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Role of Na^+-K^+ ATPase in cyclic GMP-mediated relaxation of canine pulmonary artery smooth muscle cells

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1 Sodium-potassium adenosine triphosphatase (Na^+-K^+ ATPase) plays a role in the regulation of vascular tone, but contribution of this enzyme to nitrovasodilator-induced pulmonary vasodilatation remains uncertain. We thus studied the interaction between guanosine 3′:5′-cyclic monophosphate (cyclic GMP) and Na^+-K^+ ATPase in smooth muscle cells isolated from canine pulmonary artery.

2 To assess the contractile properties, changes in smooth muscle cell length were determined microscopically. Application of potassium chloride (KCl) shortened the cell length, an effect which was reduced by sodium nitroprusside and 8-bromo-cyclic GMP in a concentration-dependent manner. Pretreatment of cells with the cyclic GMP-dependent kinase inhibitor KT 5823 (2 μM) abolished the effects of sodium nitroprusside and 8-bromo-cyclic GMP.

3 Ouabain (0.3 μM) did not alter the KCl-induced muscle shortening, but inhibited the relaxant responses to sodium nitroprusside and 8-bromo-cyclic GMP.

4 Incubation of smooth muscle cells with sodium nitroprusside concentration-dependently increased intracellular cyclic GMP levels and ouabain-sensitive ^{86}Rb uptake, and these values were significantly correlated. In the presence of KT 5823, sodium nitroprusside increased cyclic GMP levels but did not alter ouabain-sensitive ^{86}Rb uptake.

5 These results suggest that there is a link between accumulation of intracellular cyclic GMP and activation of sarcolemmal Na^+-K^+ ATPase in pulmonary artery smooth muscle cells and that this link may be involved in the sodium nitroprusside-induced pulmonary vasodilatation.

Keywords: Vascular smooth muscle; sodium nitroprusside; ouabain; ^{86}Rb uptake; pulmonary vasodilatation

Introduction

The sarcolemmal sodium-potassium adenosine triphosphatase (Na^+-K^+ ATPase) has been implicated in the mechanism of β-adrenoceptor agonist-induced relaxation of airway and vascular smooth muscle (Webb & Bohr, 1981; Gunst & Stropp, 1988). Stimulation of enzymatic activity of Na^+-K^+ ATPase by adenosine 3′:5′-cyclic monophosphate (cyclic AMP) may lead to generation of the Na gradient necessary to exclude Ca^{2+} via the Na^+/Ca^{2+} exchange or hyperpolarization of the membrane, which in turn reduces Ca^{2+} influx through membrane potential-dependent Ca^{2+} channels (Fleming, 1980). We have recently shown that hypoxia inhibits Na^+-K^+ ATPase activity and impairs the relaxant responses of canine pulmonary artery to sodium nitroprusside, an agent that generates guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Tamaoki *et al*., 1996). However, a link between intracellular cyclic GMP and sarcolemmal Na^+-K^+ ATPase has not been demonstrated and, hence, the role of Na^+-K^+ ATPase in the nitrovasodilator-induced pulmonary vasodilatation remains to be elucidated.

Many studies have used isolated arteries to study the contractile and relaxant responses without the influence of extravascular tissues. Whereas much information has been gained from this experimental preparation, contributions from the vascular endothelium still complicate interpretation of the results. Although endothelial cells can be mechanically removed or chemically inactivated, use of these methods raises the possibility of smooth muscle damage. The development of techniques to isolate and study smooth muscle cells offers a useful alternative to endothelium removal (Warshaw *et al*., 1986; Subramanian *et al*., 1991). Therefore, in the present study, to study the interaction between cyclic GMP and Na^+-K^+ ATPase, we examined the effects of sodium nitroprusside and the cell-permeable cyclic GMP analogue 8-bromo-cyclic GMP on the contractile responses to potassium chloride (KCl) in smooth muscle cells isolated from canine pulmonary artery.

