BAY 41-2272 [5-Cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridine-3-yl]pyrimidin-4-ylamine]-Induced Dilation in Ovine Pulmonary Artery: Role of Sodium Pump


Division of Pharmacology and Toxicology, Indian Veterinary Research Institute, Uttar Pradesh, India

Received January 16, 2005; accepted March 23, 2005

ABSTRACT

The mechanisms of relaxation to nitric oxide (NO)-independent soluble guanylyl cyclase (sGC) activator BAY 41-2272 [5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridine-3-yl]pyrimidin-4-ylamine] were investigated in isolated ovine pulmonary artery. BAY 41-2272 (1 nM–10 μM) produced concentration-dependent relaxation of endothelium-denuded pulmonary artery rings (pD2 20.17 ± 3.52%; n = 6). KT-5823 [1-oxo-9.12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid methyl ester (2 μM), a specific inhibitor of protein kinase G had no effect on 10 μM ODQ-insensitive relaxation evoked by BAY 41-2272. BAY 41-2272 (10 μM) inhibited Ca2+-induced contractions in K+-depolarized preparations. BAY 41-2272 (10 μM) caused about a 14-fold increase in the intracellular cGMP over the basal level, which was completely inhibited by 10 μM ODQ. BAY 41-2272 (0.1, 1.0, and 10 μM) significantly (P < 0.05) increased ouabain-sensitive 86Rb uptake in a concentration-dependent manner. BAY 41-2272 (10 μM) also stimulated sarcolemmal Na+-K+-ATPase activity. However, 10 μM ODQ had no significant effect on either basal or BAY 41-2272-stimulated 86Rb uptake/Na+-K+-ATPase activities. In conclusion, this study provides the first evidence of sodium pump stimulation by BAY 41-2272 independent of cGMP as an additional mechanism to sGC activation in relaxation of ovine pulmonary artery.

Endogenous nitric oxide (NO), derived from vascular endothelium, is an important regulator of vascular functions. Thus, endothelial dysfunction is associated with several vascular disorders, such as atherosclerosis, systemic and pulmonary hypertension, and angina pectoris (Ignarro et al., 1999). However, there are certain disadvantages of NO donor-based therapy, which include development of tolerance after prolonged use (Parker, 1989), peroxinitrite formation that may lead to protein S-nitrosylation (Stamler, 1994), tyrosine nitration (Beckman et al., 1994), and the absence of clinically significant antiplatelet activity as with organic nitrates (Parker, 1989). Therefore, there has been search in the recent past for NO-independent sGC activators that could be used clinically for the treatment of cardiovascular disorders in place of NO donors (Hobbs, 2002).

BAY 41-2272, a pyrazolopyridine, is an NO-independent stimulator of sGC, which increases intracellular cGMP. This is considered to be the primary mechanism through which it produces antiplatelet activity, a strong decrease in blood pressure, and increase in survival, indicating its potential for...
the treatment of cardiovascular disorders (Stasch et al., 2001). Recently, several investigators have reported that BAY 41-2272 is a potent pulmonary vasodilator in different animal models of pulmonary hypertension (canine model, Boerigter et al., 2003; awake lambs, Evgenov et al., 2004; and ovine fetus, Deruelle et al., 2005). Since the mechanism of pulmonary artery dilation by BAY 41-2272 is unclear, we used isolated ovine pulmonary artery as a model to elucidate its mechanism of relaxation.

