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Molecular mechanisms underlying rat mesenteric artery vasorelaxation induced by the NO-independent soluble guanylyl cyclase stimulators BAY 41-2272 and YC-1.

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***Abbreviations*:** ACh, acetylcholine; Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate; ANP, atrial natriuretic peptide; BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]pyrimidin-4-ylamine; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; CPA, cyclopiazonic acid; DDA, 2',5'-dideoxyadenosine; L-NAME, N^{ω}-nitro-L-arginine methyl

ester; NO, nitric oxide; ODQ, 1H-[1,2,4] oxadiazolo [4,3,-a]quinoxalin-1-one; PDE5, phosphodiesterase type-5; PE, phenylephrine; Rp-8-*p*CPT-cGMPS, guanosine 3′,5′-cyclic monophosphorothioate, 8-(4-chlorophenylthio)-Rp-Isomer; SERCA, sarco(endo)plasmic reticulum ATPase; sGC, soluble guanylyl cyclase; SNP, sodium nitroprusside; TCA, trichloroacetic acid; TEA, tetraethylammonium; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole.

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Abstract

This study aimed to investigate the mechanisms of relaxation to the nitric oxide (NO)-independent soluble guanylyl cyclase (sGC) stimulators 5-cyclopropyl-2-[1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]pyrimidin-4-ylamine (BAY 41-2272) and 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1) in the rat mesenteric artery. In endothelium-intact rings, BAY 41-2272 (0.0001-1 μM) and YC-1 (0.001-30 μM) caused concentration-dependent relaxations (pEC_{50} values of 8.21 ± 0.05 and 6.75 ± 0.06, respectively), which were shifted to the right by 6-fold in denuded rings. The sGC inhibitor ODQ (10 μM) partially atenuated the maximal responses to BAY 41-2272 and YC-1 and displaced their curves to the right by 9-10-fold in intact and 3-fold in denuded vessels. The NO synthesis inhibitor L-NAME (100 μM) and the NO scavenger carboxy-PTIO (100 μM) reduced BAY 41-2272 and YC-1 relaxations, whereas the phosphodiesterase type-5 inhibitor sildenafil (0.1 μM) potentiated these responses. The phosphatase inhibitor calyculin A (50 nM) reduced the relaxant responses and high concentrations of BAY 41-2272 (1 μM) and YC-1 (10 μM) inhibited Ca^{2+}-induced contractions in K^{+}-depolarized rings. BAY 41-2272 (0.1 μM) and YC-1 (1 μM) markedly elevated cGMP levels in an ODQ-sensitive manner. Co-incubation of BAY 41-2272 or YC-1 with NO donor resulted in a synergistic inhibition of phenylephrine-induced contractions paralleled by marked increases in cGMP levels. In conclusion, BAY 41-2272 and YC-1 relax the mesenteric artery through cGMP-dependent and independent mechanisms, including blockade of Ca^{2+} influx. The synergistic responses likely reflect the direct effects of NO and NO-independent sGC stimulators on the enzyme, thus representing a potential therapeutic effect by permitting reductions of nitrovasodilator dose.

Introduction

Endothelium-derived nitric oxide (NO) is considered a major regulator of vascular functions due to its pivotal importance in the control of blood vessel tone (Moncada et al., 1991). In vascular smooth muscle cells, NO-induced vasodilation is mediated through the stimulation of soluble guanylyl cyclase (sGC), resulting in the conversion of GTP to cGMP, which modulates the activity of several cGMP effector proteins (Hobbs, 1997; Lucas et al., 2000). Thus, the integrity of the NO-sGC-cGMP pathway is critical to the regulation of blood pressure. Conventional organic nitrates mimic the actions of endogenous NO, due to the stimulation of sGC after bioactivation, being clinically used for the treatment of diseases related to NO deficiency, such as angina pectoris and pulmonary hypertension (Sperling and Creager, 1999). However, a major drawback of this therapy is the development of tolerance upon prolonged use (Parker, 1989), making drug-free intervals necessary. Hence, pharmacological agents have been developed to directly activate sGC in an NO-independent manner, thus representing a potential therapeutic strategy in the management of cardiovascular diseases.

The benzylindazole derivative 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1) was originally identified as a potent non-NO-based sGC stimulator. This compound causes heme-dependent direct activation of purified sGC and enhances the sensitivity of the enzyme towards its native activator NO (Ko et al., 1994; Wu et al., 1995; Mülsch et al., 1997; Hoenicka et al., 1999). In several studies, YC-1 was demonstrated to relax vascular smooth muscle *in vitro*, cause vasodilation *in vivo* and inhibit platelet aggregation through cGMP accumulation (Wu et al., 1995; Mülsch et al., 1997; Friebe et al., 1998; Becker et al., 2000). More recently, the pyrazolopyridine 5-cyclopropyl-2-[1-(2-fluorobenzyl)-1H-

pyrazolo[3,4-b]pyridin-3-yl]pyrimidin-4-ylamine (BAY 41-2272), a high-affinity YC-1 analog, also sensitizes sGC to NO and is approximately two orders of magnitude more potent than YC-1 to stimulate purified sGC activity (Stasch et al., 2001; Straub et al., 2001). BAY 41-2272 lowers mean arterial pressure in spontaneously hypertensive rats, increases survival in a low-NO rat model of hypertension and causes potent dilation of pulmonary vasculature in different models of pulmonary hypertension, thus consisting of a potential candidate for the treatment of cardiovascular disorders (Stasch et al., 2001; Boerrigter et al., 2003; Evgenov et al., 2004; Deruelle et al., 2005). *In vitro*, BAY 41-2272 causes potent relaxation of rabbit aortic rings and ovine pulmonary artery through elevation of intracellular cGMP levels (Priviero et al., 2005; Bawankule et al., 2005).

In the present investigation, we have undertaken a thorough and systematic study of the *in vitro* effects of BAY 41-2272 and YC-1. We tested the hypothesis that these drugs relax the rat mesenteric artery through cGMP-dependent and independent mechanisms, using both functional and biochemical approaches. In addition, we provided both functional and biochemical support for a synergistic interaction of these NO-independent stimulators with endogenous and exogenous NO to stimulate sGC in this vascular bed.

Materials and methods

Animals. The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, U.S.A.). All experiments were conducted in accordance with institutional guidelines and approved by the local committee on animal experiments. Experiments were performed on adult male Sprague-Dawley rats (250-275 g) obtained from Harlan Laboratories (Indianapolis, U.S.A.). The animals were housed 2 per cage on a 12 h light-dark cycle, and fed a standard chow diet with water ad lib.

Rat mesenteric artery preparation. The animals were anaesthetized with pentobarbital sodium (40 mg/kg, i.p.), killed by decapitation and exsanguinated. The main branch of the superior mesenteric artery was dissected out and cut into eight rings of approximately 3 mm in length following removal of the surrounding fat and connective tissues. In some rings, the endothelium was removed by gentle rubbing of the intimal surface with a fine tipped forceps. Each ring was mounted between two stainless steel wire hooks in a small vessel myograph for isometric force recording (Danish Myograph Technology, Aarhus, Denmark). Tissues were maintained at 37^{o}C in Krebs-Henseleit buffer of the following composition (mM): NaCl, 130; $NaHCO_3$, 14.9; dextrose, 5.5; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_47H_2O$, 1.17 and $CaCl_22H_2O$, 1.6. The bath solution was continuously bubbled with 95% O_2 and 5% CO_2 to give rise to a relatively constant pH of 7.2-7.4. All rings were placed under an optimal resting tension of 10 mN and were allowed to equilibrate for 45 min, during which time the bathing solution was replaced every 15 min and baseline

adjusted when necessary. Changes in isometric force were recorded in a PowerLab 8/SP™ data acquisition system (software Chart 5.0, ADInstruments, Colorado Springs, U.S.A.).

