at least 1000 times prior to use. Control experiments confirmed that no response was obtained when the osmolarity is preserved [30].

Statistical analysis

Acquisition and analysis of the data were accomplished using pClamp 9.2.1.9 software (Molecular Devices Corporation, Sunnyvale, CA, USA) and GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, CA, USA).

Clinical data are reported as the mean ± SEM; n is the number of cells/rings analyzed isolated from at least three animals. Statistical analyses and significance, as measured by Student’s t-test for either paired or unpaired samples (two-tailed) and repeated measures ANOVA followed by Dunnett’s or Bonferroni’s posttest, were obtained using GraphPad Prism version 5.04. Posttests were performed only when ANOVA found a significant value of F and no variance in homogeneity. In all comparisons, P < 0.05 was considered significant. The pharmacological response to ritanserin, described in terms of pIC50 (the −log of the IC50 value, i.e., the drug concentration reducing the response by 50%), was obtained by nonlinear regression analysis.

Docking simulations

The homology 3D model of the CaV1.2 channel pore domain was utilized as previously described [31]. Docking of the ligands ritanserin, ketanserin, and Bay K 8644 structures were downloaded from the PubChem database in sdf format (PubChem CID 5074, 3822, and 6603728, respectively) [33], while the pdbqt file was created by using AutoDock Vina v. 1.1.2 tools [34]. Multiple ligand–protein interaction maps were generated using the Protein–Ligand Interaction Profiler (PLIP) [35]. PyMOL was used as the molecular graphics system (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

RESULTS

Effect of ritanserin and ketanserin on I(Ca,1,2)

This series of experiments were carried out to evaluate the effect of ritanserin and ketanserin (Fig. 1a) on I(Ca,1,2). Figure 1b shows recordings of the inward current elicited with a clamp pulse to 10 mV from a Vh of −80 mV under control conditions and after the addition of cumulative concentrations of ritanserin, which inhibited peak I(Ca,1,2) in a concentration-dependent manner with a pIC50 (M) value of 5.47 ± 0.08 (n = 5; Fig. 1c). In contrast, ketanserin-induced inhibition of current amplitude was

Fig. 1 Effects of ritanserin and ketanserin on I(Ca,1,2) in single vascular myocytes. a Structure of ritanserin (left) and ketanserin (right). b Average traces (recorded from five cells) of I(Ca,1,2) elicited with 250 ms clamp pulses to 10 mV from a Vh of −80 mV, measured in the absence (control) or presence of cumulative concentrations of ritanserin. c Concentration-dependent effect of ritanserin measured in the absence (control) and presence of 100 nM Bay K 8644. The concentration-response curve of ketanserin is also shown. On the ordinate scale, the current amplitude is reported as a percentage of the value recorded just before the addition of the first concentration of ritanserin or ketanserin. The curves show the best fit of the points. Data points are the mean ± SEM (n = 5–6). *P < 0.05 vs. ritanserin, Student’s t test for unpaired samples.
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![Image]

As shown in Fig.1c, pretreatment with Bay K 8644 caused a significant rightward shift of the ritanserin concentration-response curve (pIC$_{50}$ (M) value of 4.57 ± 0.13, *P = 0.003 vs. control). Characterization of the effects of ritanserin on I$_{Ca1.2}$ significantly lower than that of its analog ritanserin and, at the maximal concentration assessed (100 µM), ketanserin inhibited the current amplitude by only ~50%.

To investigate whether the dihydropyridine-binding site on the channel protein was involved in the Ca$^{2+}$ antagonistic activity of ritanserin, the potential functional interaction of ritanserin and the CaV1.2 channel stimulator Bay K 8644 was assessed. In myocytes challenged with 100 nM Bay K 8644, I$_{Ca1.2}$ increased to 524% ± 36% of the control (n = 5). As shown in Fig. 1c, pretreatment with Bay K 8644 caused a significant rightward shift of the ritanserin concentration-response curve (pIC$_{50}$ (M) value of 4.57 ± 0.13, n = 5; P = 0.003 vs. control).

