Role of Low Ouabain-Sensitive Isoform of Na^+-K^+-ATPase in the Regulation of Basal Tone and Agonist-Induced Contractility in Ovine Pulmonary Artery

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Abstract: Although Na^+-K^+-ATPase plays an important role in vascular smooth muscle function, it is unknown which isoforms of the enzyme are present in the pulmonary vasculature and whether they possess different affinities for ouabain. Unlike rodents, Na^+-K^+-ATPase in sheep and humans displays greater affinity for ouabain. Thus, the present study examined the presence of various isoforms of the enzyme by Western blot analysis and their sensitivity to inhibition by ouabain (biochemical estimation of enzyme activity/K^+-relaxations) in the ovine pulmonary artery. Further, we studied the effect of ouabain on the basal tone and agonist-induced contractions in this vessel. Our results show the presence of both α_1 and α_2 isoforms of Na^+-K^+-ATPase in this vessel. The biphasic shape of the ouabain inhibition curve indicates that the α_1 and α_2 isoforms of the enzyme may possess low and high affinity, respectively, for the cardiac glycoside. Concentrations of ouabain <1 μM had no significant effect on the basal tone of the vessel. At 1 μM concentration, however, there was a significant increase in the basal tension (55% of 5-HT 1 μM contraction). Ouabain (0.1 μM) selectively increased the vasoconstrictor potency of 5-HT (pD_2 6.81 ± 0.10 versus control pD_2 5.95 ± 0.07), but not that of phenylephrine (pD_2 5.80 ± 0.07 versus control pD_2 6.05 ± 0.05). Neither endothelium removal nor treatment with PKG inhibitor KT 5823 (2 μM) had any effect on the sodium pump function. These results indicate that the low, but not the high, ouabain-sensitive isoform of Na^+-K^+-ATPase regulates basal tone and agonist-induced contractility in the ovine pulmonary artery.

Key Words: Na^+-K^+-ATPase, ouabain, 5-HT, pulmonary artery, α-isoforms

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INTRODUCTION

The Na^+-K^+-ATPase has been hypothesized to play an important role in the regulation of vascular tone.[1,2] For instance, stimulation of the plasmalemmal Na^+-K^+-ATPase in vascular smooth muscle causes membrane hyperpolarization that consequently reduces Ca^{2+} influx through voltage-gated calcium channels leading to vasodilation. Alternately, vascular smooth muscle relaxation through stimulation of the Na^+-K^+-ATPase is mediated by the activation of Na-Ca exchange mechanism, which in turn reduces intracellular Ca^{2+}. On the contrary, inhibition of the enzyme produces vascular contractility. Thus, ouabain and endogenous ouabain-like compounds that specifically inhibit Na^+-K^+-ATPase increase total peripheral resistance and blood pressure in several experimental animals.[3,4] In pulmonary circulation, hypothalamic inhibitory factor, an endogenous inhibitor of Na^+-K^+-ATPase with ouabain-like properties, has been implicated in the pathogenesis of pulmonary hypertension in spontaneously hypertensive rats.[5] Na^+-K^+-ATPase is considered to be an important target of nitric oxide (NO) and other vasodilators in pulmonary arteries.[6–9] It is known that endogenous NO determines the low pulmonary vascular resistance in several species, including man.[10,11] Thus, impaired endothelial NO production resulting from sustained hypoxia has been suggested to cause pulmonary hypertension, partly as a result of inhibition of Na^+-K^+-ATPase.[12]

Four isoforms of the catalytic α-subunit (α_1, α_2, α_3, and α_4) of the sodium pump have been identified in different tissues. Recent studies in mice show that the α_2-subunit is crucial in determining the vascular tone and ouabain-induced hypertension.[13–15] It is, however, unknown which isoform/s of Na^+-K^+-ATPase is present in the pulmonary vasculature. Although rat is the preferred animal model in vascular research, this species is considered less relevant for studying the modulation of Na^+-K^+-ATPase signaling by ouabain in vascular smooth muscle due as a result to high content of ouabain-insensitive α_1-isoform in its arteries.[16] Further, it is well known that Na^+-K^+-ATPase in ovines,[17] like that of the humans,[18] is highly sensitive to ouabain in comparison to rat and mouse.[19] Therefore, the first objective of the present study was to examine the presence of different α-isoforms of Na^+-K^+-ATPase and their sensitivity to ouabain in ovine pulmonary artery. The second objective was to study the inhibition of the enzyme by ouabain and its effect on vascular tone and agonist-induced contractions in this vessel. In the absence of specific ligands for different isoforms of sodium pump, ouabain sensitivity has been used as an important parameter in

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delineating the role of high-sensitive α_2 and low-sensitive α_1 subunits in cell function (endothelium,[20] rat testis[21]). We have used this experimental tool to determine whether ouabain possessed different affinity for inhibition of various isoforms of Na^+-K^+-ATPase in ovine pulmonary artery. Further, we used K^+-induced relaxation in arteries incubated in K^+-free solution plus 5-HT as a functional indicator of vascular Na^+-K^+-ATPase.[22]

METHODS

Blood Vessel Preparation

Lungs from freshly sacrificed adult male sheep were collected from the local slaughterhouse in cold (4°–6°C) oxygenated modified Krebs-Henseleit solution (PSS) of the following composition: (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 11.9, KH_2PO_4 1.2 and D-glucose 11.1 (pH 7.4). Second-generation intralobar pulmonary arteries were dissected from lungs, cleared of connective tissue, and cut into rings of about 2–3 mm length. Unless otherwise mentioned, endothelium was routinely removed by rubbing with a wet cotton swab. Adult male Wistar rats were anesthetized by pentobarbitone sodium (60 mg/kg) and killed by exsanguination. Kidney was isolated for Western blot analysis.