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Methods

Preparation of pulmonary artery smooth muscle cells

Adult mongrel dogs of either sex (17–31 kg) were anaesthetized with sodium pentobarbitone (35 mg kg^{-1}, i.v.). After thoracotomy, the lungs were removed and second branches of pulmonary lobar arteries (2–3 mm in diameter) were dissected free from surrounding tissue and immersed in Krebs-Henseleit (Krebs) solution of the following composition (in mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25.5 and D-glucose 5.6 (pH 7.4, 4°C). The arteries were then transferred to 5 ml of Krebs solution containing 157 u ml^{-1} collagenase, 50 u ml^{-1} elastase, 5 u ml^{-1} deoxyribonuclease, 1.5% bovine serum albumin, 4 mM adenosine 5′-triphosphate (ATP) and 0.1% soybean trypsin inhibitor, and incubated for 90 min at 37°C to isolate smooth muscle cells. Enzymatic digestion was terminated by adding an equal volume of medium 199 plus 10% foetal bovine serum, and the solution was filtered to remove connective tissue and cell debris. The filtrate containing a mixture of smooth muscle cells, endothelial cells and fibroblasts was centrifuged at 360 *g* for 6 min. The pellet was suspended in medium 199 plus 10% foetal bovine serum to adjust the cell concentration to 10^5 cells ml^{-1}. The cell mixture (0.1 ml) was plated on sterilized glass cover slips coated with 100 μg ml^{-1} poly-L-lysine, which were then put into culture dishes containing 2.5 ml of medium 199 plus 10% foetal bovine serum and placed in a water-jacketed incubator at 37°C for 24 h. After the 24-h incubation period, smooth muscle cells that had adhered to the coverslips were used in contraction studies.

The viability of the cells was 75–91%, as determined by the trypan blue exclusion test. Because endothelial cells died almost immediatley after plating, the cells on the coverslips were a mixture of smooth muscle cells and fibroblasts. The smooth muscle cells comprised at least 65% of a given cell culture, and could be distinguished from fibroblasts by their spindle-shape morphology and distinct plasma membrane. These cells were confirmed to be smooth muscle cells based on the positive staining for smooth muscle-specific mouse anti-myosin antibody.

Contraction studies

The method for the measurement of smooth muscle cell contraction has been described in detail elsewhere (Madden *et al*., 1992). Briefly, a cover slip containing smooth muscle cells was mounted in a specially constructed chamber and placed on the stage of an inverted microscope (NFX-II, Nikon, Tokyo). The cells were continuously perfused (0.5 ml min^{-1}) with Krebs solution (pH 7.4, 37°C) gassed with a mixture of 95% O_2 and 5% CO_2. To measure contractile responses of smooth muscle cells, the image during contraction was recorded on a video camera (Sony, VO-5800, Tokyo) with a videocassette recorder capable of freeze-frame replay, and changes in smooth muscle cells length were determined. Normally, five or six smooth muscle cells were seen within the visual field of the microscope at a magnification of $\times 200$ and these cells were measured throughout the study.

After the equilibration of smooth muscle cells for 30 min, KCl (50 mM) was added to the chamber and the maximal contraction of the cells was determined. To determine the relaxation of KCl-contracted cells, smooth muscle cells were incubated with various concentrations of sodium nitroprusside or 8-bromo-cyclic GMP (1, 10 and 100 μM) and 15 min later the muscle shortening induced by 50 mM KCl was measured. To assess the involvement of cyclic GMP-dependent protein kinase and a change in Na^+-K^+ ATPase activity, cells were incubated for 15 min with KT 5823 (2 μM), a specific inhibitor of cyclic GMP-dependent protein kinase (Kase *et al*., 1987), or ouabain (0.3 μM), an inhibitor of Na^+-K^+ ATPase, then the relaxant responses of KCl-contracted cells to sodium nitroprusside and 8-bromo-cyclic GMP were similarly determined. In our preliminary experiments, KT 5823 or ouabain at the concentrations used in the present study did not change the resting length or the KCl-induced contraction of pulmonary artery smooth muscle cells.

Measurement of cyclic GMP levels

Using paired cell cultures from the same dogs, we assessed the link between cyclic GMP levels and Na^+-K^+ ATPase activity in pulmonary artery smooth muscle cells. To evaluate cellular cyclic GMP synthesis, the cells were incubated with sodium nitroprusside (1, 10 and 100 μM) in the absence and presence of KT 5823 (2 μM), and 15 min later the cells were sonicated and placed in ice-cold 10% trichloroacetic acid with [^{3}H]-cyclic GMP added as a tracer of recovery determination. After the extraction of trichloroacetic acid with ether, the residue was dissolved in acetate buffer. The cyclic GMP levels were determined in duplicate by radioimmunoassay according to the method of Brooker *et al*. (1979), corrected for ether extraction of 87% recovery, and normalized for protein content of the cells as determined by the method of Lowry *et al*. (1951).

