British Journal of Pharmacology (2008) 154, 787–796

0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Vardenafil, but not sildenafil or tadalafil, has calcium-channel blocking activity in rabbit isolated pulmonary artery and human washed platelets

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Background and purpose: Phosphodiesterase type-5 (PDE5) inhibitors constitute a novel and important therapeutic option for the treatment of pulmonary hypertension. The effects of the PDE5 inhibitors sildenafil, tadalafil and vardenafil on rabbit isolated pulmonary artery ring preparations and on intracellular Ca^{2+} concentration of thrombin-stimulated human platelets were investigated.
Experimental approach: Rabbit pulmonary artery rings were mounted in 10 mL organ bath containing Krebs solution. Tissues were connected to force-displacement transducers, and changes in isometric force were recorded. Ca^{2+} flux in human washed platelets was measured.
Key results: Sildenafil, tadalafil and vardenafil (0.0001–10 μM) concentration-dependently relaxed endothelium-intact and endothelium-denuded pulmonary artery rings. Endothelium denudation caused rightward shifts in the concentration–response curves to sildenafil, tadalafil and vardenafil (9-, 12- and 123-fold, respectively). Incubation with N^{ω}-nitro-L-arginine methyl ester (100 μM) or ODQ (1*H*-[1,2,4] oxadiazolo [4,3,-a]quinoxalin-1-one) (10 μM) caused similar reductions of PDE5-induced vasorelaxations in intact rings. Sildenafil and tadalafil did not affect the phenylephrine-induced contractions, whereas vardenafil reduced the maximal responses, and shifted the phenylephrine-induced contraction curves to the right in endothelium-denuded rings (5- and 19-fold for 1 and 10 μM, respectively). Vardenafil (but neither sildenafil nor tadalafil) caused a marked rightward shift and a decrease of maximal contractile response to $CaCl_2$. Vardenafil, but neither sildenafil nor tadalafil, significantly reduced the Ca^{2+} mobilization and Ca^{2+} influx in thrombin-stimulated washed platelets.
Conclusions and implications: Our results indicate that vardenafil, in contrast to sildenafil or tadalafil, also blocked Ca^{2+} fluxes, thus enhancing its vasorelaxation of the pulmonary artery.
British Journal of Pharmacology (2008) **154,** 787–796; doi:10.1038/bjp.2008.141; published online 21 April 2008

Keywords: pulmonary artery; PDE5 inhibitors; NO; cGMP; calcium blockade; endothelium; relaxation

Abbreviations: GTN, glyceryl trinitrate; L-NAME, N^{ω}-nitro-L-arginine methyl ester; ODQ, 1*H*-[1,2,4] oxadiazolo [4,3,-a] quinoxalin-1-one; PDE5, phosphodiesterase type-5

Introduction

Pulmonary hypertension is associated with altered vascular function characterized by vasoconstriction, smooth muscle cell proliferation and vascular remodelling, which brings morbidity and shortens survival. NO has emerged as an important signalling molecule playing a crucial role in a number of cellular functions, including the regulation of vascular smooth muscle tone (Furchgott and Vanhoutte, 1989). The physiological target of NO is the enzyme soluble guanylate cyclase, which catalyses the conversion of GTP to the intracellular second messenger cGMP, mediating NO-induced relaxation (Murad, 1988). Intracellular cGMP is rapidly inactivated to GMP by the activity of cyclic nucleotide phosphodiesterases (PDEs). Therefore, cGMP concentration in smooth muscle cells is mainly dependent on the balance between the production by soluble guanylate cyclase and the breakdown by PDEs, which represents the unique degradation pathway for this second messenger (Maurice *et al*., 2003; Rybalkin *et al*., 2003). There are 11 distinct PDE isoenzymes, differing in their substrate specificity, selective inhibition or stimulation by cofactors and/or standard drugs, and gene homology (Bender and Beavo, 2006). Phosphodiesterase type-5 (PDE5) selectively degrades cGMP, and is the predominant isoform responsible for the metabolism of cGMP in the corpus cavernosum (Carson and Lue, 2005). Three commercially available PDE5 inhibitors sildenafil (Viagra), vardenafil (Levitra) and tadalafil (Cialis) are currently

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Received 7 December 2007; revised 21 January 2008; accepted 4 March 2008; published online 21 April 2008

approved for the treatment of erectile dysfunction (Boolell *et al*., 1996; Rosen and Kostis, 2003; Montorsi *et al*., 2004).

These inhibitors are now receiving attention for their activity in the pulmonary vasculature (Ghofrani *et al*., 2002a), specifically sildenafil, due to the observed beneficial effects in the treatment of pulmonary hypertension (Wilkens *et al*., 2001; Ghofrani *et al*., 2002b; Ghofrani and Grimminger, 2006). PDE5 is highly expressed in pulmonary vasculature (Hanson *et al*., 1998; Giordano *et al*., 2001), and its inhibition has been associated with pulmonary vasodilatation (Thusu *et al*., 1995; Ziegler *et al*., 1998; Tsai *et al*., 2005). However, there are few reports regarding the effect of vardenafil or tadalafil on the pulmonary vasculature. In the present investigation, we have performed a systematic study of the *in vitro* effects of sildenafil, tadalafil and vardenafil in the rabbit pulmonary artery. We aimed to explore the mechanisms by which sildenafil, tadalafil and vardenafil induced relaxation in rabbit isolated pulmonary artery, focusing on the contribution of the NO–cGMP pathway and blockade of Ca^{2+} entry in modifying the contraction of arterial smooth muscle.

Materials and methods

Animals used

The animal procedures and experimental protocols in this study were approved by the Ethics Committee for Experimental Research of the State University of Campinas (UNICAMP).

Preparation of rabbit pulmonary artery rings

Briefly, male New Zealand white rabbits (2–2.5 kg) were anaesthetized with pentobarbital sodium (Hypnol; 40 mg kg^{-1}, i.v.) and exsanguinated via the carotid artery. The heart and lungs were removed *en bloc* from the thoracic cavity and placed in fresh Krebs solution containing the following (in mM): NaCl 118; $NaHCO_3$ 25; glucose 5.6; KCl 4.7; KH_2PO4 1.2; $MgSO_4 \cdot 7H_2O$ 1.17; $CaCl_2 \cdot 2H_2O$ 2.5. The proximal right and left branches of the main pulmonary artery were isolated, cleaned of all visible fat and connective tissue and cut into segments (2.5–3.5 mm in length) for use in tissue bath studies. In some rings, the endothelium was removed mechanically by rubbing the intimal surface of the vessels. The absence of the endothelium was confirmed by the loss of a relaxant response to ACh at the beginning of the experiments.