Characterization of the effects of ritanserin on I$_{Ca1.2}$ A biophysical and pharmacological analysis was carried out to clarify the mechanism underlying the ritanserin-induced inhibition of I$_{Ca1.2}$ and its activity was described at the channel protein. Figure 2a illustrates the time course of the effects of 3 µM ritanserin or vehicle (0.1% DMSO) on the current recorded at 0.067 Hz from a $V_h$ of −50 mV to a test potential of 10 mV. After I$_{Ca1.2}$ reached steady values, the addition of ritanserin to the bath solution produced a gradual decrease in the current amplitude that reached a plateau in ~5 min. In contrast, DMSO had no effect on the current amplitude. Ritanserin-induced inhibition of I$_{Ca1.2}$ was partially reversed upon drug washout (Fig. 2a) and was not affected by the membrane potential. In fact, when $V_h$ was shifted to −80 mV, the residual current amplitude (47.2% ± 3.5% of control, n = 5) was similar to that recorded at a $V_h$ of −50 mV (45.5% ± 8.6% of control, n = 5; P = 0.6999).

I$_{Ca1.2}$ evoked at 10 mV from a $V_h$ of either −50 mV or −80 mV was activated and then declined with a time course that could be fitted by a monoeponential function. Ritanserin (3 µM) significantly accelerated only the $\tau$ of inactivation recorded at a $V_h$ of −80 mV (Fig. 2b).

The current–voltage relationship (Fig. 3a) shows that 3 µM ritanserin significantly decreased the peak inward current in the range of membrane potential values from −30 to 50 mV, shifting the apparent maximum by 5 mV in the hyperpolarizing direction without varying the threshold at approximately −40 mV. The voltage dependence of ritanserin inhibition was further investigated by analyzing the steady-state inactivation and
activation curves for $I_{Ca_{1.2}}$. The steady-state activation curves (Fig. 3b), calculated from the current–voltage relationships shown in Fig. 3a, were fitted with the Boltzmann equation. Ritanserin neither shifted the 50% activation potential ($−17.0 ± 5.1$ mV for the control, and $−17.1 ± 4.5$ mV for 3 µM ritanserin; $n = 5$; $P = 0.8818$, Student’s $t$ test for paired samples) nor affected the slope factor ($10.3 ± 1.0$ and $9.9 ± 1.0$ mV, respectively; $P = 0.0865$).

The apparent dissociation constant of ritanserin for inactivated channels ($K_i$) was determined by the shift of $Ca_{1.2}$ channel steady-state availability as a function of ritanserin concentration at a $V_m$ of $−80$ mV [36]. Ritanserin significantly shifted the steady-state inactivation curve to more hyperpolarizing potentials in a concentration-dependent manner (Fig. 3b; $P = 0.0003$, repeated measures ANOVA). The 50% inactivation potential changed from $−22.5 ± 1.1$ mV ($n = 5$, control) to $−27.2 ± 0.9$ mV (1 µM ritanserin, $P > 0.05$), $−31.5 ± 2.2$ mV (3 µM ritanserin, $P < 0.05$), and $−35.6 ± 2.7$ mV (10 µM ritanserin, $P < 0.05$, Dunnett’s posttest). The slope factor, however, was not affected by ritanserin ($−7.2 ± 0.6$, $−7.1 ± 1.0$, $−6.3 ± 0.6$, and $−8.5 ± 1.1$ mV, respectively; $P > 0.05$). The $K_i$ was estimated by plotting the 50% inactivation potentials as a function of ritanserin concentration. This relationship was fitted to the equation

$$V_{50} = K_{control} \times \ln \left[1/(1 + ([\text{drug}] / K_i))\right] + V_{50\ control}$$

where $V_{50\ control}$ and $K_{control}$ are the values of the 50% inactivation potential and the slope measured under control conditions, respectively. The value of $K_i$ thus determined was $1.6 ± 0.9$ µM ($n = 5$), which was not significantly different from the $K_i$ reported above ($3.6 ± 0.7$ µM, $n = 5$; $P = 0.1201$, Student’s $t$ test for unpaired samples).