Measurement of ouabain-sensitive ^{86}Rb uptake

The Na^+-K^+ ATPase activity was determined by measuring ouabain-sensitive ^{86}Rb uptake (Gupta *et al*., 1992). After the incubation of cells with various concentrations of sodium nitroprusside (1, 10 and 100 μM) for 15 min in the absence or presence of KT 5823 (2 μM), ^{86}RbCl (final concentration, 1 μCi ml^{-1}) was added and 10 min later the cells were washed with ice-cold unlabelled Krebs solution to remove radioisotope from the extracellular compartment. The cells were disrupted with 10% trichloroacetic acid and the radioactivity incorporated into cells was measured by scintillation counting. To assess Na^+-K^+ ATPase activity, the maximally-effective concentration of ouabain (0.2 mM) was added 10 min before the introduction of ^{86}RbCl into the medium, and the ouabain-sensitive portion of the ^{86}Rb uptake was calculated by subtracting the ouabain-insensitive ^{86}Rb uptake from the total ^{86}Rb uptake. The results are expressed as nmol min^{-1} mg^{-1} protein. In our preliminary experiments, KT 5823 *per se* did not change ouabain-sensitive ^{86}Rb uptake.

Materials

Elastase, deoxyribonuclease, smooth muscle-specific mouse anti-myosin antibody, sodium nitroprusside, 8-bromo-cyclic GMP and ouabain were purchased from Sigma (St. Louis, MO, U.S.A.) and collagenase and soybean trypsin inhibitor were from Wako Pure Chemical Company (Osaka, Japan). KT 5823 (N-methyl-(8**R***, 9**S***, 11**S***)-(−)-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H, 8H, 11H-2,7b,11a-triazadibenzo[a,g]cycloocta[cde] trinden-1-one)) was a gift from Kamiya Biomedical Company (Thousand Oaks, CA, U.S.A.), and medium 199 was obtained from GIBCO (Grand Island, NY, U.S.A.). [^{3}H]-cyclic GMP and ^{86}RbCl were obtained from Amersham (Tokyo, Japan). All other chemicals were of reagent grade and obtained from common commercial sources.

Statistics

All values are presented as means ± s.e.mean. Statistical analysis was performed by ANOVA followed by multiple comparisons or by Student's t test. $P<0.05$ was considered statistically significant.

Results

Contractile responses

The changes in length of pulmonary artery smooth muscle cells are shown in Figure 1. These data were obtained from at least 19 different dogs and 96 separate cultures. After the equilibration period, the length of smooth muscle cells was 126 ± 11 μm ($n=296$) in Krebs solution. The smooth muscle cells began to decrease in length within 1 min after the addition of KCl. The maximal shortening was 26.8 ± 3.0% ($n=19$) of their initial length and was observed within 5 min of exposure to KCl. Incubation of the smooth muscle cells with sodium nitroprusside or 8-bromo-cyclic GMP had no effect on the resting length, but decreased the contractile response to the subsequent addition of KCl in a concentration-dependent manner. A significant decrease was noted at concentrations of 10 and 100 μM for each drug, and this decrease was abolished by preincubation with KT 5823.

When the smooth muscle cells were exposed to KCl in the presence of ouabain, mean cell length was, on average, 3% shorter, but it was not significantly different from the value in the absence of ouabain. In ouabain-treated smooth muscle cells, relaxant responses to 10 μM sodium nitroprusside and 8-bromo-cyclic GMP were abolished, and the effectiveness of these drugs at 100 μM was reduced in comparison to that in cells which had not been treated with ouabain (compare Figures 1 and 2).