Experimental protocols. In order to verify the viability of the preparations, a high extracellular potassium solution (80 mM; achieved by the substitution of NaCl in Krebs buffer with an equimolar concentration of KCl) was added to the organ baths at the end of the equilibration period. Next, functional removal of the endothelium was verified by the lack of relaxation to 1 μM acetylcholine (ACh) in vessels precontracted with phenylephrine (PE, 1 μM). Rings were then washed several times to restore vessel tension to the baseline level. In the first set of experiments, concentration-response curves for BAY 41-2272 (0.0001-1 μM) or YC-1 (0.001-30 μM) were constructed in the absence and in the presence of inhibitors in order to investigate the molecular mechanisms leading to their vasorelaxing responses. In these experiments, the inhibitors were incubated for 30 min prior to the generation of concentration-response curves to BAY 41-2272 or YC-1. Cumulative additions were made as soon as the response leveled off to the preceding addition. One concentration-response curve to either BAY 41-2272 or YC-1 was obtained in each segment. Hence, control rings (treated with the appropriated vehicles) were run in parallel with experimental rings. The second set of experiments consisted of concentration-response curves to PE (0.001-10 μM) in endothelium-denuded vessels in the absence and in the presence of different concentrations of BAY 41-2272 (0.0001-0.1 μM), YC-1 (0.001-1 μM), sodium nitroprusside (SNP, 0.0001-0.1 μM), atrial natriuretic peptide (ANP, 0.0001-0.1 μM) or their combination so as to explore the synergistic or additive nature of this interaction. The third set of experiments was performed using nominally Ca^{2+}-free medium

(containing 1 mM EGTA to chelate trace Ca^{2+}), and consisted of concentration-response curves to $CaCl_2$ (0.01-10 mM) obtained in the absence and in the presence of BAY 41-2272 (0.1-1 μM) or YC-1 (1-10 μM). In the last set of experiments, cyclic nucleotide measurements were performed in response to BAY 41-2272 and YC-1 to provide biochemical support to the functional experiments.

Nonlinear regression analysis to determine the pEC_{50} was done using GraphPad Prism (GraphPad Software, San Diego, CA) with the constraint that Φ = zero. All concentration-response data were evaluated for a fit to a logistics function in the form: $E = E_{max}/((1+(10^c/10^x)^n) + \Phi)$, where E is the effect of above basal; E_{max} is the maximum response produced by BAY/YC-1; c is the logarithm of the EC_{50}, the concentration of drug that produces half-maximal response; x is the logarithm of the concentration of the drug; the exponential term, n, is a curve fitting parameter that defines the slope of the concentration-response line, and Φ is the response observed in the absence of added drug.

Determination of cyclic nucleotide levels. To determine the vascular cyclic nucleotide contents of rat mesenteric artery under experimental conditions, endothelium-intact or denuded rings were equilibrated for 20 min in warmed and oxygenated Krebs solution. Tissues were then stimulated for 10 min with BAY 41-2272 (0.1 μM), YC-1 (1 μM), SNP (0.1 μM), forskolin (0.1 μM) or their combination in the absence of in the presence of ODQ (10 μM). Next, rings were collected immediately by freezing the segments in liquid nitrogen. Some tissues were frozen following addition of vehicle to obtain baseline readings. Frozen rings were pulverized, homogenized in trichloroacetic acid (TCA, 5% wt/vol) and then centrifuged for 10 min at 4°C at 1,500 *g*. TCA was extracted from the

samples with three washes of water-saturated ether. The weights of the dried pellets were used in order to standardize the different samples. Preparation of tracer, samples, standards, and incubation with antibody were performed as described in commercially available kits (Cayman Chemical Cyclic GMP/Cyclic AMP EIA kit, Ann Arbor, MI, U.S.A.). The assays were performed in duplicates using different dilutions of samples.

Drugs and chemicals. Acetylcholine, apamin, atrial natriuretic peptide (ANP), calyculin A, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), charybdotoxin, cromakalim, cyclopiazonic acid (CPA), 2',5'-dideoxyadenosine (DDA), forskolin, glybenclamide, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1), iberiotoxin, indomethacin, KT 5823, nifedipine, N^{ω}-nitro-L-arginine methyl ester (L-NAME), ouabain, 1H-[1,2,4] oxadiazolo [4,3,-a]quinoxalin-1-one (ODQ), phenylephrine, rolipram, sodium nitroprusside (SNP), tetraethylammonium (TEA) and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). The compounds DT-3, guanosine 3′,5′-cyclic monophosphorothioate, 8-(4-chlorophenylthio)-Rp-Isomer (Rp-8-*p*CPT-cGMPS) and 1L-6-hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (Akt inhibitor) were acquired from Calbiochem (San Diego, U.S.A.). The compound 5-cyclopropyl-2-[1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]pyrimidin-4-ylamine (BAY 41-2272) was obtained from Axxora, LLC (San Diego, U.S.A.). Sildenafil was obtained from Pfizer (New York, U.S.A.). All other reagents used were of analytical grade. Stock solutions were prepared in deionized water and stored in aliquots at -20^{o}C; dilutions were made up in deionized water immediately before use. BAY 41-2272, YC-1, ODQ, forskolin, carboxy-PTIO, DDA, sildenafil, glybenclamide, KT 5823,

rolipram, CPA, calyculin A, DT-3, Rp-8-*p*CPT-cGMPS and Akt inhibitor were prepared in dimethyl sulphoxide (DMSO). Nifedipine, indomethacin and cromakalim were dissolved in ethanol. Apamin was prepared in 5% acetic acid. The final concentration of the solvents employed did not exceed 0.1%. Preliminary experiments ascertained the lack of response to either vehicle in the concentrations assayed.

Statistical analysis. All values of relaxation are shown as a percentage of the level of precontraction. Contractile responses were calculated as a percentage of KCl (80 mM)-induced contraction. All data are expressed as means ± S.E.M. (*n*). Statistical comparisons were made using one-way ANOVA followed by Student-Newman-Keuls multiple comparison test with $p<0.05$ taken as significant in each case. EC_{50} values are presented as the negative logarithm (pEC_{50}) and calculated by fitting concentration-response relationships to a sigmoidal model of the form log-concentrations *vs* response using GraphPad software.

Results

Role of endothelium in vasorelaxation induced by BAY 41-2272 and YC-1. Phenylephrine (PE, 1 μM) caused a sustained contraction in mesenteric artery ring preparations with intact or denuded endothelium, and generated active force of 20 ± 4 mN and 27 ± 5 mN, respectively.

When added cumulatively to the bathing medium, BAY 41-2272 (0.0001-1 μM) and YC-1 (0.001-30 μM) caused sustained relaxations of PE-contracted endothelium-intact rings in a concentration-dependent manner with pEC_{50} values of 8.21 ± 0.05 (n=26) and 6.75 ± 0.06 (n=30) and maximal responses of 96 ± 1% and 95 ± 1%, respectively. Figure 1 shows that mechanical endothelial cell removal caused significant rightward shifts in the curves to BAY 41-2272 (7.43 ± 0.04; n=21) and YC-1 (5.99 ± 0.05; n=19) of approximately 6-fold along with a 30% reduction in maximal responses. In both circumstances, BAY 41-2272 was approximately 30 times more potent than YC-1 to cause vasorelaxation ($p<0.01$).

Effects of ODQ, L-NAME, carboxy-PTIO and sildenafil. In PE-contracted arteries, acetylcholine (ACh, 0.001-10 μM) induced reproducible relaxations, which were abolished by endothelium denudation (results not shown) and significantly reduced by the sGC inhibitor ODQ (10 μM), the NO synthesis inhibitor L-NAME (100 μM) and the NO scavenger carboxy-PTIO (100 μM).

In endothelium-intact mesenteric artery, addition of ODQ caused marked rightward shifts in the concentration-response curves to BAY 41-2272 (10-fold; n=7) and YC-1 (9-

fold; n=6) along with significant reductions of 27 ± 3% and 22 ± 2% in their maximal responses, respectively (Table 1, Figure 2). Furthermore, the inhibitory effect of ODQ was less pronounced in denuded rings, as evidenced by an approximate 3-fold shift to the right on the curves to both BAY 41-2272 and YC-1. However, maximal responses to BAY 41-2272 (42 ± 4% inhibition) were more sensitive to ODQ than those to YC-1 (11 ± 3% inhibition) in denuded vessels ($p<0.01$; Figure 2). In the concentration used in this study, ODQ virtually abolished relaxations induced by the NO donor sodium nitroprusside (SNP, 0.0001-1 μM; data not shown).