In vascular smooth muscles, Na\(^+\)-K\(^+\)-ATPase has an important role in the maintenance of electrochemical gradient of Na\(^+\) and K\(^+\) across the cell membrane and is therefore critical in the regulation of vascular tone. Sarcolemmal Na\(^+\)-K\(^+\)-ATPase has been implicated in both cGMP-dependent (rat aorta, Rapoport et al., 1985; canine pulmonary artery, Tamaoki et al., 1997) and -independent vasodilation (rabbit aorta, Rapoport et al., 1985; canine pulmonary artery, Boerrigter et al., 2003; awake lambs, Evgenov et al., 2004; and young newborn lambs, Evgenov et al., 2004). Recently, several investigators have reported that Na\(^+\)-K\(^+\)-ATPase has an implication in both cGMP-dependent and K\(^+\)-ATPase has an implication in mediating the response of BAY 41-2272, arterial strips were exposed to 10 M ODQ for 30 min before exposure to BAY 41-2272. After exposure to 86Rb, the tissues were then washed in ice-cold (4°C) unlabeled PSS for 2 min to remove radioisotope from the extracellular compartments, blotted on the filter paper, and dried overnight in an oven maintained at 100°C. 86Rb content of the tissue was determined by gamma counting. Ouabain-sensitive 86Rb uptake, which is known to be an index of Na\(^+\)-K\(^+\)-ATPase activity, was calculated by subtracting 86Rb uptake in the presence of maximally effective concentration of 0.2 mM ouabain from total 86Rb uptake.

**Estimation of Na\(^+\)-K\(^+\)-ATPase Activity.** Isolation of sarcolemmal membranes from pulmonary arteries was performed as per the procedure described by Matlib et al. (1985). Na\(^+\)-K\(^+\)-ATPase activity was determined by measuring the liberation of inorganic phosphate (Pi) from ATP in the medium containing 50 mM Tris-Cl buffer, pH 7.5, 140 mM NaCl, 14 mM KCl, 5 mM MgCl\(_2\), 6 mM L-0.5 mM EDTA, 1 mM ouabain, and 10 µl of requisite volume of membrane homogenate in a final volume of 1 ml. This reaction mixture was preincubated for 5 min at 37°C. The reaction was started by the addition of 3 mM ATP solution. For total ATPase assay, ouabain was omitted from the reaction mixture, which was included for Mg\(^2+\)-ATPase assay. After 1 h of incubation at 37°C in both the cases, the reaction was stopped by adding 0.1 ml of ice-cold 5% sodium dodecyl sulfate, and color was developed with 3 ml of acidic ammonium molybdate and 0.1 ml of ANSA reagent (25 mg of 1-amino-2 naphthol-4-sulfonic acid, 1.2 g of sodium metabisulfite, and 120 mg of sodium sulfite dissolved in 10 ml of distilled water). The Pi in the reaction mixture was assayed according to the method of Yohatalou (1975). A standard phosphate (10 µg/ml) and blank were run simultaneously. The absorbance of the enzyme activity in the absence and presence of 1 mM ouabain was taken as Na\(^+\)-K\(^+\)-ATPase activity. Protein content in the membrane fraction was determined by Lowry's method (Lowry et al., 1951). Specific enzyme activity is expressed as nanomoles of Pi liberated per minute per milligram of protein. To determine the effect of BAY 41-2272 on Na\(^+\)-K\(^+\)-ATPase activity, tissues were exposed to BAY 41-2272 for 30 min and then the Na\(^+\)-K\(^+\)-ATPase activity was determined as described above. Basal activity of the enzyme was measured after incubation of the tissues for 30 min in the solvent DMSO (0.01%).

**cGMP Measurement.** To determine the vascular cGMP content, pulmonary artery rings were equilibrated for 90 min in PSS at 37°C, continuously aerated with carbogen. Then, the tissues were exposed for 3 min (brief) or 30 min (prolonged) to either 10 M BAY 41-2272 or to the solvent DMSO (solvent control). To study the influence of sGC inhibition on BAY 41-2272-stimulated increase in cGMP, the rings were pretreated with 10 µM ODQ for 30 min before exposure to BAY 41-2272. After the drug treatment, the tissues were quickly frozen in liquid nitrogen and ground in 1.07 N perchloric acid. The suspension was then sonicated and centrifuged at 10,000 g for 1 min, and the supernatant was collected for the assay. The pellets were used for protein determination (Lowry et al., 1951). The amount of cGMP was assayed by radioimmunoassay with an \(^{125}\)I-cGMP RIA kit (Immunotech, Marseille, France), and cGMP levels were expressed as nanomoles per milligram of protein. Control levels of cGMP were

Ca\(^2+\)-Induced Contraction in 60 mM K\(^+\)-Depolarized Pulmonary Artery Rings. To study the effect of BAY 41-2272 on Ca\(^2+\)- influx/Ca\(^2+\)-sensitivity of the ovine pulmonary artery rings, concentration-dependent contractions to cumulatively added CaCl\(_2\) were elicited in nominally Ca\(^{2+}\)-free (PSS containing zero Ca\(^{2+}\)), K\(^+\) (60 mM)-depopolarized preparations in the absence and presence of the sGC activator.