The shift of the inactivation curve caused by 3 µM ritanserin led to a marked reduction in the Ca$^{2+}$-window current that peaked at approximately $−20$ mV (with a relative amplitude of 0.11) compared with the peak at approximately $−25$ mV (relative amplitude 0.19) observed under control conditions.

To assess whether ritanserin inhibition of $I_{Ca_{1.2}}$ was frequency-dependent, the current was recorded during 20 depolarizing pulses 50 ms in length to 10 mV from a $V_m$ of $−50$ mV applied at a stimulation frequency of 1 Hz. Ritanserin, at a concentration of 3 µM, produced a frequency-dependent block of $I_{Ca_{1.2}}$ (Fig. 4). This frequency dependence, calculated by normalizing the current amplitude evoked by the 20th applied stimulus against that induced by the first step pulse, was significantly greater than that observed under control conditions.

Molecular docking simulation

In silico docking was carried out to define the interaction of ritanserin with the rat $Ca_{1.2}$ channel $α_{1C}$ subunit. The lowest energy poses of ritanserin, ketanserin, and Bay K 8644 showed Gibbs free-energy values (ΔG) of $−8.7$, $−8.6$, and $−8.4$ kcal·mol$^{-1}$, respectively. The computational analysis established that the three compounds were placed in the same binding region, though in different binding pockets (Fig. 5). In particular, ritanserin and ketanserin bound to the same site with good superposition; however, differences in their structure gave rise to different residue interaction networks. PLIP analysis indicated that ritanserin formed hydrophobic interactions with Leu-427, Val-430, Leu-1500, a $π$-stacking interaction with Phe-1190, and halogen bonds with Thr-391 and Thr-1443. Conversely, Bay K 8644 gave rise to hydrophobic interactions with Leu-427, Met-1186, Tyr-1489, Ile-1497, Phe-1500, a $π$-stacking interaction with Phe-1190, and a halogen bond with Thr-1443. Conversely, Bay K 8644 gave rise to hydrophobic interactions with Thr-1066, Phe-1070, Phe-1483, and Ile-1486, and formed a strong hydrogen bond with Tyr-1489 (Fig. 6b).

The ligand–protein interaction networks showed that the orientation of Tyr-1489 present in the binding pockets accommodating either Bay K 8644 or ritanserin plays a key role in the binding of the latter to the $Ca_{1.2}$ channel $α_{1C}$ subunit. Only the Tyr-1489 orientation shown in Fig. 6c was able to form a stable 3.8 Å-long hydrogen bond with ritanserin; in contrast, the Tyr-1489 orientation shown in Fig. 6d, which gave rise to the formation of a stronger 2.3 Å-long hydrogen bond with Bay K 8644, was favored by the presence of Bay K 8644 in the pocket.

Effect of ritanserin on freshly isolated rat caudal artery myocytes

The contractile effect of ritanserin was evaluated by recording the morphological changes of freshly isolated cells (shortening with formation of membranous evaginations; see ref. [37]). As shown in Fig. 7a, under the experimental conditions used for patch-clamp recordings, i.e., in the presence of 5 mM Ca$^{2+}$ and 30 mM TEA, the addition of ritanserin in the range concentration of 25–50 µM
caused cell contraction. TEA did not evoke any evident contraction per se, which is in line with what was previously observed with 60 mM K\(^+\) [24]. Conversely, the addition of ritanserin did not elicit contractions in cells dialyzed under the whole-cell configuration. Similar results were obtained under the experimental conditions used for functional experiments (i.e., 2.7 mM Ca\(^{2+}\) and no TEA in the bath; Fig. 7b). Superfusion with the sole vehicle, i.e., 0.1% DMSO, did not affect the length of the myocytes (56.6 ± 6.8 μm, control; 54.7 ± 6.3 μm, 0.1% DMSO 5 min; 53.2 ± 6.0 μm, 0.1% DMSO 10 min; P > 0.05); however, it shortened significantly upon the addition of 0.5 mM ATP (39.6 ± 3.7 μm, n = 7; P < 0.05).