Isometric tension recording

Each ring was suspended between two wire hooks and mounted in isolated organ baths under resting force of 7.5 mN in 10 mL organ chambers filled with Krebs solution at 37 °C, pH 7.4, 95% O_2 and 5% CO_2. To record the development of isometric tension, hooks were fixed to the bottom of the chamber and to a force transducer (UgoBasile, Varese, Italy) connected to a PowerLab 400 data-acquisition system (Software Chart, version 4.2; ADInstruments, Colorado Springs, MA, USA). Ring preparations were equilibrated for 1 h before the start of the experiments.

Experimental protocols

After the equilibration period, pulmonary artery rings were challenged with 80 mM KCl (the same composition as Krebs solution with NaCl replaced by equimolar KCl) to check tissue viability. Next, the endothelial integrity of the preparations or the absence of the endothelium was determined by verifying the responsiveness to ACh (1 μM) in vessels precontracted with phenylephrine (1 μM). This phenylephrine concentration was chosen after preliminary experiments in pulmonary artery rings where it causes a sub-maximal contraction (about 70%). Tissues were then washed several times to restore tension to the baseline level.

Cumulative concentration–response curves to sildenafil, tadalafil or vardenafil (0.0001–10 μM) were obtained after precontraction with phenylephrine (1 μM) in endothelium-intact or endothelium-denuded preparations in the absence or presence of either a NO synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME; 100 μM), or an inhibitor of soluble guanylyl cyclase, 1*H*-[1,2,4] oxadiazolo [4,3,-a]quinoxalin-1-one (ODQ; 10 μM). One concentration–response curve to sildenafil, vardenafil or tadalafil was obtained in each segment. Hence, control rings (treated with the appropriate vehicles) were run in parallel with experimental rings. Concentration–response curves to glyceryl trinitrate (GTN, 0.0001–10 μM) were obtained in endothelium-denuded preparations. Then, rings were washed three times every 15 min during 1 h. Next, PDE5 inhibitors (0.1 μM) were incubated for 30 min before a second curve was generated.

Concentration–response curves to phenylephrine (0.001–10 μM) were constructed in the absence or presence of different concentrations of sildenafil, tadalafil or vardenafil (0.01–10 μM each), and contractile responses were calculated as a percentage of the KCl (80 mM)-induced contraction.

Concentration–response curves to each PDE5 inhibitor were also constructed in endothelium-intact rings in the absence or presence of either the endothelin ET_A/ET_B receptor antagonist tezosentan (100 μM, 20 min incubation) or the ACE inhibitor captopril (10 μM, 20 min incubation).

$CaCl_2$-induced pulmonary artery contractions

Concentration–response curves to $CaCl_2$ (0.01–5 mM) were constructed after the addition of KCl (80 mM) in nominally Ca^{2+}-free medium (containing 1 mM EGTA) in the absence or presence of sildenafil, tadalafil or vardenafil (0.1, 1 or 10 μM each). Briefly, tissues were initially precontracted with KCl (80 mM). After washing, Krebs solution was replaced by a Ca^{2+}-free Krebs solution in the presence of EGTA (1 mM). Next, phenylephrine (1 μM) and the Ca^{2+} ATPase inhibitor cyclopiazonic acid (10 μM) were used to deplete the intracellular Ca^{2+} stores and to prevent Ca^{2+} uptake to the sarcoplasmic reticulum, respectively. The Ca^{2+}-free Krebs solution was replaced by a Ca^{2+}-free depolarizing solution (KCl, 80 mM). Then, contractile responses to $CaCl_2$

(1–10 mM) were constructed and calculated as percentage of the KCl contraction (Lagaud *et al.*, 1999; Priviero *et al.*, 2006).

Platelet preparation

Blood was obtained from healthy volunteers who had not taken any drugs for at least 10 days. Whole blood was drawn from the antecubital vein and mixed with acid-citrate dextrose buffer (85 mM trisodium citrate, 71 mM citric acid and 111 mM dextrose) at 9:1 v v^{-1}. Platelet-rich plasma was then prepared by centrifugation at 200 *g* for 15 min at room temperature. To obtain washed platelets, PRP was centrifuged at 800 *g* for 12 min at room temperature. The supernatant was discarded and the pellet was carefully resuspended in Ca^{2+}-free Krebs solution and the number of platelets was adjusted to 3×10^8 cells mL^{-1}.

Measurement of intracellular Ca^{2+} mobilization

Washed platelets (3×10^8 cells mL^{-1}) were incubated with 2 μM of fura2-AM for 45 min at room temperature (Pollock *et al.*, 1986). Iloprost (0.8 μM) was added and the suspension was centrifuged at 600 *g* for 12 min. The pellet was resuspended in calcium-free Krebs-Ringer solution and the number of platelets was adjusted to 1.2×10^8 mL^{-1}. Aliquots of platelets (1 mL) were incubated with vehicle, sildenafil, tadalafil or vardenafil (1 and 10 μM) for 20 min and then were dispensed into cuvettes (Hitachi F-2000, Japan) equipped with a stirring device. To obtain total calcium mobilization, the external Ca^{2+} concentration was adjusted to 1 mM with $CaCl_2$, following equilibration for at least 30 s. Then, thrombin (100 mU mL^{-1}) was added to induce platelet activation. To verify the Ca^{2+} mobilization from internal storage sites alone, 2 mM EGTA was added to chelate the extracellular Ca^{2+}. The fura2-AM fluorescence was monitored continuously with monochromator settings of 339 nm (excitation) and 500 mm (emission). The external influx of Ca^{2+} was calculated by subtracting the mobilization from internal stores from the total Ca^{2+} mobilization. The intracellular Ca^{2+} levels were calculated by use of a general formula as described by Pollock *et al* (1986).