**Measurement of Ouabain-Sensitive 86Rb Uptake.** 86Rb uptake by the pulmonary artery was determined as per the method described by Gupta et al. (1994). Arterial rings were equilibrated in PSS (37°C) for 2 h and aerated with carbogen. After the equilibration, the tissues were exposed to BAY 41-2272 (0.1, 1.0, or 10 M) for 30 min along with 2 µCi/ml 86RbCl. To examine the role of sGC activation in mediating the response of BAY 41-2272, arterial strips were exposed to 10 µM ODQ for 30 min before exposure to BAY 41-2272. After exposure to 86Rb, the tissues were then washed in ice-cold (4°C) unlabeled PSS for 2 min to remove radioisotope from the extracellular compartments, blotted on the filter paper, and dried overnight in an oven maintained at 100°C. 86Rb content of the tissue was determined by gamma counting. Ouabain-sensitive 86Rb uptake, which is known to be an index of Na\(^+\)-K\(^+\)-ATPase activity, was calculated by subtracting 86Rb uptake in the presence of maximally effective concentration of 0.2 mM ouabain from total 86Rb uptake.

**Materials and Methods**

**Blood Vessel Preparation and Tension Recording.** Lungs from adult sheep were collected from the local slaughter house within 20 to 30 min of slaughter in ice-cold aerated modified Krebs-Henseleit solution (PSS) of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 11.9 mM NaHCO\(_3\), 1.2 mM KH\(_2\)PO\(_4\), and 11.1 mM glucose. After careful exposure of the intrapulmonary vascular network, the fourth generation intrapulmonary arteries were dissected out and placed in aerated PSS. Arteries were cleared of fat and connective tissue and cut into 2- to 3-mm ring segments. Vascular endothelium was routinely removed mechanically. Some endothelium intact preparations were also used to elucidate the influence of tonic nitric oxide release on the vasodilator responses of BAY 41-2272. The external diameter of the arterial rings was 0.6 to 1.0 mm. The rings were mounted between two stainless steel “L-shaped” hooks under a resting tension of 1 g in a 20-ml organ bath containing PSS at 37°C and continuously aerated with carbogen (95% O\(_2\) and 5% CO\(_2\)). The arterial rings were equilibrated for 60 min before recording the muscle tension. A high-sensitivity isometric force transducer (model ML T0202/D; PowerLab, Castle Hill, NSW, Australia) measured the change in tension, and the data were recorded in a PC using Chart version 4.1.2 software program (PowerLab).

The vessels were preconstricted submaximally with 1 µM 5-HT and when the contraction reached equilibrium, 100 µM acetylcholine (ACh) was added. The lack of relaxant response to ACh confirmed the absence of a functional endothelium. The preparations were then washed with PSS to restore baseline tension. The tissues were then contracted submaximally with 1 µM 5-HT and when the contraction was stable, BAY 41-2272 was added cumulatively, until maximal reversal of 5-HT-induced contraction was obtained. To study the role of sGC in mediating relaxation by BAY 41-2272, arteries were treated with 10 M ODQ (an inhibitor of sGC) for 30 min before 5-HT contraction was elicited. Contribution of plasmalemmal Na\(^+\)-K\(^+\)-ATPase to BAY 41-2272-induced vasodilation was assessed in the presence of 100 µM ODQ. Experiments with KT compounds were conducted in dark.
Effect of Endothelium Denudation on Vasodilator Responses of BAY 41-2272. Cumulative addition of BAY 41-2272 (1 nM–10 μM) produced concentration-dependent relaxation of endothelium intact [endo (+)] and endothelium-denuded [endo (−)] pulmonary artery rings precontracted with 5-HT (1 μM; Fig. 1). However, BAY 41-2272 was significantly more effective in dilating endo (+) compared with endo (−) vascular rings. To further study the mechanism of BAY 41-2272-induced vasodilation without the interference of endothelium, we routinely used endo (−) rings.