Table 1 shows that the relaxant responses mediated by BAY 41-2272 and YC-1 were significantly inhibited in intact rings treated with L-NAME (4- and 3-fold rightward shifts respectively; n=6), without any appreciable effects on maximal responses. Interestingly, although carboxy-PTIO caused similar decreases in the sensitivity to BAY 41-2272 and YC-1, significant inhibition of maximal responses was observed (30 ± 3% and 25 ± 2%, respectively; n=5, Table 1). Addition of the selective phosphodiesterase type-5 (PDE5) inhibitor sildenafil (0.1 μM) to endothelium-intact rings significantly potentiated ($p<0.05$) the vasorelaxations induced by either BAY 41-2272 (n=6) or YC-1 (n=5). Sildenafil also enhanced the relaxant responses in denuded preparations evoked by YC-1 (~2-fold) and BAY 41-2272 (~3-fold). Similar results were obtained with SNP (8.57 ± 0.04 in the absence and 8.95 ± 0.06 in the presence of sildenafil; $p<0.05$, n=6).

Lack of effect of rolipram and DDA. In an attempt to investigate the involvement of the cAMP cascade in the vasorelaxations induced by these agents, mesenteric artery rings were treated with the adenylyl cyclase inhibitor 2',5'-dideoxyadenosine (DDA, 100 μM; n=4) or

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the PDE4 inhibitor rolipram (10 μM; n=4). At the concentrations employed, DDA significantly reduced relaxations elicited by the adenylyl cyclase activator forskolin (0.0001-1 μM; 8.40 ± 0.03 in the absence and 7.78 ± 0.06 in the presence of DDA; $p<0.01$, n=4), whereas rolipram enhanced these responses (8.28 ± 0.04 in the absence and 8.63 ± 0.05 in the presence of rolipram; $p<0.05$, n=4). Nevertheless, the vasorelaxations induced by BAY 41-2272 or YC-1 were not significantly affected following treatment with either DDA or rolipram (Table 2).

Lack of effect of Akt and PKG inhibitors. Pre-incubation of intact preparations with 1L-6-hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (Akt inhibitor, 10 μM; n=4) caused significant rightward shifts in the curves to ACh (7.69 ± 0.09 in the absence and 7.19 ± 0.05 in the presence of Akt inhibitor; $p<0.05$), but failed to antagonize those elicited by BAY 41-2272 or YC-1 (Table 3). Similarly, the cGMP-dependent protein kinase (PKG) inhibitors KT5823 (1 μM), guanosine 3′,5′-cyclic monophosphorothioate, 8-(4-chlorophenylthio)-Rp-Isomer (Rp-8-*p*CPT-cGMPS; 30 μM) and DT-3 (1 μM) had no significant effect on BAY 41-2272- and YC-1-induced relaxations, either in intact or denuded rings (n=4, each; Table 3). Relaxations to SNP were also unchanged by the PKG inhibitors assayed (n=4).

Effects of selective K^+ channel blockers. The relaxant responses of endothelium-denuded arteries to BAY 41-2272 or YC-1 remained unchanged following treatment with selective K^+ channel blockers (n=4-6; not shown), such as the K_{ATP} blocker glybenclamide (10 μM), the SK_{Ca} blocker apamin (1 μM), the BK_{Ca} blockers charybdotoxin and iberiotoxin (0.1

μM) as well as the non-selective blocker tetraethylammonium (1 mM). To ensure that appropriate concentrations were used in this study, relaxations to ACh (10 μM) were obtained in PE-contracted intact vessels treated with a combination of L-NAME (100 μM) plus indomethacin (10 μM), to exclude the participation of NO and prostanoids, respectively. Under these conditions, ACh evoked a sustained relaxation that was promptly reversed by apamin, charybdotoxin, iberiotoxin, TEA, but not glybenclamide (n=6, each). On the other hand, relaxations elicited by the K_{ATP} opener cromakalim (0.01-10 μM; n=4) were virtually abolished by glybenclamide.

Effects of ouabain, cyclopiazonic acid and calyculin A. In order to investigate the mechanisms underlying the ODQ-resistant component of the vasorelaxation evoked by BAY 41-2272 and YC-1 in denuded arteries, we examined the effects of the Na^+-K^+-ATPase inhibitor ouabain (10 μM), the sarco(endo)plasmic reticulum ATPase (SERCA) inhibitor cyclopiazonic acid (CPA, 10 μM) and the phosphatase inhibitor calyculin A (50 nM) on these responses, alone or in combination with ODQ (n=5-6, each). Neither ouabain nor CPA had any significant effect on the relaxations mediated by BAY 41-2272 or YC-1, and further addition of ODQ caused rightward shifts similar to those obtained when this inhibitor was tested alone (~3-fold). Nevertheless, calyculin A caused smaller, but significant ($p<0.05$) 2.5-fold displacement of the curves to BAY 41-2272 (7.44 ± 0.04 in the absence and 7.09 ± 0.06 in the presence of calyculin A) or YC-1 (6.02 ± 0.05 in the absence and 5.60 ± 0.04 in the presence of calyculin A) to the right. Moreover, the co-incubation of calyculin A plus ODQ shifted the curves to these agents by approximately 5-fold to the right (Figure 3; $p<0.01$).

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Inhibitory effects of BAY 41-2272 and YC-1 on $CaCl_2$-induced contractions. Cumulative addition of $CaCl_2$ (0.01-10 mM) in the presence of high K^+-depolarized endothelium-denuded rings was used to evaluate contractile responses dependent on Ca^{2+} influx. Pretreatment with the L-type Ca^{2+} channel blocker nifedipine (1 μM; n=4) markedly depressed maximal contractions to $CaCl_2$ (65 ± 6% reduction; $p<0.01$) along with a significant rightward shift in the curves (2.94 ± 0.03 in the absence and 2.43 ± 0.01 in the presence of nifedipine; $p<0.01$). In presence of ODQ, neither BAY 41-2272 at 0.1 μM nor YC-1 at 1 μM was able to change the $CaCl_2$-induced contractions. However, higher concentrations of BAY 41-2272 (1 μM; n=4) and YC-1 (10 μM; n=4) considerably depressed maximal contractions to $CaCl_2$ (38 ± 3% and 52 ± 6%, respectively; $p<0.01$) and caused an approximate 4-fold shift in the curves to the right ($p<0.01$), as shown in Figure 4.

Contractile responses to PE in BAY 41-2272-, YC-1-, SNP- and ANP-treated rings. Cumulative addition of PE (0.001-10 μM) to the bathing medium caused concentration-dependent contractions in denuded preparations with pEC_{50} and maximum response values averaging 7.50 ± 0.08 and 120 ± 3%, respectively. With the intention of investigating the nature of the interaction between BAY 41-2272 or YC-1 with SNP or ANP, curves for PE were constructed in the absence and in the presence of different concentrations of the aforementioned compounds, alone or in combination (Figure 5). When applied alone (n=5, each), BAY 41-2272 (0.0001-0.1 μM), YC-1 (0.001-1 μM), SNP (0.0001-0.1 μM) and ANP (0.0001-0.1 μM) caused significant rightward shifts in the curves for PE. Thereafter, concentrations that did not cause significant shifts were selected. Incubation of BAY 41-2272 (0.0001 μM) or YC-1 (0.01 μM) with SNP (0.0001 μM) resulted in marked rightward

shifts or approximately 14- (BAY 41-2272 plus SNP; n=4) and 8-fold (YC-1 plus SNP; n=4). However, incubation of ANP (0.001 μM) with BAY 41-2272 or YC-1 caused much smaller shifts (~2.5-fold; n=4, each).

Determination of cyclic nucleotide levels. The basal cGMP content averaged 0.23 ± 0.06 and 0.09 ± 0.01 pmol/mg in endothelium-intact and denuded mesenteric artery rings, respectively (n=4). In rings treated with BAY 41-2272 (0.1 μM) or YC-1 (1 μM), the cGMP levels were significantly increased above control values in both intact (34.7- and 26.5-fold, respectively) and denuded (16.9- and 13.2-fold, respectively) preparations (n=4). Treatment with ODQ (10 μM) reduced BAY 41-2272- and YC-1-evoked increases in cGMP levels by approximately 90% (Figure 6). Increases in cGMP concentration induced by SNP (0.1 μM) were significantly potentiated by BAY 41-2272 (5.5-fold) and YC-1 (4.2-fold) in denuded arteries. Forskolin (0.1 μM) significantly increased cAMP, but not cGMP levels ($p<0.01$). Neither BAY 41-2272 nor YC-1 affected baseline cAMP readings in rat mesenteric artery (Table 4).