Statistical analysis

Experimental values of relaxation or contraction were calculated relative to the maximal changes from the contraction produced by phenylephrine and KCl, respectively, taken as 100% in each tissue. The pEC_{50} values for sildenafil, tadalafil, vardenafil and GTN were determined as – log of the molar concentration to produce 50% of the maximal relaxation in phenylephrine-contracted tissues. Data are shown as the percentage of relaxation of *n* experiments, expressed as the mean ± s.e.mean. Statistical comparisons were made using one-way analysis of variance (ANOVA) and Bonferroni method was chosen as a *post hoc* test. Student's paired *t*-test was also used when appropriate. $P<0.05$ was considered to indicate significance. A program package was used for the statistical analysis of all data (GraphPAD Instat, 1997, version 3.00; GraphPad Prism Software, San Diego, CA, USA).

Drugs and chemicals

ACh, L-NAME, ODQ, cyclopiazonic acid, EGTA, nifedipine, Fura2-AM, thrombin, trichloroacetic acid and phenylephrine were purchased from Sigma Chemical (St Louis, MO, USA). Sildenafil citrate and Hypnol were obtained from Cristalia Laboratories (Itapira, Brazil). Vardenafil and tadalafil were obtained from commercially available sources. GTN (Nitronal; 50 mL clear glass vials filled with colourless isotonic solution containing 1 mg mL^{-1} GTN) was acquired from Lipha Pharmaceuticals (London, UK). Stock solutions of ACh, L-NAME, GTN and phenylephrine were prepared in deionized water and stored in aliquots at –20 °C; dilutions were made immediately before use. ODQ, cyclopiazonic acid, sildenafil, tadalafil and vardenafil were diluted in dimethylsulphoxide and stored at −20 °C. Nifedipine was initially prepared as a stock solution in ethanol and stored in aliquots at −20 °C; these were diluted in deionized water before use. The final concentration of ethanol did not exceed 0.1%. Preliminary experiments ascertained the lack of response to either vehicle in the concentrations employed.

Results

Role of endothelium in arterial responses to PDE5 inhibitors

The selective PDE5 inhibitors sildenafil and tadalafil (0.0001–10 μM; $n=10$ each) evoked relaxations of endothelium-intact pulmonary artery rings in tissues precontracted with phenylephrine (1 μM) in a concentration-dependent manner (Figure 1a), with pEC_{50} values of 7.68 ± 0.06 and 7.86 ± 0.05 (variable slope). Vardenafil produced biphasic relaxing responses in endothelium-intact pulmonary artery preparations, with pEC_{50} values of 8.39 ± 0.05 (first phase) and 5.89 ± 0.3 (second phase; $n=10$). The transition from the first phase to the second phase occurred at 10^{-8} M (according to two-site competition fitting analysis; Figure 1a). In this first phase, vardenafil was significantly more potent than the other two inhibitors (one-way ANOVA; $P<0.05$).

Figure 1b shows that removal of the pulmonary artery endothelium caused a significant rightward shift (one-way ANOVA; $P<0.001$) of the concentration–response curves to sildenafil, tadalafil and vardenafil (9-, 12- and 123-fold; respectively) with pEC_{50} values of 6.72 ± 0.09, 6.77 ± 0.1 and 6.30 ± 0.15, respectively; $n=10$). The biphasic relaxing response to vardenafil seen in endothelium-intact rings was not observed in these denuded preparations (one-site fitting analysis). No differences in maximal response to vardenafil were observed by removal of endothelium (107 ± 3 and 101 ± 3% in intact and denuded preparations, respectively), whereas those elicited by sildenafil (97 ± 2%) and tadalafil (96 ± 4%) in endothelium-intact preparations were significantly reduced ($P<0.05$) by endothelium removal (69 ± 4 and 75 ± 5%, respectively; $n=10$). At the two highest concentrations (3 and 10 μM), vardenafil produced greater relaxation (one-way ANOVA, $P<0.01$) than the other two inhibitors in endothelium-denuded preparations (vardenafil: 92 ± 3 and 101 ± 3%; sildenafil: 61 ± 5 and 69 ± 4%; tadalafil: 67 ± 4 and 75 ± 5%, respectively); there were no significant

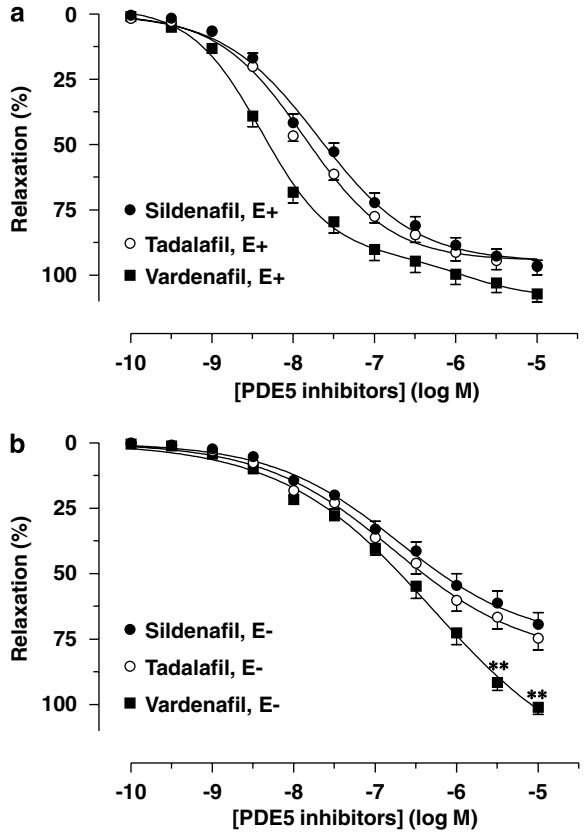

Figure 1 Concentration–response curves to sildenafil, tadalafil and vardenafil in endothelium-intact (E+(**a**)) and denuded (E−(**b**)) rabbit pulmonary artery rings precontracted with phenylephrine (1 μM). Experimental values were calculated relative to the maximal changes from the contraction produced by phenylephrine in each tissue, which was taken as 100%. Data represent the mean ± s.e.mean of 10 experiments. $^{**}P<0.01$ compared with respective concentrations of either sildenafil or tadalafil.

differences in the relaxant responses between the PDE5 inhibitors at any other concentration.

Role of the NO–cGMP pathway in PDE5 inhibitor-induced relaxations

In endothelium-intact pulmonary artery preparations, the addition of either the NO synthesis inhibitor L-NAME (100 μM) or the soluble guanylate cyclase inhibitor ODQ (10 μM) caused a further increase in tone (17 ± 4 and 15 ± 2%, respectively), whereas in endothelium-denuded rings, ODQ (or L-NAME) had no effect on the tone of the preparations. At the concentrations used, L-NAME and ODQ nearly abolished the endothelium-dependent relaxations mediated by ACh (0.001–10 μM; $n=4$ each).