Effect of BAY 41-2272 on 5-HT-Constricted Pulmonary Artery. 5-HT (1 nM–10 μM), added cumulatively at increments of 0.5 log unit, elicited concentration-dependent contraction in endothelium-denuded pulmonary artery rings (n = 4). A concentration of 1 μM 5-HT that produced approximately 90% of the maximal response induced a sustained contraction of 1.00 ± 0.12 g (n = 8). BAY 41-2272 (1 nM–10 μM), added cumulatively at increments of 1 log unit, relaxed the vascular rings in a concentration-dependent manner (pD2 = 6.82 ± 0.16; Emax = 92.30 ± 2.31%; n = 8; Fig. 2A). Because both the stock solution and the serial dilutions of BAY 41-2272 were made in DMSO, the per se effect of identical volume of DMSO was also studied on 5-HT contraction. The highest concentration of DMSO (0.46%) used as vehicle had a small relaxant effect on arterial rings (Emax = 15.83 ± 1.72%; n = 6). Pretreatment of the tissues with 10 μM ODQ for 30 min caused a small increase (0.02 g) in basal tension and the contraction produced by 1 μM 5-HT was 0.87 ± 0.11 g (n = 6), which was not statistically significant compared with the control. ODQ partially inhibited BAY 41-2272-evoked relaxation (Emax = 57.10 ± 3.10%; n = 6; Fig. 2A). Pretreatment of the tissues with 1 μM ouabain alone had a marked inhibitory effect on concentration-dependent vasodilation produced by BAY 41-2272 (Fig. 2B), thus decreasing the Emax to 20.17 ± 4.55% (n = 6). To examine whether Na\(^+\)-K\(^+\)ATPase and cGMP together contributed to the relaxant response of BAY 41-2272, concentration-responses to BAY 41-2272 (1 nM–10 μM) were elicited in the combined presence of 10 μM ODQ and 1 μM ouabain. Pretreatment of the tissues with this combination for 30 min caused a small increase in basal tension (0.06 g). In the presence of both 10 μM ODQ

![Fig. 2. A, effect of 10 μM ODQ on relaxation induced by BAY 41-2272 (1 nM–10 μM) in endothelium-denuded isolated ovine pulmonary arteries precontracted with 1 μM 5-HT. Pretreatment of the tissues for 30 min with 10 μM ODQ partially attenuated BAY 41-2272-evoked relaxations. B, effect of 1 μM ouabain and 1 μM ouabain plus 10 μM ODQ on concentration-dependent vasodilation induced by BAY 41-2272 (1 nM–10 μM). Data represent mean ± S.E.M. (n = 6–8; * P < 0.05). Student’s paired t test after two-way ANOVA was used for comparison of results.](image-url)
and 1 μM ouabain, the contraction caused by 5-HT was 1.39 ± 0.42 g (n = 6). BAY 41-2272-evoked relaxations were almost abolished (Emax = 12.1 ± 3.76%; n = 6) by this combined treatment (Fig. 2B). To further elucidate the role of Na⁺-K⁺-ATPase in BAY 41-2272-induced relaxation, vasodilator responses to BAY 41-2272 were elicited in K⁺-free PSS. In 5.9 mM K⁺ PSS, the absolute tension produced by 5-HT was 0.83 g (n = 6). BAY 41-2272 (1 nM–10 μM) produced concentration-dependent relaxation of the 5-HT-constricted arterial rings with pD2 and Emax values of 6.47 ± 0.44 and 90.66 ± 2.32%, respectively (n = 6). Equilibration of tissues with K⁺-free PSS for 30 min caused a small increase in basal tension (0.17 ± 0.08 g; n = 6) without having any significant effect on 5-HT-induced preconstriction (0.87 ± 0.09 g). However, there was a marked inhibition (Emax decreased to 39.97 ± 3.52%; n = 6) in the vasodilator response of BAY 41-2272 (Fig. 3A). Whether it is a rise in tissue cGMP or and stimulation of sarcolemmal Na⁺-K⁺-ATPase by BAY 41-2272, they can influence the Ca²⁺ influx/Ca²⁺ sensitivity of the pulmonary artery smooth muscle. The results in Fig. 3B show that 10 μM BAY 41-2272 markedly inhibited (Emax) the contraction elicited by CaCl₂ (10 μM–3 mM) in 60 mM K⁺-depolarized pulmonary artery rings.