Western Blot Analysis

Pulmonary artery rings were homogenized (10% W/V) on ice in homogenization buffer (250 mM sucrose in 10 mM MOPS, pH 7.4) using polytron homogenizer. The homogenate was then centrifuged at 10,000 g for 10 min at 4°C. The supernatant was subjected to ultracentrifugation at 112,000 g at 4°C for 1 hour. The pellet consisting of microsomes was resuspended in appropriate (depending on pellet size) volume of homogenization buffer, and protein concentration was determined by Lowry's method.[23] The same protocol was followed for rat brain and kidney.

For Western blot, microsomal fractions were electrophoresed at a constant voltage (100 V) on a linear gradient polyacrylamide gel (5%–15%) with a stacking gel of 3.3% polyacrylamide.[24] Proteins were transferred from gel onto nitrocellulose membrane (NCM), and electroblot was performed at 200 V for 1.5 hours in a water-cooled transfer apparatus. The membrane was then blocked in blocking buffer [5% skim milk 0.5% PBS (phosphate buffered saline)-Tween] for 12 hours at 4°C. The NCM was incubated in diluted primary polyclonal antibodies at a dilution (α_1, 1:500, α_2, 1:1000) with PBS-Tween for 5 hours at room temperature. After 3 washings of NCM in PBS-Tween, detection was achieved using horseradish peroxidase (HRPO)-conjugated rat antigoat immunoglobulin (IgG; Sigma-Aldrich, St. Louis, Missouri) and diaminobenzidine (DAB) substrate solution.

Estimation of Na^+-K^+-ATPase Activity

Isolation of sarcolemmal membranes from pulmonary arteries was performed as per the procedure described.[25] Na^+-K^+-ATPase activity was determined by measuring the liberation of inorganic phosphate (Pi) from adenosine triphosphate (ATP) in the medium containing (mM): Tris HCl buffer, 50, NaCl, 140; KCl, 14; $MgCl_2.6H_2O$, 5; ethylenediamine tetraacetic acid (EDTA), 0.5; ouabain, 1; pH 7.5 and requisite volume (100 μL) of membrane homogenate in a final volume of 1 mL. This reaction mixture was equilibrated for 15 minutes at 37°C, and the reaction was started by the addition of 3 mM ATP solution. For total ATPase assay, ouabain was omitted from the reaction mixture, which also included Mg^{2+}-ATPase. After 1 hour incubation in both the cases, the reaction was stopped with 0.1 mL of 5% sodium dodecyl sulphate, and color was developed with 3 mL of acidic ammonium molybdate and 0.1 mL of ANSA (1-amino-2-naphthol-4-sulfonic acid) reagent. The Pi in the reaction mixture was estimated as per the method.[26] The Na^+-K^+-ATPase activity was deduced from the difference in the activity in the absence and presence of 1 mM ouabain. In ouabain concentration-inhibition experiments, Na^+-K^+-ATPase activity was measured at different ouabain concentrations (0.1 nM–10 μM). Specific enzyme activity is expressed as nmol of Pi liberated per minute per milligram of protein. To determine the effect of 5-HT on the Na^+-K^+-ATPase activity, tissues were exposed to 1 μM 5-HT for 30 minutes and then the Na^+-K^+-ATPase activity was determined.

Tension Experiments

Arterial rings of 2–3 mm were prepared from second-generation intrapulmonary arteries. Rings were mounted between 2 "L"-shaped stainless steel hooks under a resting tension of 1.5 g in a thermostatically controlled (37.0° ± 0.5°C) organ bath of 10 mL capacity containing PSS and continuously aerated with carbogen (95% O_2 + 5% CO_2). Most of our experiments were done in endothelium-denuded pulmonary artery rings. Endothelium was removed by mechanical rubbing with a cotton swab. Endothelium removal was confirmed by demonstrating the absence of relaxation to ACh (10 μM). Following endothelium removal, most of the tissues contracted with ACh. Some arterial rings were used with intact endothelium. The arterial rings were equilibrated for 90 minutes with a repeated replacement of bath solution every 15–20 minutes. A high sensitivity force displacement transducer (model MLT0202/D; PowerLab, Castle Hill, Australia) measured the change in tension, and the data were recorded in a personal computer using chart version 4.1.2 software program (PowerLab).

Experimental Protocol

Western Blot Analysis

Primary polyclonal antibodies against α_1 (goat polyclonal IgG against epitope mapping within an internal region of Na^+-K^+-ATPase α_1 of human origin) and α_2 (goat polyclonal IgG against epitope mapping within a cytoplasmic domain of Na^+-K^+-ATPase α_2 of human origin) isoforms of Na^+-K^+-ATPase were used to characterize the respective proteins in ovine pulmonary artery. Rat kidney, known to be rich in α_1 isoform of Na^+-K^+-ATPase, was used either as positive or negative control for probing the α_1 and α_2 subunits, respectively.

Ouabain Inhibition Curve

Ouabain sensitivity of Na^+-K^+-ATPase was used as a parameter to determine the affinity of different α isoforms of the enzyme for the cardiac glycoside.[21] Microsomes prepared

from endothelium-denuded arterial rings were incubated with increasing concentrations (log pattern) of ouabain in Tris buffer for 15 minutes. The data were plotted with log concentrations ouabain versus per cent inhibition of Na^+-K^+-ATPase activity.

Functional Sodium Pump

KCl-induced relaxation in vascular rings contracted with K^+-free solution plus agonist has been used for the assessment of functional sodium pump.[22] We used this protocol in the ovine pulmonary artery. In brief, after equilibration, pulmonary arterial rings were exposed to nominally K^+-free solution (PSS containing no K^+ as KCl and KH_2PO_4 were replaced with equimolar concentration of NaCl and NaH_2PO_4, respectively) for 30 minutes. At the declining phase of the K^+-free contracture, 5-HT (0.1 μM) was added to maintain a steady level of contraction. At the plateau phase of contraction, KCl (0.01 mM–10 mM) was added cumulatively at an interval of 0.5 log unit to elicit relaxation, which was expressed as percentage reversal of contraction elicited with K^+-free solution plus 5-HT. To study the influence of ouabain, barium, and KT-5823 in modifying sodium pump function, vessels were incubated with these drugs in K^+-free solution before steady-state contraction with 5-HT was elicited.