Figure 2 shows that preincubation of endothelium-intact pulmonary artery rings with L-NAME (100 μM; $n=5$) or ODQ (10 μM; $n=5$) shifted the concentration–response curves to sildenafil, tadalafil and vardenafil to the right (one-way ANOVA, $P<0.001$). Both L-NAME and ODQ significantly reduced ($P<0.01$) the maximal relaxations to sildenafil (28 ± 2 and 36 ± 2% reductions, respectively) and tadalafil (27 ± 4 and 32 ± 3% reductions, respectively), without affecting those elicited by vardenafil in intact vessels (Figures 2a–c). Neither L-NAME nor ODQ had any significant effect on the potency or the maximal responses to sildenafil, tadalafil and vardenafil in endothelium-denuded rings (Figures 2d–f).

Effect of PDE5 inhibitors on GTN-induced vasorelaxation

The NO donor GTN (0.0001–10 μM) induced concentration-dependent relaxations of endothelium-denuded pulmonary artery rings, which were fully blocked by ODQ (10 μM; $n=4$). Figure 3 shows that, at pEC_{50} levels, the relaxant responses induced by GTN were shifted approximately 3-, 4- and 5-fold to the left by sildenafil, tadalafil and vardenafil (0.1 μM each; $P<0.01$, one-way ANOVA; $n=8$ each). The PDE5 inhibitors did not affect the maximal responses induced by GTN (Table 1).

Effect of PDE5 inhibitors on the contractile response to phenylephrine

Concentration–response curves to phenylephrine (0.001–10 μM) were constructed in the absence or presence of different concentrations of the PDE5 inhibitors (0.01–10 μM). Endothelium removal caused a significant increase ($P<0.01$) in the contractile response to phenylephrine (E_{max} 150 ± 2%) compared with endothelium-intact preparations (E_{max} 86 ± 5%). Addition of incremental concentrations of PDE5 inhibitors (0.1–10 μM) in endothelium-intact pulmonary artery preparations caused a rightward shift in the concentration–response curves to phenylephrine. Interestingly, vardenafil at 10 μM caused a decrease in the maximal responses in the contractile response to phenylephrine ($P<0.05$), whereas sildenafil and tadalafil had no effect (Figures 4a–c).

In endothelium-denuded rings, the potency of phenylephrine was not affected by tadalafil ($n=5$). At the highest concentration, sildenafil shifted the curve to the right by 2.5-fold ($n=5$; $P<0.01$). Neither sildenafil nor tadalafil at the highest concentration (10 μM) reduced the maximal response to phenylephrine (Figures 4d and e). In contrast, vardenafil (at 1 and 10 μM) shifted the curve to the right by 5- and 19-fold ($n=5$) and reduced the maximal response to phenylephrine ($P<0.001$ and $P<0.0001$ for 1 and 10 μM, respectively; Figure 4f). GTN at 0.1 and 1 μM concentration caused a rightward shift in the curve to phenylephrine by 3.6- and 5.9-fold ($n=4$; $P<0.01$), respectively, and reduced the maximal response to phenylephrine at 1 μM concentration (E_{max} control: 150 ± 2 to 119 ± 6%; $P<0.001$).

Effect of PDE5 inhibitors on $CaCl_2$-induced contractions

Cumulative addition of $CaCl_2$ (0.01–5 mM) in the presence of high-K^+-depolarized endothelium-denuded pulmonary artery rings was used to investigate contractile responses dependent on Ca^{2+} influx. These experiments were carried out in the presence of ODQ. Neither sildenafil nor tadalafil was able to affect the $CaCl_2$-induced contractions ($n=5$ each). In contrast, vardenafil (1 and 10 μM; $n=5$) caused a 2- and 2.9-fold shift in the curves to the right ($P<0.05$; Figure 5) and markedly reduced maximal contractions to $CaCl_2$ (25 ± 4%, $P<0.05$ and 39 ± 2%, $P<0.001$, respectively). Pretreatment with the L-type Ca^{2+} channel blocker nifedipine (1 μM) had a similar profile to vardenafil (3.1-fold