Effects of ritanserin and ketanserin on vascular rings

In rat caudal artery rings, the addition of cumulative concentrations of ritanserin did not evoke an increase in basal tone (data not shown). In preparations precontracted with 90 mM KCl (1364 ± 158 mg, n = 4), causing a relaxation of 67.9% ± 4.6% (n = 3; P = 0.0149). Drug washout for ~30–60 min caused a partial recovery of the 10 μM phenylephrine-induced contraction, which amounted to 42.1% ± 4.7% of the control (ritanserin; n = 10) and 76.1% ± 0.7% of the control (ketanserin; n = 3; P = 0.0028). Noticeably, 90 mM KCl-induced contraction recovered to only 10.7% ± 1.2% of the control (n = 10) following a 10–45 min washout of ritanserin (P < 0.0001 vs. phenylephrine).

Ritanserin concentration-dependently relaxed rings precontracted by 100 nM Bay K 8644 (785 ± 106 mg, n = 7); however, the pIC\textsubscript{50} (M) value was lower than that recorded in the depolarized preparations (4.66 ± 0.14, n = 7; P < 0.0001).

**DISCUSSION**

The present investigation provides the first direct electrophysiological evidence that ritanserin is a vascular Cav1.2 channel blocker, as demonstrated by the inhibitory effect produced by the drug on I\textsubscript{Ca,1.2}. The major findings supporting this conclusion are as follows: (1) in single vascular myocytes, ritanserin inhibited I\textsubscript{Ca,1.2} in a concentration-dependent manner; (2) this inhibition was antagonized by the Cav1.2 stimulator Bay K 8644 and was likely due to the interaction of ritanserin with the channel protein; (3) ritanserin stabilized the Cav1.2 channel in its inactivated state; and (4) since ritanserin relaxed vascular smooth muscle contraction resulting from the opening of Cav1.2 channels, the I\textsubscript{Ca,1.2} blockade is supposed to have functional relevance and supports previous data obtained in vascular and nonvascular tissues [15–17], where such mechanism of action was hypothesized to account for the relaxant effects of the drug.

The potency of ritanserin was diminished when Cav1.2 channels were stimulated by Bay K 8644, in analogy with what was observed with nifedipine and Bay K 8644, which are both dihydropyridines and share the same binding site on Cav1.2 [38]. This observation provides compelling evidence for a mutual interaction of ritanserin and Bay K 8644 at the channel protein. The rightward shift of the ritanserin inhibition curve by Bay K 8644 might be because Bay K 8644 and ritanserin bind to channel receptor sites that are in close proximity to each other or are allosterically linked [39]. Support for this hypothesis is aroused from the in silico analysis (see below).

Ritanserin-induced inhibition of I\textsubscript{Ca,1.2} observed at 0.067 Hz—a frequency that allows full recovery between pulses from Cav1.2 channel inactivation in rat tail artery myocytes [40]—was tonic in nature and developed independently of channel activation [41]. This is interpreted as a consequence of the selective inhibition of the resting channel state. Furthermore, ritanserin, like verapamil [42], inhibited I\textsubscript{Ca,1.2} in a frequency-dependent fashion (use-dependent block), and similarly to nicardipine [43] shifted the channel availability toward more hyperpolarizing potentials. These effects are conventionally interpreted as a consequence of a high affinity drug binding and stabilization of inactivated channels [43]. This phenomenon, however, was not voltage-dependent. In fact, the ritanserin apparent dissociation constant for the inactivated state followed a Boltzmann fit without a voltage shift and developed independently of the single-channel conductance. This hypothesis is further supported by the similar current inhibition recorded at V\textsubscript{h} values of −50 and −80 mV. Stabilization of the inactivated state, however, seemed to follow a slow kinetics, since the I\textsubscript{Ca,1.2} inactivation, observed during the 250 ms long depolarizing step, was slightly affected by ritanserin.
only at a $V_h$ of $-80$ mV. Finally, it is conceivable that ritanserin could cause CaV1.2 channel inactivation by increasing basal cytoplasmic Ca$^{2+}$ levels. However, this hypothesis can be ruled out, as the drug did not increase the resting smooth muscle tone of artery rings when added to the organ bath in the absence of other stimulating agents. Taken together, these findings indicate that within the frame of the "state-dependent pharmacology" of the channel, three different mechanisms, operated simultaneously by ritanserin, are responsible for CaV1.2 channel block: state-independent (tonic), weak state-dependent open channel inhibition (the faster CaV1.2 channel inactivation kinetics observed in the presence of the drug, reported also for other CaV1.2 channel blockers such as dihydropyridines and phenylalkylamines) [42], and state-dependent inactivated channel stabilization.