Effect of Ouabain on Basal Tone and Agonist-Induced Contractions

Effect of ouabain on basal tone was determined by exposure of the arterial rings to different concentrations of the cardiac glycoside for at least 90 minutes. To elucidate the contribution of endogenous catecholamines to ouabain-induced increase in the basal tone of the vessel, some experiments were done in the presence of alpha-adrenergic blocker phenoxybenzamine (0.1 μM). The influence of sodium pump in modifying the vasoconstrictor responses to 5-HT and phenylephrine was assessed by eliciting concentration-response curves to these agonists in the presence or absence of different concentrations of ouabain. Only 1 concentration of the inhibitor was used for each ring.

Drugs and Chemicals

Stock solutions of ACh (10 mM), 5-HT (10 mM), phenylephrine (10 mM), and ouabain (10 mM) were prepared in triple distilled water. Phenoxybenzamine (100 mM) was prepared in 95% ethanol containing 0.001 mL 10 N HCl/mL. KT 5823 was prepared in dimethyl sulfoxide (DMSO). Ketanserin (10 mM) was prepared in DMSO, and further dilutions were made in double distilled water. The drugs were purchased from Sigma-Aldrich (St. Louis, Missouri). Primary polyclonal antibodies against α_1 (goat polyclonal IgG against epitope mapping within an internal region of Na^+-K^+-ATPase α_1 of human origin) and α_2 (goat polyclonal IgG against epitope mapping within a cytoplasmic domain of Na^+-K^+-ATPase α_2 of human origin) isoforms of Na^+-K^+-ATPase were purchased from Santa Cruz Biotechnology (Santa Cruz, California).

Statistical Analysis

Results are expressed as means ± standard error of the mean (SEM), and multiple comparisons were done using 2-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Student's *t*-test was used when comparisons were made between control and drug treatment. $P < 0.05$ was considered statistically significant. EC_{50} and E_{max} were determined using nonlinear regression analysis of GraphPad Prism (La Jolla, California). pD_2 is expressed as $-\log EC_{50}$ of the agonist.

RESULTS

Detection of α_1 and α_2 Isoforms of Na^+-K^+-ATPase by Western Blotting

Western blot was used to detect the type of alpha-subunit isoforms (α_1 and α_2) of Na^+-K^+-ATPase expressed in ovine pulmonary artery. The rat kidney (positive control for α_1 and negative for α_2) sample was prepared and blotted along with the ovine pulmonary artery samples to assess the specificity of used polyclonal antibodies and integrity of the blotting procedure. As shown by the presence of specific bands (approximate molecular mass 110 kDa) for respective proteins (Fig. 1), expression of both α_1 and α_2 isoforms is confirmed in the ovine pulmonary artery. Rat kidney homogenate showed the presence of α_1, but not the α_2 isoform of the enzyme.

Ouabain Concentration-Response Curve for Na^+-K^+-ATPase

The expression of both α_1 and α_2 isoforms necessitated to examine whether the pulmonary artery plasmalemmal Na^+-K^+-ATPase isoforms were differently sensitive to inhibition by ouabain. The inhibition by ouabain (10^{-10} M–10^{-5} M) of plasma membrane Na^+-K^+-ATPase activity was biphasic in nature (Fig. 2). Accordingly, from 1 nM to 30 nM the percentage inhibition increased by 10% with a step elevation of ouabain concentration by log 0.5 units. The concentration response then showed a tendency of saturation between 30 and 50 nM of ouabain. However, between 50 and 100 nM concentrations of the cardiac glycoside, the percentage increase in the inhibition was about 30% at 70 and 100 nM, and the enzyme activity was almost completely inhibited.

Sensitivity of KCl-Induced Relaxation to Ouabain

Figure 3 depicts the concentration-dependent inhibition of K^+-induced relaxation in the ovine pulmonary artery.

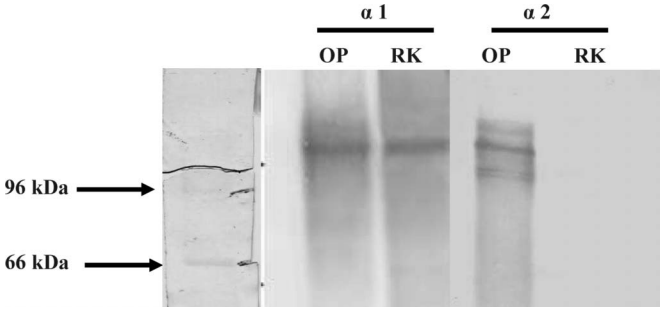

FIGURE 1. Representative Western blot of the α_1 and α_2 Na^+-K^+-ATPase of ovine pulmonary artery and rat kidney homogenates performed with goat polyclonal IgG for respective isoforms.

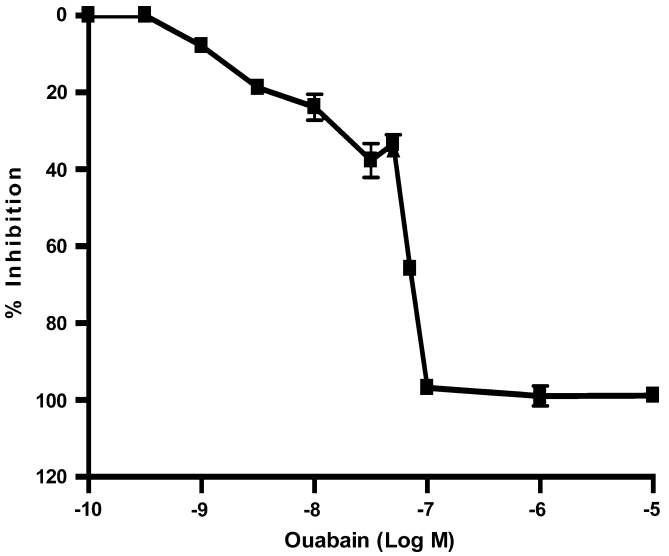

FIGURE 2. Concentration-response curve for the ouabain inhibition of Na^+-K^+-ATPase from the ovine pulmonary artery plasma membranes. The membrane preparations were pre-incubated for 15 minutes at 37°C with indicated concentrations of ouabain in the reaction mixture containing (mM) 140 NaCl, 14 KCl, 5 $MgCl_2.6H_2O$, 0.5 EDTA, 50 Tris HCl buffer (pH 7.5). Thereafter, the reaction was started by the addition of 3 mM ATP and terminated at 60 minutes. Values are expressed as percentage inhibition at different concentrations of the cardiac glycoside. Vertical bars represent SEM. n = 4–7 at each concentration of ouabain.