Discussion

The present study describes a comparative investigation of the pharmacological profiles of two NO-independent sGC stimulators in rat mesenteric artery, BAY 41-2272 and YC-1. Most notably, the results obtained suggest that both drugs signal through cGMP-dependent and independent mechanisms to cause vascular smooth muscle relaxation, confirming the original hypothesis. The main cGMP-independent signaling pathway includes inhibition of Ca^{2+} influx and activation of protein phosphatases.

BAY 41-2272 (Stasch et al., 2001; Straub et al., 2001) represents a promising compound obtained from a new series of potent pyrazolopyridine derivatives synthesized using the YC-1 chemical lead structure (Ko et al., 1994; Wu et al., 1995). Consistent with their ability to directly activate sGC, BAY 41-2272 and YC-1 concentration-dependently relax both endothelium-intact and denuded rings of rat mesenteric artery, along with significant increases in vascular cGMP levels. Our results show that BAY 41-2272 is approximately 30-fold more potent than YC-1 to induce vasorelaxation, an observation that clearly differentiates this compound from YC-1. Straub et al. (2001) demonstrated the effects of variations on the YC-1 structure in the chemical processes leading to BAY 41-2272 synthesis. While variation of the *N*-substituent on the YC-1 structure had little effect on relaxation of aortic rings, the variation of the pyrazole type core heterocycle as well as the attached heterocycle clearly enhanced the relaxant effects. Moreover, the introduction of a cyclopropyl group as a pyrimidine substituent, resulted in a more potent compound (Straub et al., 2001). The BAY 41-2272 structure comprises these variations, which most certainly reflect its higher potency over YC-1 in the rat mesenteric artery.

The relaxant activity of either compound comprises an endothelium-dependent component, since a significant decrease in potency was noted in rubbed rings. In addition, L-NAME significantly inhibited the vasorelaxations elicited by BAY 41-2272 and YC-1, suggesting that endogenous NO is required for their effects, consistent with previous studies wherein L-NAME-sensitive relaxations to BAY 41-2272 have been demonstrated in rabbit aorta (Priviero et al., 2005) and corpus cavernosum (Baracat et al., 2003). NO scavenging with carboxy-PTIO caused similar shifts in the curves to BAY 41-2272 and YC-1, further confirming the involvement of endogenously released NO in their responses. It is unclear from the present results whether these drugs actually release NO or synergize with endothelium-derived NO to evoke their relaxant responses. Interestingly, YC-1 has been shown to stimulate NO production through activation of eNOS in bovine endothelial cells (Wohlfart et al., 1999). Nevertheless, the mechanisms accounting for BAY 41-2272 and YC-1 dependence on endogenous NO warrant further investigation. Phosphorylation of eNOS by Akt increases the activity of eNOS in a Ca^{2+}-independent manner through enhancing its sensitivity to Ca^{2+}-calmodulin (Dimmeler et al., 1999). Apparently, the relaxant responses to BAY 41-2272 or YC-1 do not seem to involve Akt-mediated eNOS phosphorylation, since the Akt inhibitor used did not affect their relaxations.

Studies with purified sGC revealed that ODQ binds in an NO-competitive manner, oxidizes the heme iron and leads to the inhibition of the enzyme (Garthwaite et al., 1995; Schrammel et al., 1996). More recently, it was demonstrated that BAY 41-2272 (Stasch et al., 2001) and YC-1 (Koglin and Behrends, 2003) bind to the N-terminus of the α1-subunit of sGC to stimulate the enzyme. Treatment with ODQ caused marked shifts in the curves to BAY 41-2272 and YC-1 in endothelium-intact or denuded rings, consistent with the fact

that these drugs stimulate sGC in a heme-dependent manner. Furthermore, the findings that ODQ virtually abolished cGMP increases elicited by BAY 41-2272 and YC-1 are in agreement with previous work wherein ODQ blocked BAY 41-2272-induced stimulation of recombinant sGC (Stasch et al., 2001). An interesting finding was the evident discrepancy between the inhibitory effects of ODQ on relaxant responses and cGMP levels. Priviero et al. (2005) demonstrated that while ODQ seemingly blocked BAY 41-2272-evoked cGMP increases in endothelium-denuded rabbit aorta, the relaxant curves were only shifted to the right. In this study, similar results were obtained, since ODQ inhibited BAY 41-2272- and YC-1-induced cGMP increases by approximately 90% whereas their relaxant responses were only partially attenuated by ODQ, suggesting that in addition to stimulation of sGC, BAY 41-2272 and YC-1 relax the mesenteric artery through additional mechanisms. This is in agreement with reports showing ODQ-insensitive relaxations to BAY 41-2272 like rabbit aorta (Priviero et al., 2005), ovine pulmonary artery (Bawankule et al., 2005) as well as human and rabbit corpus cavernosum (Baracat et al., 2003).

YC-1 stimulates increases in cGMP concentration through both the stimulation of sGC as well as the inhibition of PDE5 in human platelets and aortic extracts (Friebe et al., 1998; Galle et al., 1999). In contrast, Stasch et al. (2001) reported that BAY 41-2272 is devoid of any PDE inhibitory activity, whereas a more recent study demonstrated that this compound inhibits PDE5 in platelets (Mullershausen et al., 2004). It is apparent from our results that BAY 41-2272 shares similar pharmacological properties as YC-1, although the cGMP-specific PDE5 inhibitor sildenafil caused a greater enhancement of BAY 41-2272 relaxant responses in denuded arteries. This observation suggests that YC-1 effects might include a PDE5 inhibitory component in mesenteric arteries as opposed to BAY 41-2272. In addition, the finding that ODQ reduces BAY 41-2272 maximum response in denuded

rings as opposed to YC-1 further supports the above observation. The ability of these compounds to stimulate sGC directly results in increased production of cGMP, which in turn exert a variety of effects via PKG (Friebe and Koesling, 2003), leading to vascular relaxation. Surprisingly, PKG inhibitors were unable to affect the relaxant activity evoked by BAY 41-2272 or YC-1. However, a role for PKG cannot be excluded based on these results, since the concentrations of KT5823 (1 μM), Rp-8-*p*CPT-cGMPS (30 μM) and DT-3 (1 μM) used in this study were even higher than their corresponding IC_{50} values for the native enzyme (234 nM, 0.5 μM and 25 nM, respectively) as reported previously (Grider, 1993; Butt et al., 1994; Dostmann et al., 2000). As a matter of fact, KT5823 does not affect BAY 41-2272-induced relaxations of ovine pulmonary artery (Bawankule et al., 2005) and has also been shown to inhibit PKG activity *in vitro* but not in intact human platelets or rat mesangial cells (Burkhardt et al., 2000), suggesting that interpretation of PKG inhibitor effects in intact cells or tissues require additional considerations, rather than assuming that PKG is or is not involved based solely on tools like PKG inhibitors.

Cyclic GMP mediates vascular smooth muscle relaxation through different mechanisms, including K^+ channel opening, presumably involving BK_{Ca} and K_{ATP} (Archer et al., 1994; Murphy and Brayden, 1995). Neither BAY 41-2272 nor YC-1 relaxed the mesenteric artery through mechanisms dependent on K^+ channel opening, since blockers of the different K^+ channel subtypes (SK_{Ca}, BK_{Ca} and K_{ATP}) failed to affect their vasorelaxant responses. We also investigated the role of cAMP in the relaxations induced by BAY 41-2272 and YC-1, since recent studies have shown that both drugs increase cAMP levels in leukocytes (Thomazzi et al., 2005; Hwang et al., 2003). Neither the adenylyl cyclase inhibitor DDA nor the PDE4 inhibitor rolipram affected the relaxations induced by BAY

41-2272 and YC-1, excluding the involvement of cAMP in their responses. This is further reinforced by the findings that only forskolin, but not BAY 41-2272 or YC-1, increased cAMP levels in the mesenteric artery. Accordingly, rolipram also failed to influence BAY 41-2272-induced relaxations in the corpus cavernosum (Baracat et al., 2003).