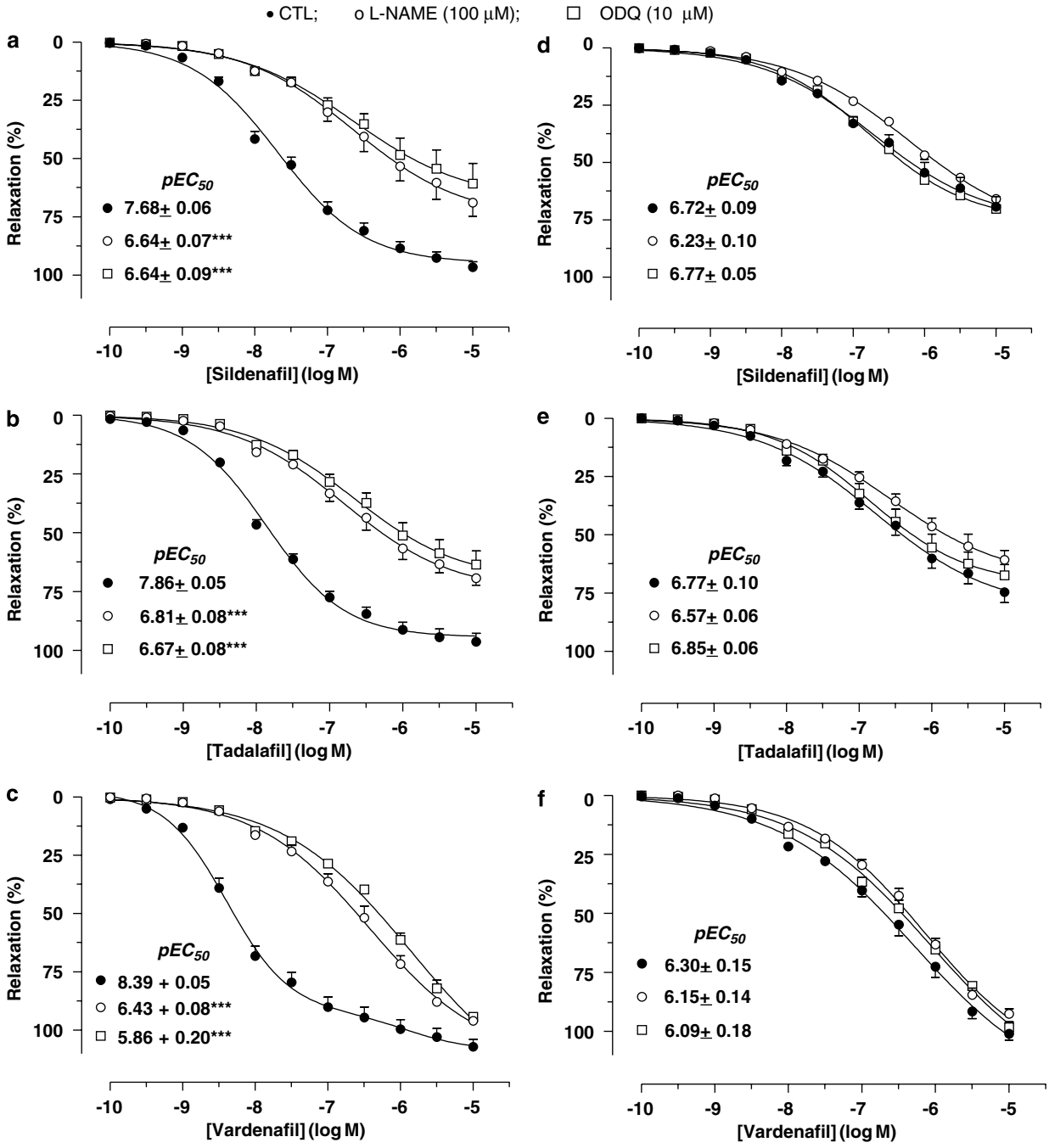

Figure 2 Concentration–response curves to sildenafil, tadalafil and vardenafil in the absence or presence of either L-NAME (100 μM) or ODQ (10 μM). Panels **a–c** represent endothelium-intact preparations, whereas panels **d–f** represent endothelium-denuded preparations of rabbit pulmonary artery rings precontracted with phenylephrine (1 μM). The inset in all panels shows the pEC_{50} values for each PDE5 inhibitor. Data were calculated relative to the maximal changes from the contraction produced by phenylephrine in each ring, which was taken as 100%. Data are the mean ± s.e.mean of 5 experiments. ***$P<0.001$ (one-way ANOVA) compared with respective untreated preparations. L-NAME, N^{ω}-nitro-L-arginine methyl ester; ODQ, 1*H*-[1,2,4] oxadiazolo [4,3,-a]quinoxalin-1-one; PDE5, phosphodiesterase type-5.

rightward shift and reduction of the maximal responses (control: 133 ± 3; treated: 78 ± 8%; $P<0.01$)).

Effects of tezosentan and captopril on relaxations induced by PDE5 inhibitors

To further explore the mechanisms underlying the relaxations of endothelium-intact pulmonary artery rings induced by PDE5 inhibitors, we investigated the effects of the endothelin ET_A/ET_B receptor antagonist tezosentan (100 μM) and the ACE inhibitor captopril (10 μM) on the curves for sildenafil, tadalafil and vardenafil. Preincubation with tezosentan nearly abolished the contractions evoked by endothelin-1 (0.0001–3 μM; $n=4$). There were no significant differences in the treatment with tezosentan or captopril in either pEC_{50} or E_{max} values between the different PDE5 inhibitors ($n=4$ each; Figure 6).

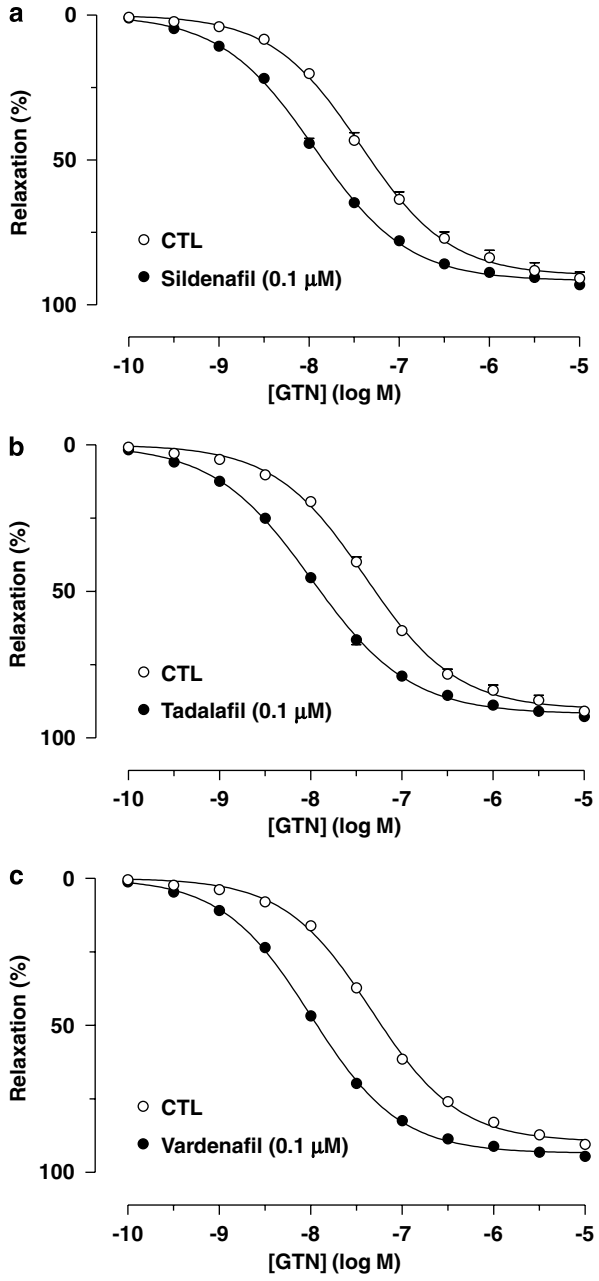

Figure 3 Concentration–response curves to GTN in the absence or presence of 0.1 μM of sildenafil (**a**), tadalafil (**b**) and vardenafil (**c**). Curves were obtained in endothelium-denuded rabbit pulmonary artery rings precontracted with phenylephrine (1 μM). Data are the mean ± s.e.mean of 8 experiments. GTN, glyceryl trinitrate.