To examine whether cGMP stimulates sarcolemmal sodium pump to dilate the ovine pulmonary artery, 8-Br-cGMP, a cell-permeable analog of cGMP was used. Surprisingly, 100 μM 8-Br-cGMP caused a very small relaxation (6.00 ± 1.4%; n = 4) of the pulmonary artery rings preconstricted with 1 μM 5-HT. It was therefore not technically feasible to assess the effect of either ouabain or any other pharmacological intervention on 8-Br-cGMP-induced vasodilation.

**Effect of Protein Kinase G and Protein Kinase A Inhibitors on ODQ-Insensitive Relaxation Caused by BAY 41-2272.** Since elevation in intracellular cGMP levels may occur both due to activation of sGC as well as phosphodiesterase inhibition, we examined the role of cGMP-protein kinase G pathway in ODQ-resistant relaxation mediated by BAY 41-2272 in ovine pulmonary artery. Figure 4A depicts the results of the effect of KT-5823, a specific inhibitor of protein kinase G on the vasodilator responses of BAY 41-2272 resistant to 10 μM ODQ. Pretreatment of the tissues with 2 μM KT-5823 for 30 min in the presence of 10 μM ODQ had no effect on ODQ-insensitive BAY 41-2272-induced relaxations (pD2 = 5.38 ± 0.15; Emax = 65.56 ± 6.29%; n = 5 in comparison with ODQ control, pD2 = 5.33 ± 0.14; Emax = 62.94 ± 2.78%; n = 8). As shown in Fig. 4B, KT-5720 a selective inhibitor of protein kinase A, caused a small leftward shift in the concentration-response curve produced by BAY 41-2272. Inhibitory effect of KT-5823 (2 μM) (A) and protein kinase A inhibitor KT-5720 (0.5 μM) (B) on 10 μM ODQ-resistant relaxations induced by BAY 41-2272 (1 nM–10 μM). The data were analyzed by Student’s paired t test. Results are expressed as mean ± S.E.M. ∗P < 0.05 was considered statistically significant.

**Fig. 3. A, inhibitory effect of K⁺-free PSS on concentration-dependent relaxation of ovine pulmonary artery rings by BAY 41-2272 (1 nM–10 μM; n = 6). Concentration-responses to BAY 41-2272 were first elicited in 1 μM 5-HT-constricted tissues in normal PSS (K⁺; 5.9 mM). The tissues were then equilibrated in K⁺-free PSS for 30 min before the second concentration-response curve to BAY 41-2272 was constructed. B, inhibitory effect of 10 μM BAY 41-2272 on Ca²⁺ (10 μM–3 mM) contractions elicited on K⁺ (60 mM)-depolarized ovine pulmonary artery preparations (n = 4). The data were compared by Student’s paired t test. Results are expressed as mean ± S.E.M. ∗P < 0.05 was considered statistically significant.**
Effect of BAY 41-2272 on Ouabain-Sensitive $^{86}$Rb Uptake. To further confirm the contribution of sarcolemmal Na$^+$/K$^+$-ATPase to the dilator responses of BAY 41-2272 in ovine pulmonary artery, ouabain-sensitive $^{86}$Rb uptake was measured. BAY 41-2272 (0.1, 1, and 10 μM) caused concentration-dependent increase in $^{86}$Rb uptake (Fig. 5). ODQ (10 μM) had no significant effect on either basal or BAY 41-2272-stimulated ouabain-sensitive $^{86}$Rb uptake (Fig. 5). 8-Br-cGMP (100 μM), a cell-permeable analog of cGMP, had no effect on ouabain-sensitive $^{86}$Rb uptake (0.20 ± 0.03 nmol $^{86}$Rb/min/mg tissue dry wt; n = 6 versus basal. 0.24 ± 0.02 nmol $^{86}$Rb/min/mg tissue dry wt; n = 9).