It is very well established that cGMP mediates vascular smooth muscle relaxation by lowering intracellular Ca^{2+} levels through increasing Ca^{2+} efflux, promoting Ca^{2+} sequestration in the sarcoplasmic reticulum and inhibiting Ca^{2+} influx (Lucas et al., 2000). It is unlikely that the ODQ-resistant component of the relaxant response induced by BAY 41-2272 and YC-1 involves stimulation of either the plasma membrane Na^{+}-K^{+}-ATPase or SERCA, since ouabain and CPA had no effect in the relaxations elicited by these drugs. On the other hand, the ODQ-resistant relaxations evoked by BAY 41-2272 and YC-1 seem to involve the inhibition of Ca^{2+} entry. This is evident from BAY 41-2272- and YC-1-induced inhibition of Ca^{2+} contraction in K^{+}-depolarized preparations. Accordingly, BAY 41-2272 and YC-1 have been previously shown to inhibit Ca^{2+} entry in a cGMP-independent manner (Wang et al., 2001; Bawankule et al., 2005). Our findings also suggest that BAY 41-2272 and YC-1 induce relaxations through stimulation of protein phosphatases in the vascular smooth muscle, since the phosphatase inhibitor calyculin A partially antagonized their relaxant effect.

Similar to the findings obtained with the purified sGC (Mülsch et al., 1997; Stasch et al., 2001), a synergism between BAY 41-2272 and SNP as well as YC-1 and SNP was observed with regard to cGMP increases in mesenteric artery rings. This effect accounted for their inhibitory responses on the contractions mediated by phenylephrine in denuded vessels. In contrast, a combination of BAY 41-2272 or YC-1 with ANP resulted only in an additive inhibition, thus revealing a specific and strong synergistic effect between direct

NO application and NO-independent sGC stimulation. These findings indicate the possibility to lower the dose of nitrovasodilators to achieve a hypotensive response in patients, thus reducing the risks of adverse effects stemming from NO-based therapies. Indeed, the potential therapeutic benefits of YC-1 have been demonstrated in hypertensive animals *in vivo* (Rhotermund et al., 2000).

In conclusion, this study demonstrates that in addition to the stimulation of sGC, inhibition of Ca^{2+} entry and stimulation of protein phosphatases by BAY 41-2272 and YC-1 also represent important mechanisms in the relaxation of rat mesenteric artery in response to non-NO-based sGC activators. Although further studies are required to elucidate the mechanism underlying stimulation of protein phosphatases by BAY 41-2272 and YC-1, the pathways leading to arterial smooth muscle relaxation presented herein suggest that this class of compounds is of particular clinical interest in the management of cardiovascular disorders, principally in conditions where basal NO production is prejudiced.

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Footnotes

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Figure Legends

Figure 1. Concentration-response curves to the sGC stimulators BAY 41-2272 (0.0001-1 μM; n=21-26; circles) and YC-1 (0.001-30 μM; n=19-30; squares) in endothelium-intact (E+, filled symbols) and denuded (E-, open symbols) rat mesenteric artery rings contracted by phenylephrine (PE, 1 μM). Experimental values were calculated relative to the maximal changes from the contraction produced by PE in each tissue, which was taken as 100%. Data represent the mean ± S.E.M. of *n* experiments. *Inset:* Responses induced by acetylcholine (ACh, 1 μM) in E+ (open bars) and E- rings (closed bars).

Figure 2. Effects of the sGC inhibitor 1H-[1,2,4] oxadiazolo [4,3,-a]quinoxalin-1-one (ODQ, 10 μM) on the relaxations induced by BAY 41-2272 (0.0001-1 μM; top panels; n=7) and YC-1 (0.001-30 μM; bottom panels; n=6) in endothelium-intact (E+, left panels) and denuded (E-, right panels) mesenteric artery rings contracted by phenylephrine (PE, 1 μM). Experimental values were obtained in absence (control, CTL; filled symbols) and presence (open symbols) of ODQ. Data were calculated relative to the maximal changes from the contraction produced by PE in each ring, which was taken as 100%. Data represent the mean ± S.E.M. of *n* experiments.

Figure 3. Concentration-response curves to the sGC stimulators BAY 41-2272 (0.0001-1 μM; top panel; n=6) and YC-1 (0.001-30 μM; bottom panel; n=5) in endothelium-denuded rat mesenteric artery rings contracted by phenylephrine (PE, 1 μM) in the absence (control, CTL; filled circles) and in the presence of calyculin A (50 nM; open circles) or calyculin A

plus ODQ (10 μM; filled squares). Experimental values were calculated relative to the maximal changes from the contraction produced by PE in each tissue, which was taken as 100%. Data represent the mean ± S.E.M. of *n* experiments.

Figure 4. Effects of the sGC stimulators BAY 41-2272 (0.1-1 μM; top panel; n=4) and YC-1 (1-10 μM; bottom panel; n=4) on the contractions of endothelium-denuded rat mesenteric artery rings induced by $CaCl_2$ (0.01-10 mM) in K^+-depolarizing solution containing ODQ (10 μM). Experimental values were obtained in absence (filled circles) and presence of 0.1 μM or 1 μM (open circles) as well as 1 μM and 10 μM (filled squares) of BAY 41-2272 or YC-1, respectively. 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 represent the mean ± S.E.M. of *n* experiments.

Figure 5. Rightward shifts of the curves elicited by phenylephrine (PE, 0.001-10 μM) and carbachol (CCh, 0.01-100 μM) in the presence of increasing concentrations of sodium nitroprusside (SNP, 0.0001-0.1 μM), BAY 41-2272 (0.0001-0.1 μM), YC-1 (0.001-1 μM) and atrial natriuretic peptide (ANP, 0.0001-0.1 μM) in endothelium-denuded rat mesenteric artery preparations (panel a; n=5). Rightward shifts of the curves elicited by PE in presence of SNP (0.0001 μM), ANP (0.001 μM), BAY 41-2272 (0.0001 μM), YC-1 (0.01 μM) alone (open/closed bars) or in combination (hatched bars; panel b; n=4). Data represent the mean ± S.E.M. of *n* experiments.

Figure 6. Effects of BAY 41-2272 (0.1 μM) and YC-1 (1 μM) in endothelium intact (traces a-e; +) and denuded (traces f-j; -) mesenteric artery rings contracted with phenylephrine (PE, 1 μM; upward arrow) in the absence and presence of ODQ (10 μM). In these experiments, ODQ was added to the bathing medium 20 min before addition of PE. Representative tracings from these experiments as well as calculated percent relaxation values and corresponding cGMP levels (pmol/mg) are shown. The values represent mean ± S.E.M. of *n* experiments. Numbers in parenthesis represent percent of inhibition caused by ODQ. All comparisons were made by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. $^{*}p<0.05$ and $^{**}p<0.01$ compared to BAY 41-2272 and YC-1 responses in the absence of ODQ; $^{\dagger}p<0.01$ compared to basal cGMP levels; $^{\#}p<0.01$ compared to BAY 41-2272 and YC-1 cGMP levels in the absence of ODQ.

Table 1. Potency (pEC_{50}) and maximum response (E_{max}) values derived from concentration-response curves to the sGC stimulators BAY 41-2272 (0.0001-1 μM) and YC-1 (0.001-30 μM) in endothelium-intact mesenteric artery rings contracted with phenylephrine (1 μM). Curves were performed in the absence and in the presence of the sGC inhibitor ODQ (10 μM; n=6-7), NO synthesis inhibitor L-NAME (100 μM; n=6) or NO scavenger carboxy-PTIO (PTIO, 100 μM; n=5). Data represent the mean ± S.E.M. of *n* experiments.

	BAY 41-2272		**YC-1**	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}
control	8.24 ± 0.06	93 ± 3	6.76 ± 0.06	96 ± 2
+ *ODQ*	7.25 ± 0.08**	68 ± 4**	5.87 ± 0.08**	75 ± 2**
control	8.23 ± 0.07	93 ± 2	6.73 ± 0.07	96 ± 2
+ *L-NAME*	7.69 ± 0.05**	91 ± 2	6.26 ± 0.08**	91 ± 3
control	8.20 ± 0.04	91 ± 4	6.80 ± 0.06	94 ± 3
+ *PTIO*	7.80 ± 0.06**	65 ± 5**	6.23 ± 0.08**	70 ± 2**

**$p<0.01$ compared to the respective control values.

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Table 2. Potency (pEC_{50}) and maximum response (E_{max}) values derived from concentration-response curves to the sGC stimulators BAY 41-2272 (0.0001-1 μM) and YC-1 (0.001-30 μM) in endothelium-intact mesenteric artery rings contracted with phenylephrine (1 μM). Curves were performed in the absence and in the presence of the PDE5 inhibitor sildenafil (0.1 μM; n=5-6), adenylyl cyclase inhibitor 2',5'-dideoxyadenosine (DDA, 100 μM; n=4) or PDE4 inhibitor rolipram (10 μM; n=4). Data represent the mean ± S.E.M. of *n* experiments.