Exposure of the arterial rings to K^+-free solution following equilibration in PSS resulted in a slow increase in baseline tension reaching a maximal tension of 0.62 ± 0.09 g (n = 6) over a period of 25–30 minutes. The K^+-free contracture was not steady, and it declined slowly. However, when 5-HT (0.1 μM) was added to the declining phase of the K^+-free-induced contracture, a steady level of contraction (1.46 ± 0.24 g; n = 6) was achieved. KCl (10 μM–10 mM), added cumulatively at an increment of 0.5 log unit, caused concentration-dependent relaxation with an E_{max} of 90.60 ± 1.33% (n = 12) and the pD_2 was 3.62 (95% CL, 3.84–3.40). As shown in Figure 3A, ouabain (1 nM) had a small inhibitory effect on K^+-induced relaxation (E_{max}, 81.5 ± 2.4%; pD_2, 3.67, n = 6). Increasing the concentration of ouabain to 10 nM caused a very significant decrease in KCl-induced relaxation (E_{max}, 7.7 ± 5.9%; n = 6). At 0.1 μM concentration of the cardiac glycoside, KCl 1, 3 and 10 mM elicited concentration-dependent contractions.

Effect of Ba (30 μM) on KCl-Induced Relaxation

Figure 3B illustrates the effect of Ba^{2+} (30 μM) on KCl-induced relaxation. KCl (10^{-5} M–10^{-2} M), added cumulatively on vessels contracted with K^+-free solution plus 5-HT, produced a concentration-dependent relaxation (pD_2, 2.77 ± 0.12; E_{max}, 62.12 ± 7.45%, n = 4). Preincubation of the

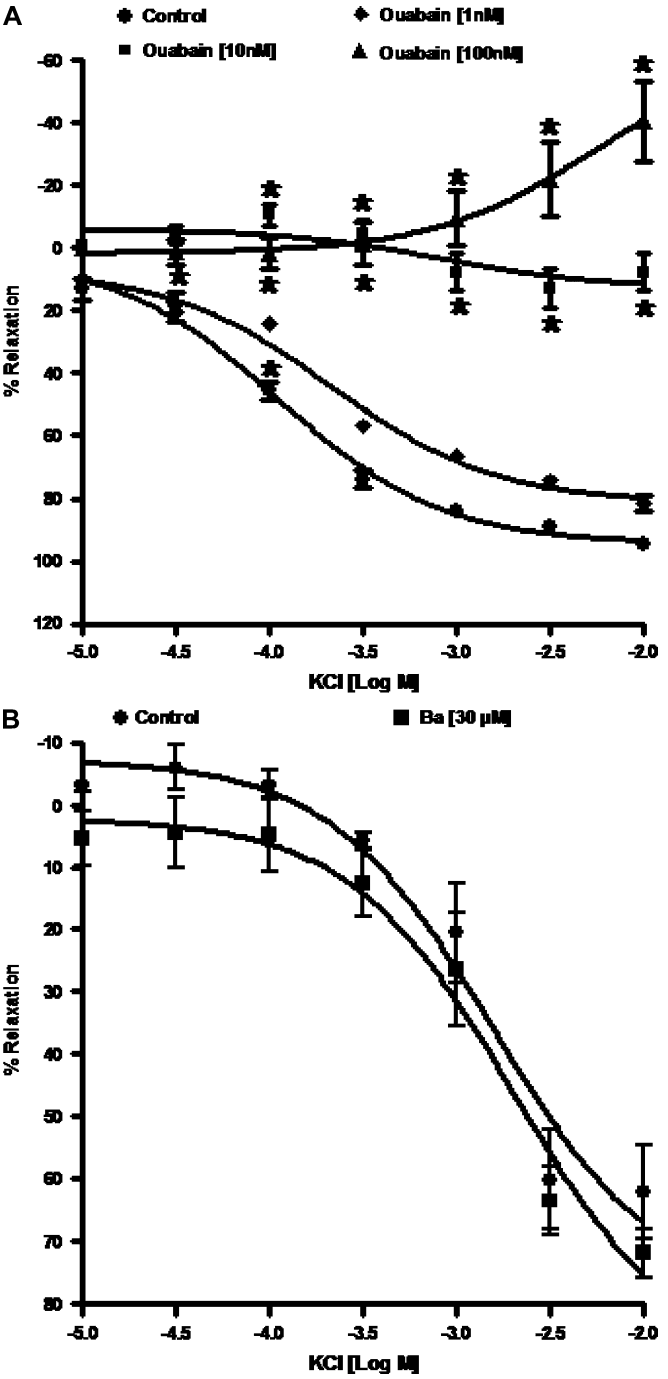

FIGURE 3. A, Effect of ouabain (1, 10, and 100 nM) on KCl (10^{-5} M–10^{-2} M)-induced relaxation of the isolated ovine pulmonary artery precontracted with 5-HT (0.1 μM) in K^+-free solution. Tissues were pretreated with ouabain for 30 minutes before exposure to K^+-free solution plus 5-HT. B, Effect of pretreatment with Ba (30 μM) for 30 minutes on K^+-induced relaxation of arterial rings contracted with K^+-free solution and 5-HT (0.1 μM). Results are presented as mean ± SEM. *$P < 0.05$

tissues with Ba^{2+} (30 μM) had no significant effect on KCl-induced relaxations (pD_2, 2.63 ± 0.19; E_{max}, 71.87 ± 3.84%, n = 4).