Effect of PDE5 inhibitors on platelets calcium mobilization

In the presence of external $CaCl_2$, the addition of thrombin to human washed platelets resulted in an immediate reduction in the Fura2-am fluorescence, indicating a rise in $[Ca^{2+}]_i$ to 150 ± 18.6 nM ($n = 4$–5 each). When the external Ca^{2+} was removed by adding 2 mM EGTA, the rise in $[Ca^{2+}]_i$ in response to thrombin was significantly reduced to 64.5 ± 12.4 nM. At the concentrations used (1 and 10 μM), neither sildenafil ($P = 0.075$) nor tadalafil ($P = 0.11$) significantly inhibited the mobilization of the external Ca^{2+} influx

Table 1 Potency (pEC_{50}) and maximal response (E_{max}) values derived from concentration–response curves to GTN (0.0001–10 μM) in endothelium-denuded rabbit pulmonary artery rings precontracted with phenylephrine (1 μM)

PDE5 inhibitors	*pEC_{50} values*		*E_{max} values (%)*	
	Absence	*Presence*	*Absence*	*Presence*
Sildenafil	7.43 ± 0.02	7.94 ± 0.02**	91 ± 5	93 ± 3
Tadalafil	7.40 ± 0.03	7.99 ± 0.01**	91 ± 4	93 ± 3
Vardenafil	7.34 ± 0.02	8.00 ± 0.02**	95 ± 3	95 ± 3

Abbreviations: GTN, glyceryl trinitrate; PDE5, phosphodiesterase-5.
Curves were performed in the absence or presence of the PDE5 inhibitors sildenafil, tadalafil or vardenafil (0.1 μM each). Data represent the mean ± s.e.mean of 8 animals.
**$P < 0.01$ (one-way ANOVA) compared with respective control values (absence of drugs).

(Table 2). In contrast, vardenafil at 10 μM reduced significantly not only the Ca^{2+} mobilization but also the external Ca^{2+} influx ($P < 0.01$; Table 2).

Discussion

Sildenafil, tadalafil and vardenafil are all classified as PDE5 inhibitors, but they differ slightly in their selectivity, pharmacokinetics and side effects (Rosen and Kostis, 2003). Vardenafil, for instance, is considered to be slightly more potent, possibly due to its different chemical structure, which allows a slower dissociation rate from PDE5 compared with sildenafil and tadalafil (Blount *et al*., 2004). Our results demonstrate that in several rabbit pulmonary artery assays, vardenafil is not only more potent than the other two PDE5 inhibitors, but also has some other pharmacological action, not related to PDE5 inhibition. The first evidence came from the results showing that neither sildenafil nor tadalafil affected the phenylephrine-induced pulmonary artery contractions in the endothelium-denuded artery whereas vardenafil markedly shifted the curve to the right. One possibility could be that vardenafil would be inhibiting the synthesis of vasoconstrictor agents in endothelial cells, such as endothelin-1 and angiotensin II. However, neither the endothelin receptor antagonist tezosentan nor the ACE inhibitor captopril affected the actions of vardenafil, thus excluding this possibility. The finding that only vardenafil markedly reduced $CaCl_2$-induced contractions in the phenylephrine-treated isolated pulmonary artery indicates that this additional mechanism could be related to blockade of Ca^{2+} channels.

Vascular tone is regulated by $[Ca^{2+}]_i$ and the sensitivity of the contractile elements to Ca^{2+} in the smooth muscle cell. Calcium influx through L-type Ca^{2+} channels represents one of the major pathways to increase $[Ca^{2+}]_i$, and its blockade causes vasorelaxation or inhibits the contraction (Vogalis *et al*., 1991; Kuriyama *et al*., 1995). Specifically, in vascular smooth muscle, removal of extracellular Ca^{2+}, blockade of L-type voltage-operated Ca^{2+} channels and depletion of intracellular Ca^{2+} stores are important tools for distinguishing between the sources of Ca^{2+} to the cell for