	BAY 41-2272		**YC-1**	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}
control	8.23 ± 0.02	91 ± 1	6.77 ± 0.02	96 ± 1
+ *sildenafil*	8.53 ± 0.05*	94 ± 2	7.20 ± 0.06*	97 ± 1
control	8.13 ± 0.05	98 ± 6	6.86 ± 0.09	99 ± 2
+ *DDA*	8.18 ± 0.04	97 ± 5	6.73 ± 0.09	100 ± 3
control	8.27 ± 0.06	94 ± 5	6.78 ± 0.07	99 ± 1
+ *rolipram*	8.16 ± 0.06	96 ± 5	6.65 ± 0.07	100 ± 2

*$p<0.05$ compared to the respective control values.

Table 3. Potency (pEC_{50}) and maximum response (E_{max}) values derived from concentration-response curves to the sGC stimulators BAY 41-2272 (0.0001-1 μM) and YC-1 (0.001-30 μM) in endothelium-intact mesenteric artery rings contracted with phenylephrine (1 μM). Curves were performed in the absence and in the presence of the Akt inhibitor 1L-6-hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (10 μM; n=4) or the PKG inhibitors KT5823 (1 μM; n=4), guanosine 3′,5′-cyclic monophosphorothioate, 8-(4-chlorophenylthio)-Rp-Isomer (Rp-8-*p*CPT-cGMPS; 30 μM; n=4) and DT-3 (1 μM; n=4). Data represent the mean ± S.E.M. of *n* experiments.

	BAY 41-2272		**YC-1**	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}
control	8.20 ± 0.06	95 ± 3	6.70 ± 0.04	95 ± 3
+ *Akt inhibitor*	8.11 ± 0.05	93 ± 2	6.61 ± 0.05	91 ± 4
control	8.08 ± 0.04	91 ± 5	6.79 ± 0.06	93 ± 4
+ *KT 5823*	8.20 ± 0.04	91 ± 6	6.68 ± 0.06	97 ± 2
control	8.28 ± 0.05	91 ± 8	6.64 ± 0.08	97 ± 2
+ *Rp-8*-p*CPT*	8.14 ± 0.05	86 ± 7	6.58 ± 0.07	93 ± 3
control	8.20 ± 0.06	95 ± 3	6.73 ± 0.05	94 ± 3
+ *DT-3*	8.06 ± 0.07	92 ± 5	6.59 ± 0.05	91 ± 4

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Table 4. Rat mesenteric artery cyclic nucleotide levels (pmol/mg tissue) in response to BAY 41-2272 (0.1 μM), YC-1 (1 μM), SNP (0.1 μM), forskolin (0.1 μM) or their combination. Data represent the mean ± S.E.M. of *n* experiments.

	cGMP	**cAMP**
Baseline	0.088 ± 0.011	5.653 ± 0.856
BAY 41-2272	1.522 ± 0.121*	5.372 ± 0.932
YC-1	1.192 ± 0.214*	6.691 ± 0.741
SNP	0.929 ± 0.088*	4.071 ± 0.656
BAY 41-2272 + SNP	5.141 ± 0.244*†	n.d.
YC-1 + SNP	3.919 ± 0.325*†	n.d.
Forskolin	0.075 ± 0.033	19.240 ± 2.340*

*$p<0.01$ compared to the respective baseline values; †$p<0.01$ compared to the sum of BAY 41-2272/YC-1 and SNP alone.

n.d., not determined.