Docking results demonstrated that, similar to dihydropyridine CaV1.2 channel blockers [45], ritanserin bound to the outer, lipid-facing surface of the pore in the intersubunit crevice formed by neighboring tilted S5–S6 helices and the P-loop of domains III and IV, a region involved in CaV1.2 inactivation [46, 47]. Noticeably, ritanserin and Bay K 8644 bound to the same region, though to different, very closely situated pockets, analogous to what was previously observed with the PKA inhibitor H-89 [48]. The Tyr-1489 residue, located in the overlapping area, was crucial for the interaction of the two molecules with the channel protein. The conformation of the residue shown in Fig. 6c allowed ritanserin binding. Conversely, when the residue assumed the conformation shown in Fig. 6d only Bay K 8644 was able to dock. Additionally, the Tyr-1489 residue conformation is important in shaping the channel pore state [46, 47] indicating that the conformation favored by ritanserin stabilizes the closed state, while that favored by Bay K 8644 stabilizes the open state of the channel. Finally, the in vitro with the isolated preparations, ritanserin may cause relaxation per se via a reduction of the window current. On the other hand, although ritanserin shifted the voltage at which the maximum of the current–voltage relationship occurred by 5 mV, neither the slope nor the 50% activation potential were affected by the drug, thus indicating that the sensitivity of the channel activation mechanism to the membrane voltage was only modestly altered.

Fig. 7 Ritanserin-induced contractility of single vascular myocytes. Cell length measured before (control) and after the addition of various concentrations of ritanserin. Myocyte shortening recorded in the presence of a 5 mM Ca$^{2+}$ and 30 mM tetraethylammonium or b 2.7 mM Ca$^{2+}$ in the bath solution. Representative video images, taken at least one minute after treatment with the drug, are shown below each column. Only one scale bar is shown for each row. Columns are the mean ± SEM (n = 4–16). a *$P$ = 0.0002 vs. control, Student’s t test for paired samples; b ****$P < 0.0001$ repeated measures ANOVA and Dunnett’s posttest.

Fig. 8 Effects of ritanserin and ketanserin on rat tail artery rings. a Original recordings of isometric tension (representative of 13 similar experiments) of the relaxation caused by cumulative concentrations of ritanserin (µM) added (lower trace) at the plateau of 90 mM K$^+$ (K90)-induced contraction in rat tail artery rings. The effect of vehicle (DMSO, in µL) is also shown (upper trace). b Concentration–response curves of the relaxing effect of ritanserin and ketanserin on rings precontracted by either 100 nM Bay K 8644 (only ritanserin) or 90 mM KCl. Bay K 8644-induced contractile response was obtained following pretreatment of the ring with 20 mM KCl. On the ordinate scale, the response is reported as a percent of the tension induced by the contracting agent (100%). Data points are the mean ± SEM (n = 3–7).
two different docking poses and hydrogen bond lengths likely explain the decreased Ca^{2+} antagonist potency of ritanserin recorded in the presence of Bay K 8644. The lower inhibitory effect of ketanserin compared to ritanserin could be due to its different interaction network with binding pocket residues and, in particular, to the lack of interaction with the key Tyr-1489 residue.