Effect of Ouabain on the Basal Tone of Ovine Pulmonary Artery

We observed that treatment of arterial rings for 90 minutes with lower concentrations of ouabain (1 nM–100 nM) had no significant effect on the basal tone (n = 6). However, ouabain (1 μM) significantly increased the basal tone that was biphasic in nature (n = 6). The first phase of contraction reached peak in about 1 hour, followed by the second phase of sustained contraction that attained peak in about 3 hours (Fig. 4A). The maximal force generated by ouabain was 1.33 ± 0.17 g (about 55% of force produced by 5-HT 1 μM). Pretreatment of the tissues with phenoxybenzamine (0.1 μM) abolished the first phase without having a significant effect on the more sustained phase of contraction (Fig. 4B, n = 3).

Effect of Different Concentrations of Ouabain on 5-HT and Phenylephrine-Induced Contractions

Figure 5A shows the effect of ouabain (10 and 100 nM) on contractions elicited by 5-HT. 5-HT (1 nM–10 μM) caused concentration-dependent contraction (pD_2, 5.95 ± 0.07; E_{max}, 112 ± 5.2%, n = 6) of the pulmonary artery rings. Pretreatment of the tissues with ouabain (10 nM; n = 6) had no significant effect on the potency (pD_2, 5.88 ± 0.06) and efficacy (E_{max}, 124 ± 5.0%) of 5-HT (n = 6). However, ouabain (100 nM; n = 6) caused almost 1 log unit shift to left in the 5-HT concentration-response curve with corresponding

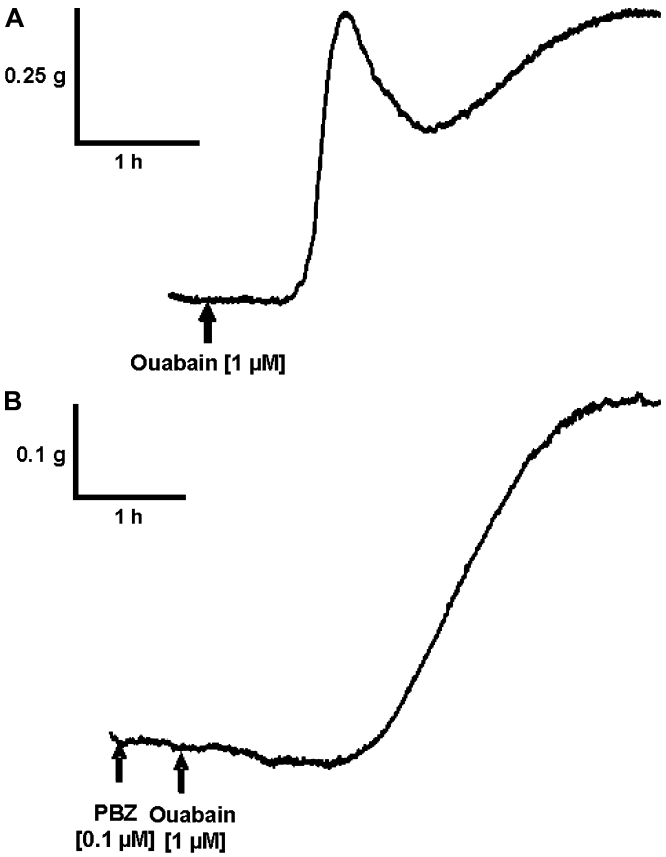

FIGURE 4. Raw tracings demonstrate the effect of ouabain (1 μM) on the basal tone of the isolated ovine pulmonary artery in the absence (A) and presence (B) of phenoxybenzamine (PBZ, 0. 1 μM). Tissues were pretreated with PBZ for 30 minutes before eliciting responses to ouabain.