Figure 1

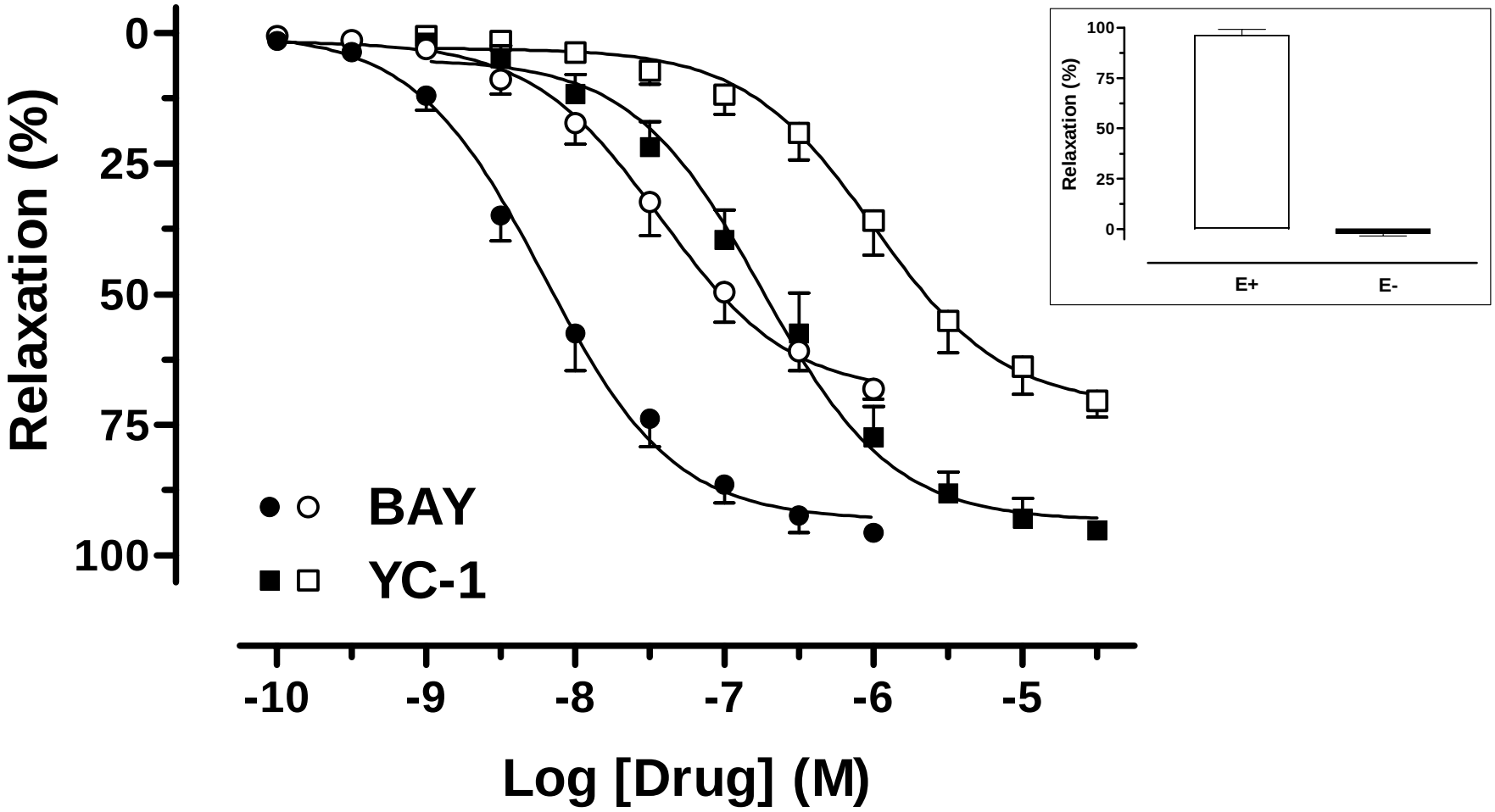

Figure 2

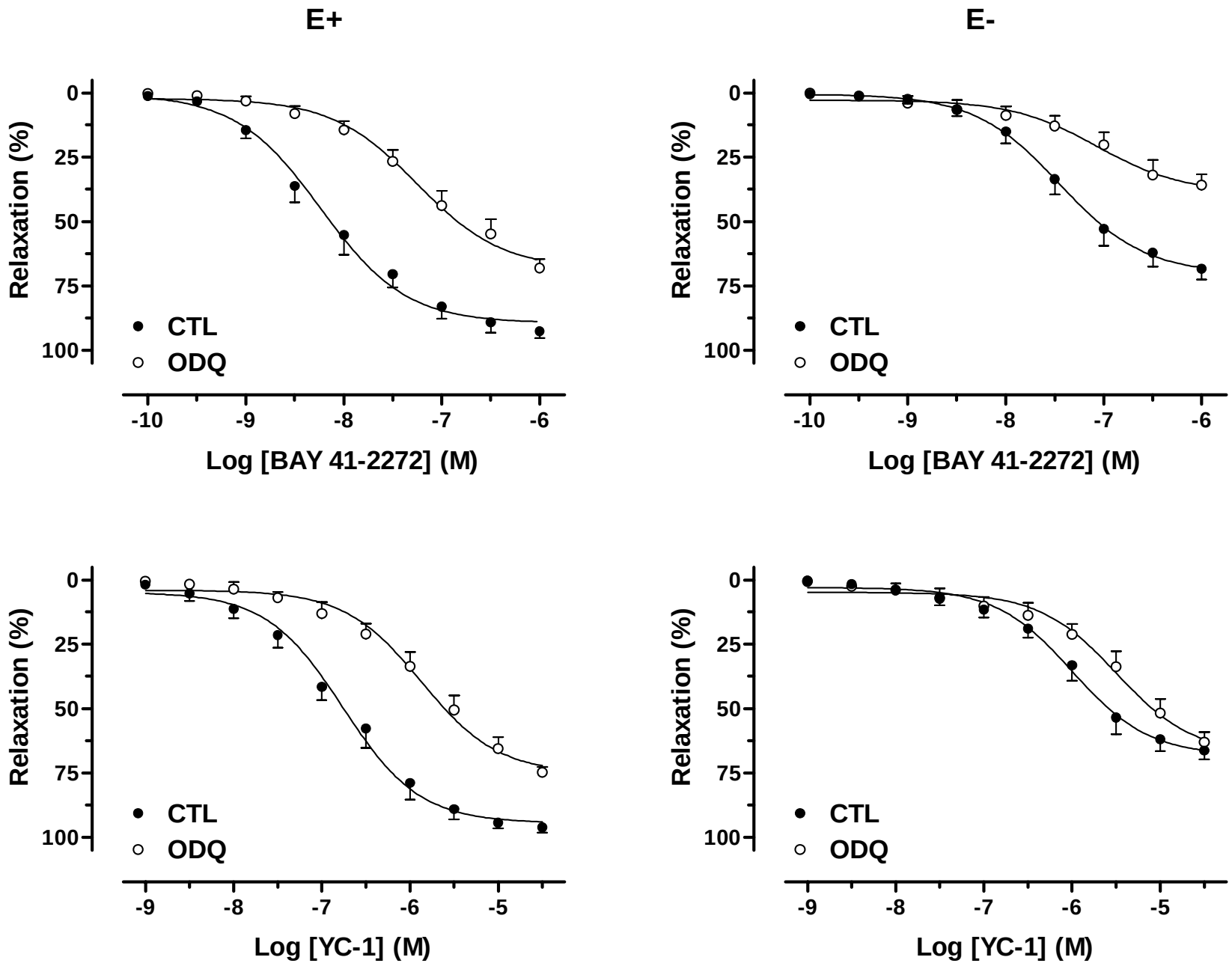

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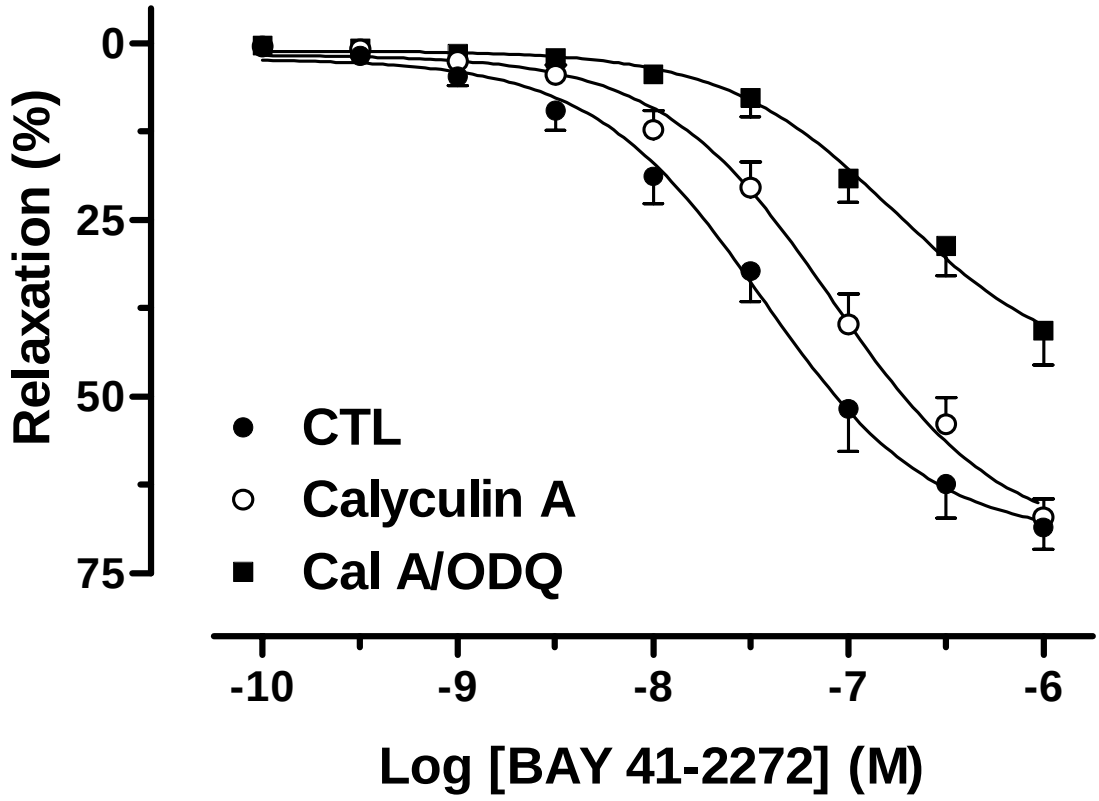
Figure 3