Ritanserin inhibited I_{Ca,1.2} in the same cells where it induced a contractile effect. Noticeably, dialysis of the cytoplasm (as in the case of the conventional whole-cell method) prevented cell contraction but not inhibition of I_{Ca,1.2}. This observation suggests that contraction was mediated by a rise in cytoplasmic Ca^{2+} concentration (which, in the whole-cell configuration, is buffered by the high level of EGTA present in the pipette solution) and/or by diffusible intracellular factors. On the other hand, ritanserin inhibition of I_{Ca,1.2} seems to be related to a direct interaction of the drug with the channel protein, although the possible involvement of intracellular signaling pathways surviving dialysis cannot be ruled out. Taken together, these results indicate that the drug is able to activate both vasoconstricting and vasorelaxant mechanisms, the former prevailing in single cells and the latter in intact tissue either in resting conditions or under the presence of stimulating agents such as high concentrations of K^+. This apparent discrepancy, previously observed with the rat toxicant nortormide [49] and described with high K^+ [24] likely follows the disruption of the extracellular matrix and ensuing isolation of single smooth muscle cells, leading to the loss of focal contact tension that in turn considerably alters cell signaling systems [50]. Moreover, this hypothesis argues that the relaxation effect elicited by ritanserin in the intact tissue may be underestimated, i.e., inhibition of the CaV1.2 channel by itself would induce a more pronounced vasorelaxation.

CaV1.2 channel inhibition was also observed in intact tissue under conditions of full membrane depolarization, i.e., in vascular rings depolarized with high K^+ concentrations. Under experimental conditions similar to those represented by voltage-clamp pulses of depolarization applied to evoke I_{Ca,1.2} ritanserin caused relaxation and its potency and efficacy were consistent with those calculated in the patch-clamp experiments. Furthermore, this vasorelaxant effect was significantly antagonized by the CaV1.2 channel agonist Bay K 8644, which is in perfect agreement with what was observed in single myocytes, thus supporting the hypothesis of a mutual interaction of ritanserin and Bay K 8644 at the channel protein. Finally, in rat caudal artery rings, the myorelaxant effect of the drug was only partially reverted by washout, similar to what was observed in the patch-clamp experiments. The partial reversibility of ritanserin I_{Ca,1.2} antagonism observed in the present study suggested a relatively strong interaction of the drug with the channel protein. In fact, recovery of high K^+-induced contraction (essentially dependent on the opening of CaV1.2 channels) was significantly lower than that correlated to phenylephrine (only partly dependent on the opening of CaV1.2 channels). Furthermore, this effect was similar to that previously reported in isolated dog cardiac tissue [6], where the effects of ritanserin could not be fully reversed even after 2 h of repeated washout with physiological solution.

Interestingly, ketanserin, when assessed on both smooth muscle active tone and I_{Ca,1.2} amplitude, showed a much lower activity compared to its analog ritanserin. In silico docking and postdocking analyses, displaying a higher number and stronger type of interactions toward the binding pocket for ritanserin compared to ketanserin, well supported these experimental observations. Taken together, these data strengthen the hypothesis of the existence of a rather specific ritanserin structure–CaV1.2 channel blocking activity relationship.

In conclusion, the present electrophysiological, functional, and in silico data point to ritanserin as a vasorelaxant and CaV1.2 channel blocking agent. This observation may open a new avenue for the treatment of Raynaud’s syndrome. In this disorder, either CaV1.2 channel blockers, such as nifedipine [21], or 5-HT_2 receptor antagonists, such as sarpogrelate [18–20], are effective therapeutic agents. Therefore, the CaV1.2 channel and 5-HT_2 receptor antagonist ritanserin may represent an interesting scaffold to develop bifunctional defense drugs for the treatment of Raynaud’s phenomenon, as opposed to ketanserin, which was previously defined as not clinically beneficial for the treatment of this disorder in progressive systemic sclerosis [51]. The discovery of a contractile pathway activated by the drug suggests that ritanserin derivatives, possibly deprived of the contractile property, would give rise to relaxing agents that are more potent than the parent compound itself. Finally, in search of effective drugs for the treatment of Raynaud’s syndrome, forthcoming analysis of some 5-HT_2 receptor antagonists will clarify whether other agents besides ritanserin emerge as scaffolds worthy of further pharmaceutical development.

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AUTHOR CONTRIBUTIONS
FF and SB designed the research; FF, AT, and SB performed the research; FF and AT analyzed the data; FF, OS, SS, and SB wrote the paper; and GS critically revised the paper.

ADDITIONAL INFORMATION
Competing interests The authors declare no competing interests.

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