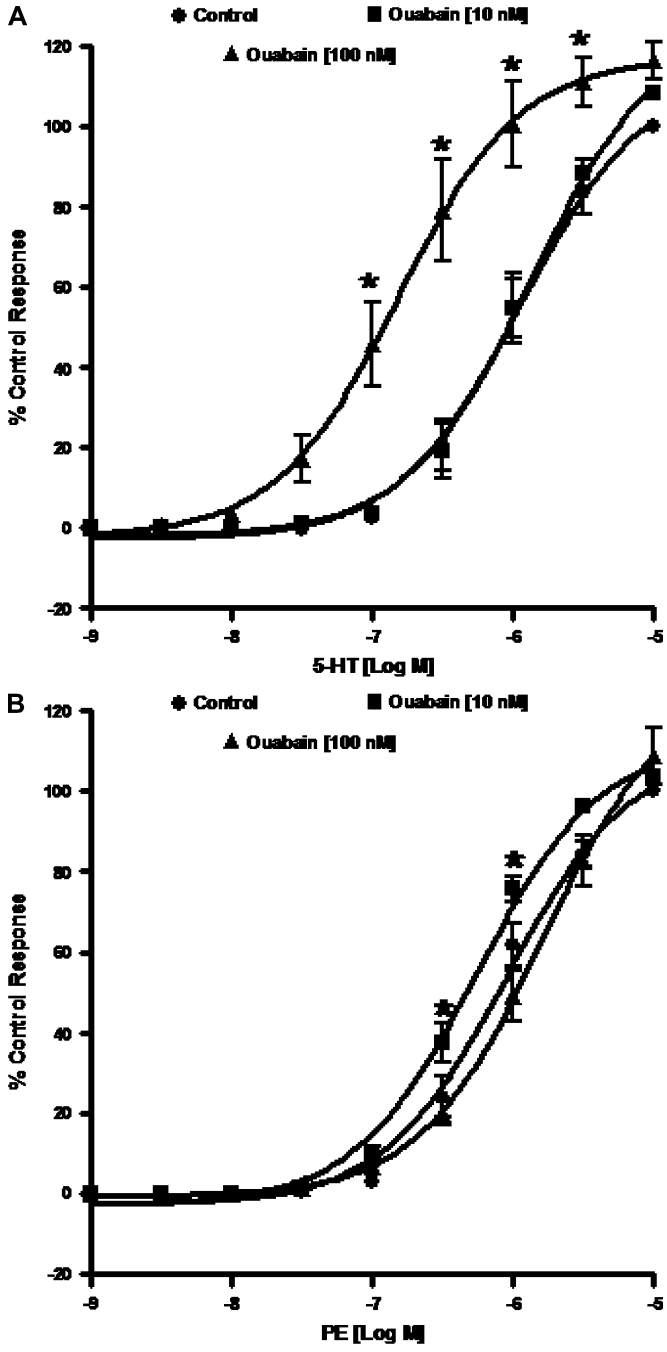

FIGURE 5. A, Effect of ouabain on concentration response to 5-HT. Contractions were elicited with 5-HT (10^{-9} M–10^{-5} M) in the absence or presence of ouabain (10 and 100 nM). Tissues were pretreated with ouabain for 30 minutes before 5-HT concentration-response curves were plotted. B, Effect of ouabain (10 and 100 nM) on concentration-dependent contractions induced by phenylephrine (10^{-9} M–10^{-5} M). Results are presented as mean ± SEM. *$P < 0.05$.

increase in the potency (pD_2, 6.81 ± 0.10) of the agonist but had no significant effect on its efficacy (E_{max}, 117 ± 5.0%). We, however, found no significant effect of 5-HT (1 μM; n = 6) per se on the plasmalemmal Na^+-K^+-ATPase activity of the pulmonary artery smooth muscle. For example, the basal Na^+-K^+-ATPase was 8.05 ± 2.45 nmol inorganic phosphate/mg protein/minute in comparison to activity (11.78 ± 3.01 nmol inorganic phosphate/min/mg protein) determined at 1 μM 5-HT. Unlike 5-HT, concentration-dependent contractions with adrenergic agonist phenylephrine (10^{-9} M–10^{-5} M) were not significantly influenced by ouabain (10 and 100 nM, Fig. 5B). For example, the pD_2 (6.05 ± 0.05) and E_{max} (110 ± 3.4%) of phenylephrine in the controls (n = 7) were not significantly different from those obtained in the presence of either 10 nM (pD_2, 6.26 ± 0.04; E_{max}, 112 ± 2.4%, n = 6) or 100 nM (pD_2, 5.80 ± 0.07; E_{max}, 125 ± 5.9%, n = 6) of ouabain. Nevertheless, a small but significant increase in contraction to phenylephrine at 0.3 and 1.0 μM was evident in the presence of ouabain 10 nM.

Effect of Ketanserin on 5-HT Contractions

Figure 6 shows the antagonism of 5-HT–induced vasoconstrictor responses by ketanserin in the pulmonary artery segments. 5-HT (10^{-9} M–10^{-5} M) produced concentration-dependent contractions (pD_2, 6.99 ± 0.07; E_{max}, 104.80 ± 3.70%; n = 8) of these vessels. Ketanserin (1 and 10 nM) concentration-dependently antagonized 5-HT responses. The pD_2 and E_{max} of 5-HT in the presence of 1 nM ketanserin were 6.64 ± 0.11 and 91.47 ± 4.70% (n = 4), respectively. Ketanserin (10 nM) caused a further rightward shift in the concentration-response curve of 5-HT (pD2, 6.22 ± 0.11, E_{max}, 94.84 ± 6.39%, n = 4). As shown in Figure 6, ketanserin at 2 concentration levels significantly decreased the potency, but not the efficacy, of 5-HT.

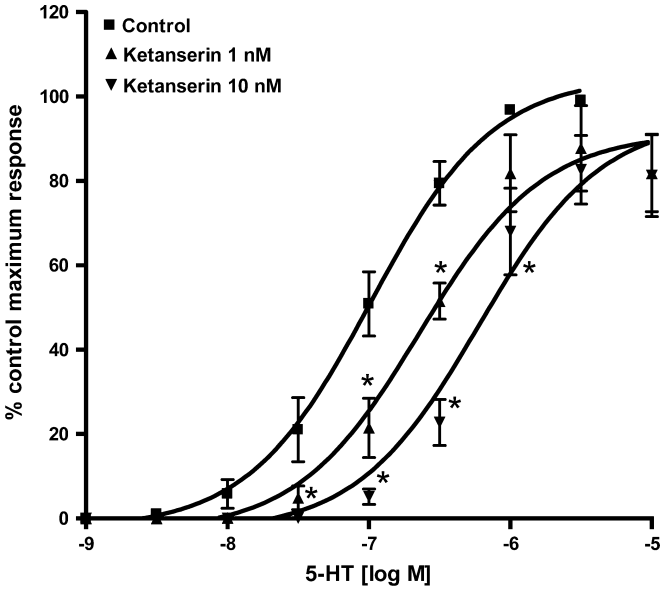

FIGURE 6. Antagonism of 5-HT (10^{-9} M–10^{-5} M)-induced concentration-dependent contractions by 1 and 10 nM of ketanserin in the sheep pulmonary artery. Results are presented as mean ± SEM. *$P < 0.05$. 5-HT contractions are expressed as per cent control maximum response.

Influence of Endothelium on KCl-Induced Relaxation

In the endothelium-intact pulmonary artery rings contracted with 5-HT (1 μM), ACh (10 μM) produced a fast relaxation of about 15%–60% with a mean value of 34 ± 4.6% (n = 6). In the endothelium-denuded rings, ACh (10 μM) always elicited a contractile response (0.20 ± 0.07 g, n = 7). As shown in Figure 7A, endothelium had no significant influence on KCl (0.3mM–10 mM)–induced relaxation. For example, in the endothelium-intact rings, the pD_2 and E_{max} were 3.08 ± 0.04 and 69.49 ± 4.92% (n = 7), respectively, which were not significantly different from those of endothelium-denuded preparations (pD_2, 2.99 ± 0.09, E_{max}, 73.58 ± 5.14%, n = 8). Similarly, KT5823 (2 μM) a specific inhibitor of protein kinase G, had no significant effect on KCl-induced relaxations (pD_2, 3.08 ± 0.11, E_{max}, 78.44 ± 5.68%, n = 6 versus control pD_2, 3.22 ± 0.14, E_{max}, 73.16 ± 6.00, n = 6; Fig. 7B).