Effect of BAY 41-2272 on Plasma Membrane Na$^+$/K$^+$-ATPase Activity. Figure 6A depicts the effect of 10 μM BAY 41-2272 on plasmalemmal Na$^+$/K$^+$-ATPase activities of ovine pulmonary artery. Exposure of the tissues to BAY 41-2272 for 30 min resulted in a significant (P < 0.05) stimulation of Na$^+$/K$^+$-ATPase activities by approximately 2-fold over the basal activity, determined in the presence of vehicle control DMSO. Pretreatment of the tissues with 10 μM ODQ for 30 min had no effect on either basal or BAY 41-2272-stimulated increase in Na$^+$/K$^+$-ATPase activity. The specificity of sodium pump stimulation by BAY 41-2272 was further confirmed by assessing its effect on ouabain-insensitive Mg$^{2+}$/ATPase activity. As shown in Fig. 6B, 10 μM BAY 41-2272 had no significant effect on ouabain-insensitive Mg$^{2+}$-ATPase activity. 8-Br-cGMP (100 μM) had no effect on the Na$^+$/K$^+$-ATPase activity (8-Br-cGMP, 3.18 ± 0.62 nmol Pi/min/mg protein versus control, 3.93 ± 0.62 nmol Pi/min/mg protein; n = 6 each for treatment and control groups).

Effect of BAY 41-2272 on Tissue cGMP Level. To examine the possibility that BAY 41-2272 could increase tissue cGMP level by the inhibition of PDE5, apart from stimulation of sGC, we did not use a PDE5 inhibitor in cGMP assay. As shown in Fig. 7, 10 μM BAY 41-2272 caused about a 14-fold increase in intracellular cGMP over the basal levels, when the tissues were exposed to the drug for 3 min. ODQ (10 μM) completely inhibited the increase in cGMP stimulated by BAY 41-2272. There was a significant decline in tissue cGMP, when the arterial strips were exposed to BAY 41-2272 for 30 min. Nevertheless, the BAY 41-2272-induced increase in intracellular cGMP was approximately 3-fold higher than the control levels. Pretreatment with 10 μM ODQ lowered the BAY 41-2272-stimulated increase in cyclic nucleotide levels to 70% of the control value.

Discussion

Results of the present study suggest two different mechanisms for BAY 41-2272-induced dilation in ovine pulmonary artery. Whereas cGMP-independent sarcolemmal sodium pump seems to be the primary target of vasodilation by BAY 41-2272, the involvement of a secondary cGMP-dependent pathway is also evident. In a recent study, using photoaffinity labeling, Stasch et al. (2001) demonstrated that BAY 41-2272 binds to close to amino acid residues cysteine 238 and cysteine 243 at the N terminus of the α1-subunit of sGC to stimulate this enzyme. Furthermore, in the same study, ODQ, a potent and selective inhibitor of sGC, was shown to completely inhibit the stimulatory effect of BAY 41-2272 on the enzyme. The pharmacological activities such as relaxation of rat and rabbit aorta in vitro, decrease in blood pressure, and antiplatelet activity reported so far have been attributed to an increase in cGMP by BAY 41-2272 (Stasch et al., 2001; Straub et al., 2001; Hobbs and Moncada, 2003). In the present study, however, we observed that ODQ partially attenuated BAY 41-2272 relaxation. This therefore suggests that in addition to stimulation of sGC, BAY 41-2272 is causing relaxation of ovine pulmonary artery through some other mechanism. The present observation is consistent with a previous study, wherein ODQ-insensitive relaxation to BAY 41-2272 has been demonstrated in human and rabbit corpus cavernosum preparations (Kalsi et al., 2003).