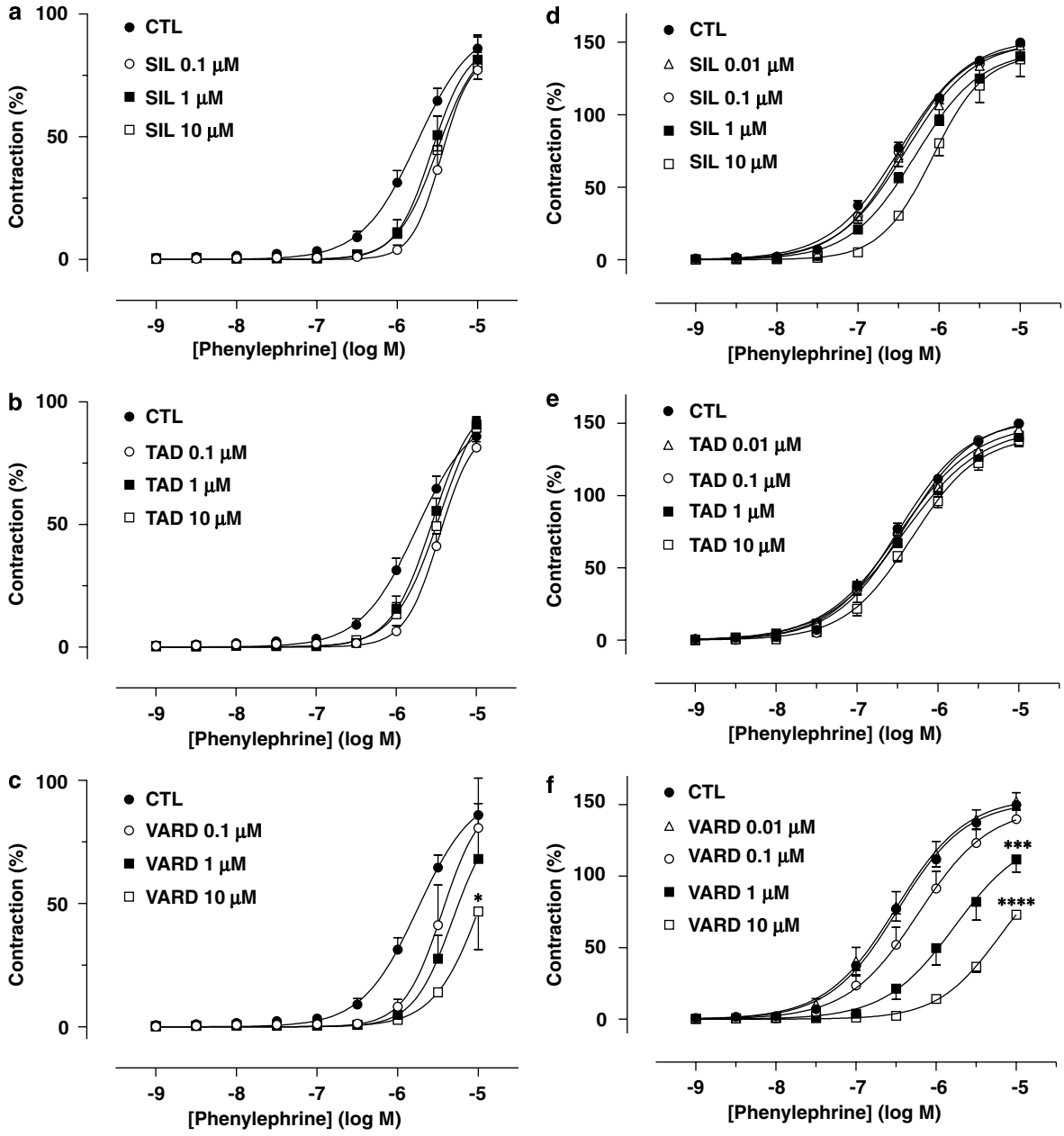

Figure 4 Concentration–response curves to phenylephrine in the absence or presence of increasing concentrations of sildenafil, tadalafil and vardenafil in endothelium-intact (0.1–10 μM each (**a**–**c**)) or in endothelium-denuded rabbit pulmonary artery rings preparations (0.01–10 μM (**d**–**f**)). Data were calculated relative to the maximal changes from the contraction produced by KCl (80 mM) in each ring, which was taken as 100%. Data are the mean ± s.e.mean of rings from 5 animals. *$P<0.05$ (one-way ANOVA) compared with control (CTL), and 0.1 or 1 μM vardenafil concentrations in endothelium-intact rings at 10^{-5} M phenylephrine. ***$P<0.001$; ****$P<0.0001$ (one-way ANOVA) compared with CTL, and 0.01 or 0.1 μM vardenafil concentrations in endothelium-denuded rings at 10^{-5} M phenylephrine.

functional responses (Fleckenstein and Fleckenstein-Grün, 1988; Godfraind, 1988). L-type voltage-operated Ca^{2+} channels are well described in vascular smooth muscle, but their presence in endothelial cells is controversial (Oshima *et al*., 2005). Our results obtained with vardenafil in endothelium-denuded pulmonary artery were similar to those obtained with the L-type voltage-operated Ca^{2+} channel blocker nifedipine, strongly suggesting that vardenafil is acting on a calcium channel located in smooth muscle.

Vardenafil, in contrast to sildenafil and tadalafil, inhibited thrombin-induced Ca^{2+} mobilization in human washed platelets, reinforcing the suggestion that vardenafil presents an additional mechanism related to blockade of Ca^{2+} channels in rabbit pulmonary artery. Although some authors have advocated a role for voltage-operated Ca^{2+} channels in Ca^{2+} entry in platelets (Palés *et al*., 1991), the balance of evidence is against this proposal (Sage, 1997). For instance, platelets lack binding sites for verapamil and nitrendipine (Motulsky *et al*., 1983) and these Ca^{2+} channel blockers are

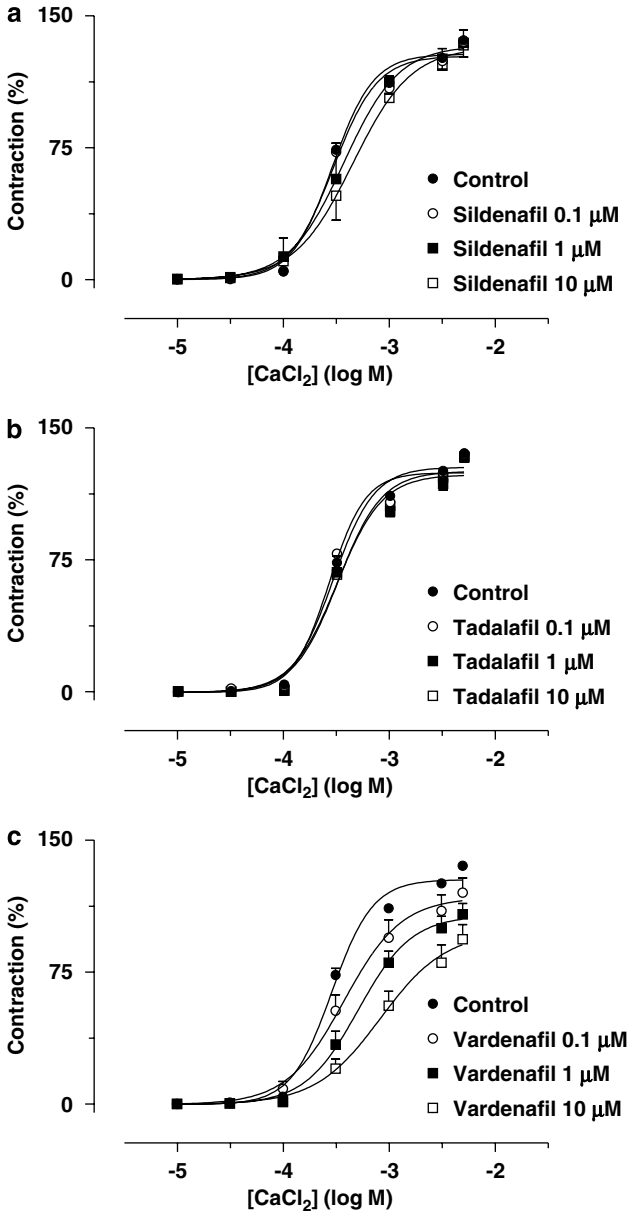

Figure 5 Concentration–response curves to $CaCl_2$ in the absence or presence of sildenafil (0.1–10 μM (**a**)), tadalafil (0.1–10 μM (**b**)) and vardenafil (0.1–10 μM (**c**)) in endothelium-denuded rabbit pulmonary artery rings. Data are the mean ± s.e.mean of rings from 4 to 5 animals, calculated as a percentage of the contractions induced by KCl (80 mM).