DISCUSSION

In the present study, we demonstrate the presence of both α_1 and α_2 isoforms of Na^+-K^+-ATPase in the ovine pulmonary artery using polyclonal antibodies for the respective isoforms. The concentration-response curve for ouabain-induced inhibition of Na^+-K^+-ATPase was biphasic in nature, indicating that α_1 and $\alpha 2$ isoforms may possess low and high affinity, respectively, for the cardiac glycoside. Concentrations of ouabain <1 μM had no significant effect on the basal tone of the vessel, but a significant increase in the basal tone was evident with either 1 μM ouabain or K^+-free solution, consistent with the inhibition of the sodium pump. A lower concentration of ouabain (0.1 μM), however, selectively increased the vasoconstrictor potency of 5-HT, but not that of phenylephrine. Endothelium removal or treatment with protein kinase inhibitor KT 5823 had no significant influence on the sodium pump function in this vessel.

Western blot studies identify the presence of α_1 and α_2 isoforms of Na^+-K^+-ATPase in the ovine pulmonary artery, which are consistent with the presence of these 2 isoforms in rodent vasculature.[27] Both α_1 and α_2 isoforms of the enzyme showed sharp clear bands of molecular mass of ~110 kDa in the ovine pulmonary artery, whereas only α_1 isoform was detected in the rat kidney homogenate. This observation is consistent with a previous report that showed that rodent tissues predominantly express the α_1 isoform in comparison to the α_2 isoform (5%–10% of the Na^+-K^+-ATPase).[16] Using ouabain inhibition curve for plasma membrane Na^+-K^+-ATPase activity, it has been demonstrated that α_1 and α_2 isoforms possess low and high sensitivity, respectively, for the cardiac glycoside.[21] Similarly, the biphasic concentration-response curve for ouabain-induced inhibition of Na^+-K^+-ATPase activity in membrane preparation from ovine pulmonary artery, as shown in the present study, suggests that α_1 and α_2 isoforms may possess low and high affinity for

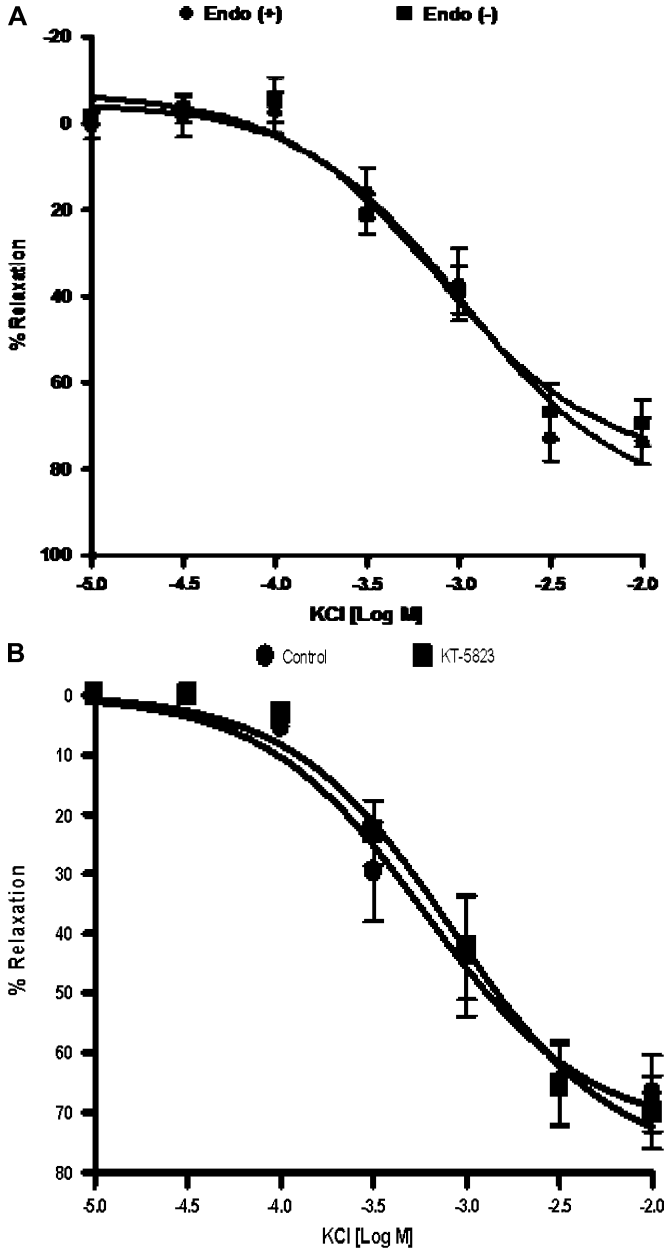

FIGURE 7. A, Influence of endothelium removal on KCl (10^{-5} M–10^{-2} M)-induced relaxation of pulmonary artery rings contracted with K^+-free solution plus 5-HT (1 μM). In the endothelium intact rings, the presence of endothelium was confirmed by the relaxation elicited with ACh (10 μM) in tissues precontracted with 5-HT (1 μM). In endothelium-denuded vessels, ACh (10 μM) always elicited a contraction over 5-HT-induced contraction. Results are presented as mean ± SEM. B, Effect of the pretreatment of endothelium-intact arterial rings with KT 5823 (2 μM) for 30 minutes on concentration-dependent relaxation elicited with KCl (10^{-5} M–10^{-2} M). Results are presented as mean ± SEM.

ouabain, respectively. This observation is in accordance with several previous reports, which show that Na^+-K^+-ATPase α-subunit isoforms have more than 1 ouabain affinity sites in nonrodent species, including humans.[28,29]