YC-1, another NO-independent sGC activator, has been shown to stimulate an increase in tissue cGMP at least through two different mechanisms, one involving the activation of sGC and the other through the inhibition of phosphodiesterase type 5 (Galle et al., 1999). The structural similarity between BAY 41-2272 and YC-1 may suggest that both the compounds have similar mechanisms of action. However, the effect of BAY 41-2272 on phosphodiesterase type 5-activating relaxation of human corpus cavernosum has been described elsewhere (Kalsi et al., 2003).
tissues were exposed either to the solvent DMSO or 10⁻⁴ M ODQ for 3 or 30 min before quickly frozen in liquid nitrogen. To study the effect of sGC inhibitor on BAY 41-2272 response, arterial strips were pretreated with 10⁻⁴ M ODQ for either 3 or 30 min before exposure to 10⁻⁴ M BAY 41-2272 for 3 or 30 min. To study the effect of sGC inhibitor on BAY 41-2272 response, arterial strips were incubated with 10⁻⁴ M ODQ during the final 30-min equilibration period before exposure to either the solvent or BAY 41-2272. Ouabain-sensitive Na⁺-K⁺-ATPase activity was expressed as Pi nanomoles per minute per milligram of protein. B, neither 10⁻⁴ M BAY 41-2272 nor 1⁻⁴ M ODQ had any effect on Mg²⁺-ATPase activity. *, significantly different from control at P < 0.05 compared with ODQ (a) (by one-way ANOVA followed by Tukey’s multiple comparison tests).

Fig. 6. A, effect of 10⁻⁴ M ODQ on 10⁻⁴ M BAY 41-2272-stimulated Na⁺-K⁺-ATPase activity. Pulmonary artery strips were incubated in PSS for 2 h at 37°C, continuously aerated with carbogen. At the end of the equilibration period, the tissues were exposed either to the solvent DMSO or 10⁻⁴ M BAY 41-2272 for 30 min. To study the influence of sGC inhibition on basal or BAY 41-2272-stimulated increase in Na⁺-K⁺-ATPase activity, the arterial strips were incubated with 10⁻⁴ M ODQ during the final 30-min equilibration period before exposure to either the solvent or BAY 41-2272. Ouabain-sensitive Na⁺-K⁺-ATPase activity was expressed as Pi nanomoles per minute per milligram of protein. B, neither 10⁻⁴ M BAY 41-2272 nor 1⁻⁴ M ODQ had any effect on Mg²⁺-ATPase activity. *, significantly different from control at P < 0.05 compared with ODQ (a) (by one-way ANOVA followed by Tukey’s multiple comparison tests).

Fig. 7. Effect of 10⁻⁴ M ODQ on 10⁻⁴ M BAY 41-2272-stimulated rise in intracellular cGMP in ovine pulmonary artery strips. The control level of the cyclic nucleotide refers to the measurements made in the presence of the solvent DMSO (0.1%). After equilibration for 90 min in PSS, the tissues were exposed either to the solvent DMSO or 10⁻⁴ M BAY 41-2272 for either 3 or 30 min before quickly frozen in liquid nitrogen. To study the effect of sGC inhibitor on BAY 41-2272 response, arterial strips were pretreated with 10⁻⁴ M ODQ for 30 min before exposure to 10⁻⁴ M BAY 41-2272. Results are expressed as a percentage of control cyclic nucleotide. Values are shown as mean ± S.E. (n = 6 for each group). *, P < 0.05 compared with BAY 41-2272 (3 or 30 min). a, P < 0.05 compared with BAY 41-2272 (3 min). Data were analyzed by one-way ANOVA followed by Tukey’s multiple comparison tests.