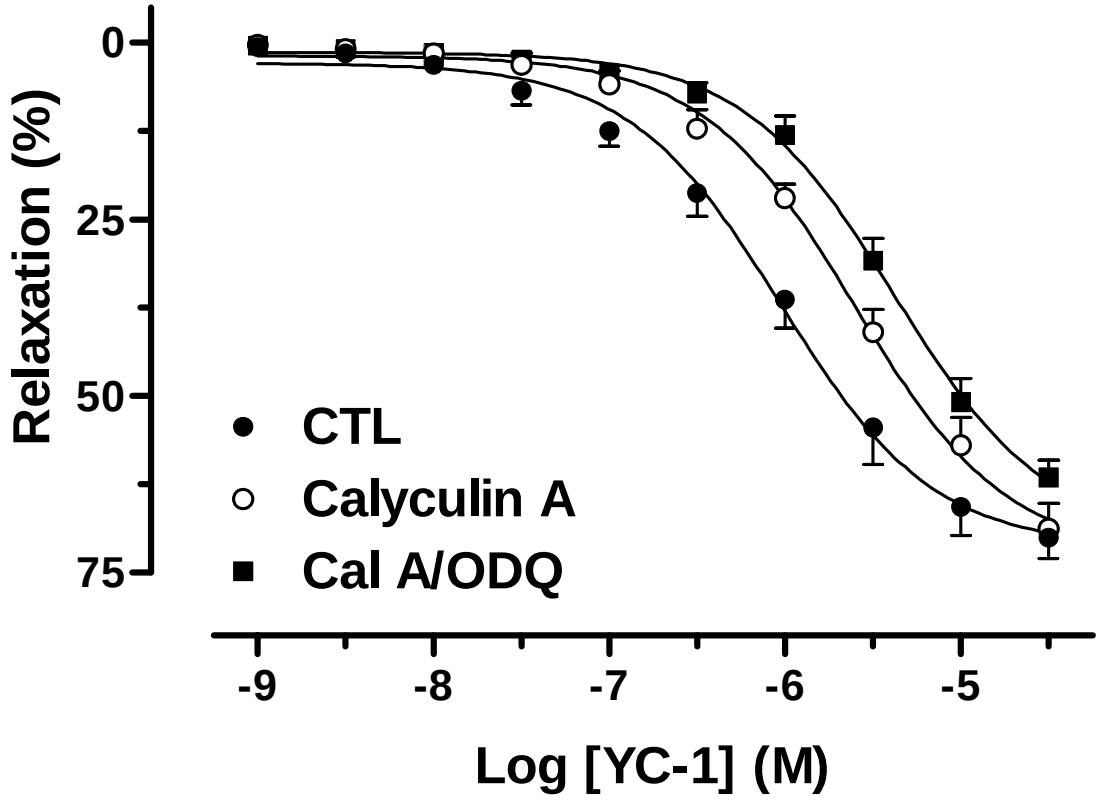


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Figure 4

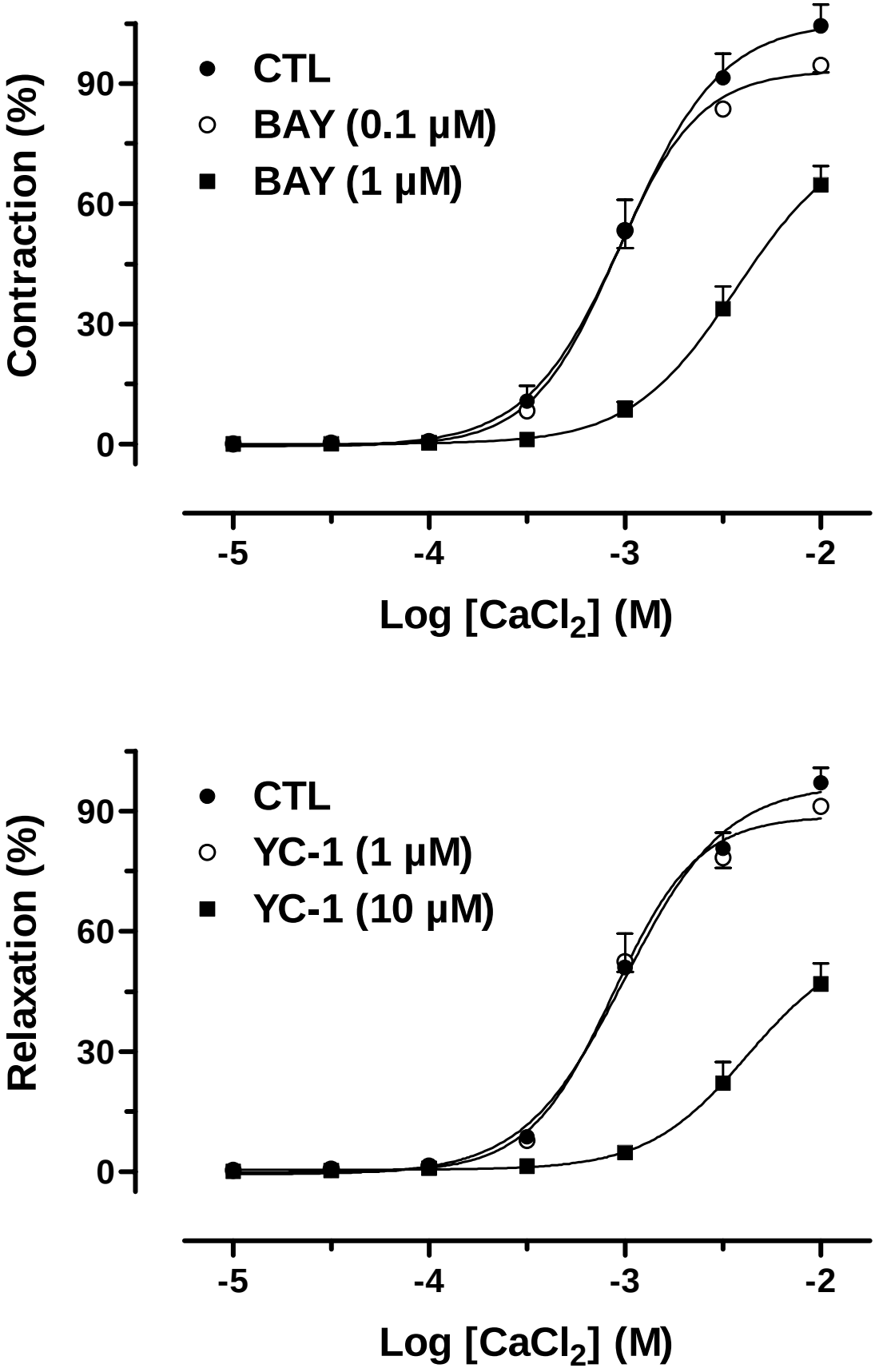

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Figure 5

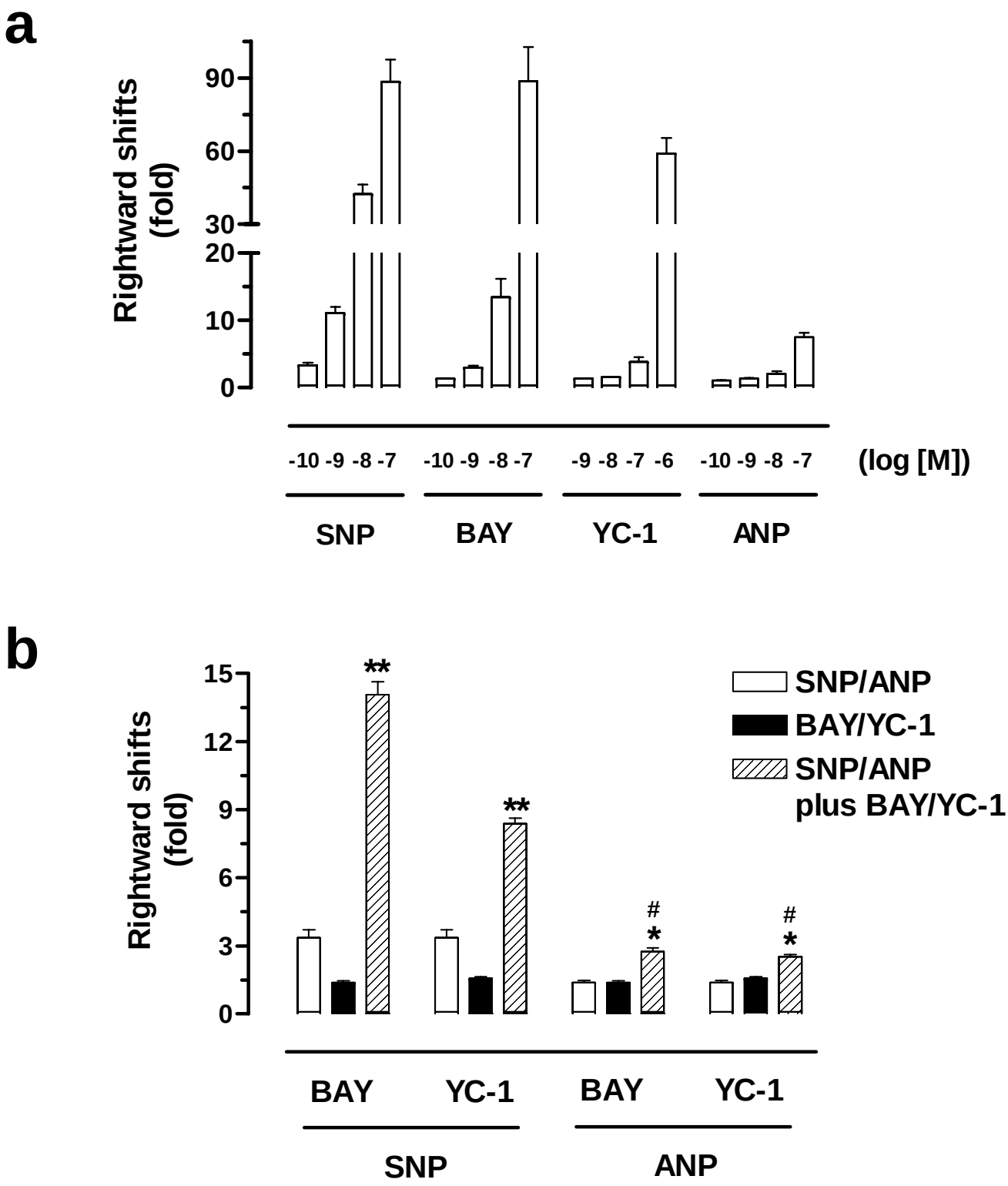

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Figure 6