In addition to assessing the effect of ouabain on the Na^+-K^+-ATPase activity, we studied its effect on the functional sodium pump, which was measured as K^+-induced relaxation in tissues contracted with K^+-free solution plus 5-HT. According to some earlier reports, 5-HT has been shown to stimulate Na^+-K^+-ATPase activity in the canine femoral artery cells[30] and human placental veins.[31] In the present study, however, we observed that 5-HT had no significant effect on the plasmalemmal Na^+-K^+-ATPase activity. Ouabain 10 nM strongly inhibited (~92%) KCl-induced relaxations, but at this concentration it inhibited plasma membrane Na^+-K^+-ATPase activity by 29%. This discrepancy can be explained in terms of different experimental protocols used for the determination of the Na^+-K^+-ATPase. For example, Na^+-K^+-ATPase activity was determined biochemically in the presence of K^+ (14 mM) in the extracellular medium, whereas functional sodium pump was measured in the absence of extracellular K^+. In an earlier study on the α_1 isoform of the Na^+-K^+-ATPase from sheep kidney, it was shown that increasing extracellular concentrations of K^+ decreased the affinity of ouabain binding to the enzyme.[32] This phenomenon is well established and studied at the molecular level, showing that phosphorylated form of Na^+-K^+-ATPase (in the absence of extracellular K^+) has greater affinity for ouabain, and extracellular K^+, which induces dephosphorylation of the enzyme, reduces its affinity for the cardiac glycoside.[33]

Low concentration of Ba^{2+} is known to block inward rectifier potassium (K_{ir}) channels in vascular smooth muscles.[34] As observed in the present study, the lack of effect of low concentration of Ba^{2+} rules out the contribution of K_{ir} channels to K^+-induced relaxation in the ovine pulmonary artery. This observation further suggests that the stimulation of the sodium pump is solely responsible for K^+-induced relaxation of the arteries exposed to K^+-free solution.

Although ouabain (0.1 μM) caused near maximal inhibition of the pump activity, an increase in basal tension was evident at 1 μM concentration of the cardiac glycoside. The response was biphasic in nature, with a first phase of contraction followed by a more slow and sustained contraction. Phenoxybenzamine abolished the first phase but had no effect on the second phase of contraction. This observation is consistent with the contribution of adrenergic neurogenic component to the overall myogenic response elicited by ouabain, as evident in other vascular tissues.[35] Regarding the concentration of ouabain inducing myogenic response, our finding is in agreement with the observation made in mouse portal vein, where 1 μM ouabain caused a significant increase in basal tension.[14] There is considerable controversy regarding the role of α_1 and α_2 subunits in the regulation of vascular membrane potential and contractility. For instance, Weston et al[27] demonstrated that a high concentration of ouabain (500 μM), which would inhibit the α_1-containing isoform of Na^+/K^+-ATPase, produced smooth muscle depolarization, indicating that this isoform is active under basal conditions in the rat mesenteric artery. However, other studies provide evidence that α_2, but not the α_1, subunit was responsible for the regulation of vascular contractility and blood pressure in mice.[14,15] The results of the present study, however, indicate that the low-affinity isoform (α_1) regulates basal tone in the sheep pulmonary artery.

5-HT is a potent constrictor of pulmonary arteries. Further, it is considered to play an important role in pulmonary hypertension. Ouabain (0.1 μM) markedly increased the vasoconstrictor potency of 5-HT in the ovine pulmonary artery, implicating again the role of α_1 isoform in regulating agonist-induced contractility. This finding is of considerable significance from the point of view that a pathophysiologic condition, such as hypoxia, that inhibits pulmonary artery sodium pump would enhance the constrictor response to endogenous 5-HT. This view is supported by previous studies showing that ouabain enhanced the pulmonary vasoconstrictor response to hypoxia in intact animals (dogs and cats,[36] rat[37]). Unlike 5-HT, we observed that ouabain had no significant effect on the potency and efficacy of phenylephrine in this vessel. As shown in a previous study, the enhancement of serotonin-induced contractions by ouabain was far greater (5- to 6-fold shift) than contractions elicited with noradrenaline (1.6-fold shift) in rabbit ear artery.[38] These authors attributed it to a change in the state of ketanserin-sensitive 5-HT receptors. In the present study, we have demonstrated the presence of ketanserin-sensitive 5-HT_{2A} receptors in the sheep pulmonary artery. Therefore, it is reasonable to believe that the specific enhancement of 5-HT responses may involve a similar mechanism. Variable effects of ouabain on vasoconstrictor responses to agonists have been reported earlier. For example, ouabain increased the pressor responses to KCl, but not to angiotensin II, in rat lungs.[37] Further reports show that ouabain inhibited alpha-adrenergic agonist responses in rat pulmonary artery[39] and rabbit aorta.[40]

To study the influence of basal NO on the functional sodium pump, we assessed the influence of endothelium removal on the relaxation response to KCl in vessels contracted with 5-HT in K^+-free medium. Endothelium removal had no significant effect on the K^+-induced relaxation in this vessel. Similarly, the attenuation of basal cyclic guanosine monophosphate (cGMP) response by protein kinase G-inhibitor KT 5823 had no significant effect on the functional sodium pump. This observation is consistent with our earlier observation in the ovine pulmonary artery, where we demonstrated that cGMP did not stimulate this pump.[8,13] Nevertheless, some reports show the modulation of vascular sodium pump by endothelium in some blood vessels (mouse aorta,[15] human placental vessels,[41] rat aorta[42]). Such a discrepancy may relate to the difference in vascular bed or the species of animal used in a particular experiment.

In conclusion, the results of the present study suggest that both α_1 and α_2 isoforms of Na^+-K^+-ATPase, present in ovine pulmonary artery, possess low and high affinity, respectively, for ouabain. Further, inhibition of the low, but not the high, affinity isoforms of Na^+-K^+-ATPase by ouabain significantly increased the basal tone and 5-HT–induced contractility in this vessel. The implications of these findings are that inhibition of this pump by either endogenous ouabain-like compounds or hypoxia may relate to a pathophysiologic condition, such as pulmonary hypertension. Further studies, however, are needed to elucidate the distinct physiologic roles of α_1 and α_2 subunits in pulmonary artery function.

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