ity is at present controversial. For example, Stasch et al. (2001) and Bischoff and Stasch (2004) observed that BAY 41-2272 was devoid of any inhibitory effect on PDE5. On the contrary, Mullershausen et al. (2004) demonstrated PDE5 inhibitory action of BAY 41-2272 in platelets. Although the present investigation cannot rule out PDE5 inhibitory mechanism, two important observations suggest that BAY 41-2272-stimulated increase in intracellular cGMP primarily involves sGC pathway. First, ODQ abolished BAY 41-2272-stimulated increase in tissue cGMP; and second, there was a substantial decay (24% at 30 min compared with 3-min level) in the cGMP level after prolonged exposure to BAY 41-2272. This is in contrast to significantly elevated tissue cGMP level for a prolonged period (52% of the maximal at 30 min) in rabbit aortic rings exposed to YC-1 (Galle et al., 1999). The fact that additional mechanisms to cGMP-mediated relaxation of ovine pulmonary artery in response to BAY 41-2272 are further evident from the observation that protein kinase G inhibitor KT-5823 had no effect on ODQ-insensitive relaxations. In a previous study, cross-activation of protein kinase A (PKA) by cGMP has been reported (Chao et al., 1994). It is therefore predicted that PKA in turn would stimulate Na⁺-K⁺-ATPase. We, however, find no evidence for stimulation of Na⁺-K⁺-ATPase by PKA in ovine pulmonary artery, because KT-5720 had no inhibitory effect on ODQ-resistant relaxation induced by BAY 41-2272.

It is very well established that sarcolemmal Na⁺-K⁺-ATPase plays an important role in regulating vascular smooth muscle tone. An increase in Na⁺-K⁺-ATPase activity may induce vascular smooth muscle relaxation through an increase in Na⁺/Ca²⁺ exchange and a reduction in Ca²⁺ influx through voltage-dependent calcium channels (Clausen and Nielsen, 1994). In the present study, we observed that relaxation response to BAY 41-2272 was nearly abolished by pretreatment with ouabain and significantly inhibited after incubation in K⁺-free buffer. Thus, it seems that BAY 41-2272 stimulates the sarcolemmal sodium pump to induce relaxation in ovine pulmonary artery. This hypothesis is further substantiated by the observations that BAY 41-2272 significantly increased ouabain-sensitive ⁸⁶Rb uptake as well as plasma membrane Na⁺-K⁺-ATPase activity in the pulmonary arterial strips. In canine pulmonary artery smooth muscle cells, cGMP-mediated relaxation was attributed to stimulation of sarcolemmal Na⁺-K⁺-ATPase (Tamaoki et al., 1997). However, we observed that BAY 41-2272-stimulated ouabain-sensitive ⁸⁶Rb uptake and Na⁺-K⁺-ATPase activity in ovine pulmonary artery were resistant to sGC inhibitor ODQ, which otherwise attenuated BAY 41-2272-induced increase in cGMP. The lack of correlation between the rise in tissue cGMP and sodium pump stimulation suggests a novel mechanism for cGMP-independent stimulation of sarcolemmal sodium pump by BAY 41-2272. The lack of effect of 8-bromo-cGMP on ⁸⁶Rb uptake is consistent with its poor dilator response in ovine pulmonary artery. Although cGMP-independent stimulation of Na⁺-K⁺-ATPase by NO/NO donors has been demonstrated in several arterial smooth muscles (rabbit aorta, Gupta et al., 1994; rat pulmonary artery, Homer and Wanstall, 2000; and ovine pulmonary artery, Sathishkumar et al., 2005), the mechanism by which BAY
41-2272 stimulates plasmalemmal sodium pump without involving sGC/cGMP pathway needs to be worked out. Whether it is a rise in tissue cGMP level and/or activation of sodium pump by BAY 41-2272, Ca\(^{2+}\) movement/sensitivity of the contractile apparatus to Ca\(^{2+}\) may be influenced. This is evident from BAY 41-2272-induced inhibition of Ca\(^{2+}\) contraction in K\(^{+}\)-depolarized pulmonary artery preparations.

In conclusion, the present study demonstrates that in addition to activation of sGC, cGMP-independent stimulation of sarcolemmal sodium pump by BAY 41-2272 is an important mechanism in the relaxation of ovine pulmonary artery. Since both cGMP and sodium pump have significant roles in maintaining vascular tone, the dual mechanism of arterial dilation by BAY 41-2272 is of clinical interest in the management of pulmonary hypertension, particularly when the endogenous production of nitric oxide is impaired.

Acknowledgments
We are grateful to Dr. J. P. Stasch for the gift sample of BAY 41-2272.

References


Address correspondence to: Dr. Santosh K. Mishra, Division of Pharmacology and Toxicology, Indian Veterinary Research Institute, Izatnagar-243122 (UP), India. E-mail: smishraivri@rediffmail.com