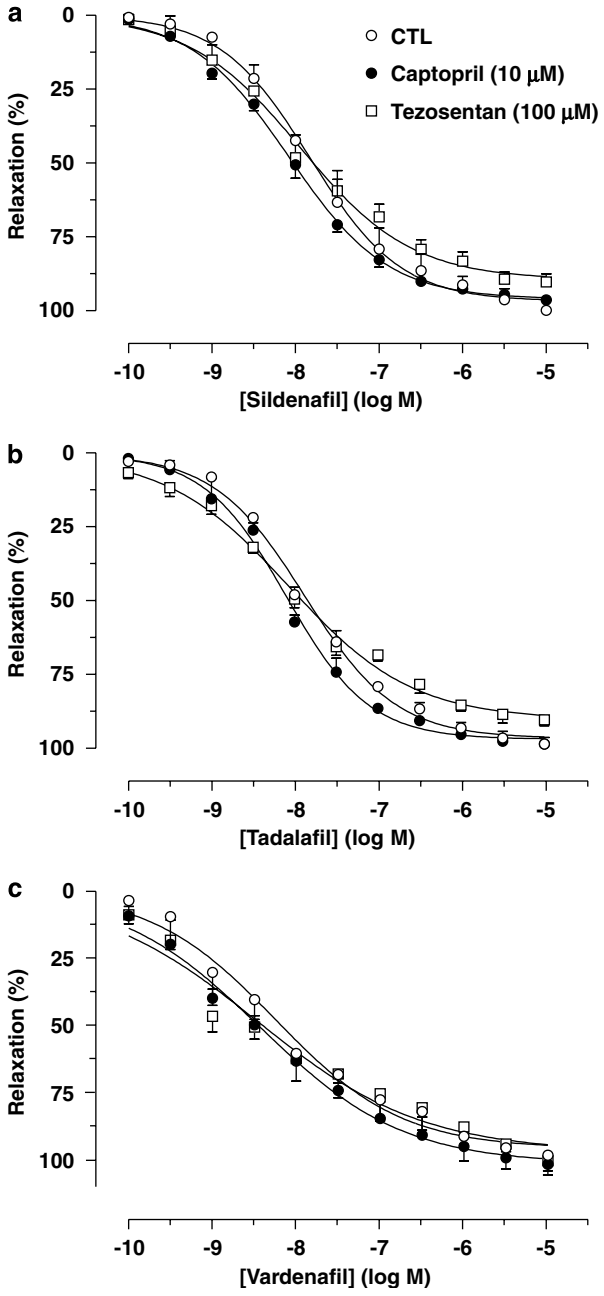

Figure 6 Concentration–response curves to sildenafil (**a**), tadalafil (**b**) and vardenafil (**c**) in the absence or presence of either the ACE inhibitor captopril (10 μM) or the endothelin ET_A/ET_B receptor antagonist tezosentan (100 μM) in endothelium-intact pulmonary artery rings precontracted by phenylephrine (1 μM). Data were calculated relative to the maximal changes from the contraction produced by phenylephrine in each ring, which was taken as 100%. Data are the mean ± s.e.mean of rings from 4 animals. CTL, control.

ineffective in platelets at concentrations that inhibit Ca^{2+} entry in excitable cells (Rink and Sage, 1990). Thus, it is unlikely that vardenafil is acting on voltage-sensitive Ca^{2+} channels in platelets. Store-operated Ca^{2+} channels play an important role in Ca^{2+} entry in platelets; however, the molecular identity of these channels has yet to be established (Authi, 2007). Human pulmonary endothelial cells express transient receptor potential channels (TRPC1), which belong to the store-operated cation channel family (Paria *et al.*, 2004; Ambudkar, 2007). Considering that the blockade of Ca^{2+} channels by vardenafil was observed both in platelets and rabbit pulmonary artery, one likely explanation is that vardenafil, besides being a PDE5 inhibitor, also blocks store-operated Ca^{2+} channels. As hypoxia causes pulmonary vasoconstriction and upregulates transient receptor potential channels leading to enhanced Ca^{2+} entry through receptor- and store-operated Ca^{2+} channels (Murray *et al.*, 2006), this extra Ca^{2+}-blocking activity may confer

Table 2 Effect of sildenafil, tadalafil and vardenafil (1 and 10 μM each) on human platelet Ca^{2+} mobilization

	$CaCl_2$ (1 mM)	*EGTA (2 mM)*	*Ca^{2+} influx ($CaCl_2$-EGTA)*
Control	150 ± 18.6	64.5 ± 12.4	85.5 ± 12.8
Sildenafil 1 μM	151.7 ± 5	77.0 ± 7.7	74.7 ± 8.9
Sildenafil 10 μM	118.7 ± 15.4	57.1 ± 13.3	61.6 ± 7.4
Tadalafil 1 μM	160.2 ± 7.5	58.7 ± 10.5	101.5 ± 7.5
Tadalafil 10 μM	150.4 ± 22.8	57.6 ± 12.8	92.8 ± 12.5
Vardenafil 1 μM	115.5 ± 18	56.7 ± 6.2	58.8 ± 11.8
Vardenafil 10 μM	72.2 ± 11*	34.3 ± 8.3*	37.9 ± 8.2**

Washed platelets were loaded with Fura2-am (2 μM) and then incubated with either $CaCl_2$ (1 mM) or EGTA (2 mM). The external influx of Ca^{2+} was calculated by subtracting the mobilization from internal stores (column headed EGTA) from the total Ca^{2+} mobilization (column headed $CaCl_2$). Data shown are the concentrations of intracellular Ca^{2+} (nM) as the means ± s.e.mean of 4–5 experiments.
*$P<0.05$; **$P<0.01$ (one-way ANOVA), compared with control values.

therapeutic advantages for vardenafil in the treatment of pulmonary hypertension.

In conclusion, our results indicate that vardenafil, in contrast to sildenafil or tadalafil, has an additional action of blocking Ca^{2+} channels, which enhances its vasorelaxant property in the rabbit pulmonary artery. This effect is observed at the lowest concentration used (100 nM), which is close to the C_{max} observed after 20 mg of vardenafil (40 nM; Wespes *et al*., 2006). Considering that the C_{max} for both tadalafil (20 mg) and sildenafil (100 mg) is approximately 1 and 1.2 μM, respectively, our data suggest that vardenafil may be more effective than sildenafil and tadalafil for the treatment of pulmonary hypertension.

Acknowledgements

HA Toque and G De Nucci are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for providing financial support.

Conflict of interest

The authors state no conflict of interest.

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