Cyclic GMP levels and Na^+-K^+ ATPase activity

Incubation of smooth muscle cells for 15 min with various concentrations of sodium nitroprusside increased cyclic GMP levels in the absence and presence of KT 5823, in a concentration-dependent manner and to a similar extent. Ouabain-sensitive ^{86}Rb uptake, which comprised approximately 40% of

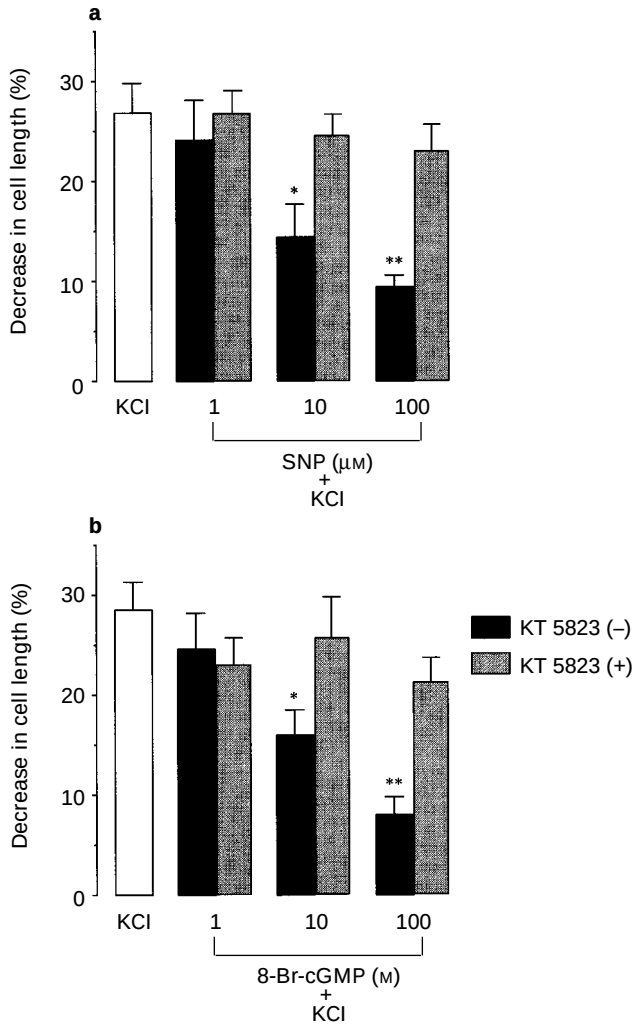

Figure 1 Relaxant responses of canine pulmonary artery smooth muscle cells to sodium nitroprusside (SNP, a) and 8-bromo-cyclic GMP (8-Br-cGMP, b). The cells were incubated for 15 min with various concentrations of SNP or 8-Br-cGMP in the absence and presence of KT 5823 (2 μM), and the contractions induced by KCl (50 mM) were determined. Responses are expressed as % decrease from the initial length. Data are means ± s.e.mean, $n=4$ cultures for each column. $^*P<0.05$, $^{**}P<0.01$, significantly different from the response to KCl alone (open columns).

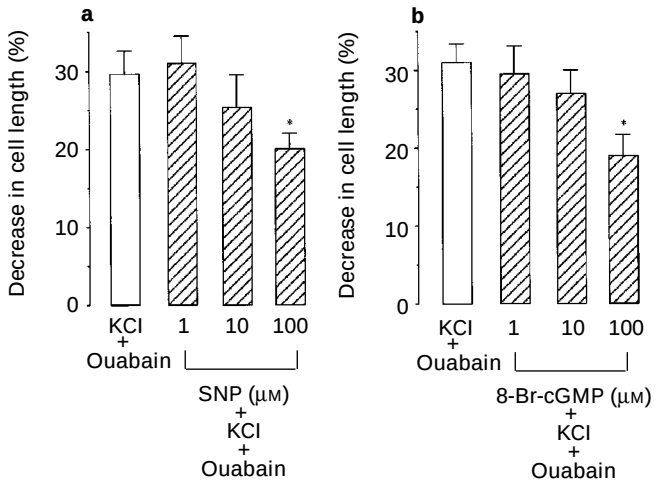

Figure 2 Effect of ouabain on relaxant responses of canine pulmonary artery smooth muscle cells to (a) sodium nitroprusside (SNP) and (b) 8-bromo-cyclic GMP (8-Br-cGMP). The cells were incubated for 15 min with various concentrations of SNP and 8-Br-cGMP in the presence of ouabain (0.3 μM), and the contractions induced by KCl (50 mM) were determined. Responses are expressed as % decrease from the initial length. Data are means ± s.e.mean, $n=4$ cultures for each column. $^*P<0.05$, significantly different from the response to KCl plus ouabain (open columns).

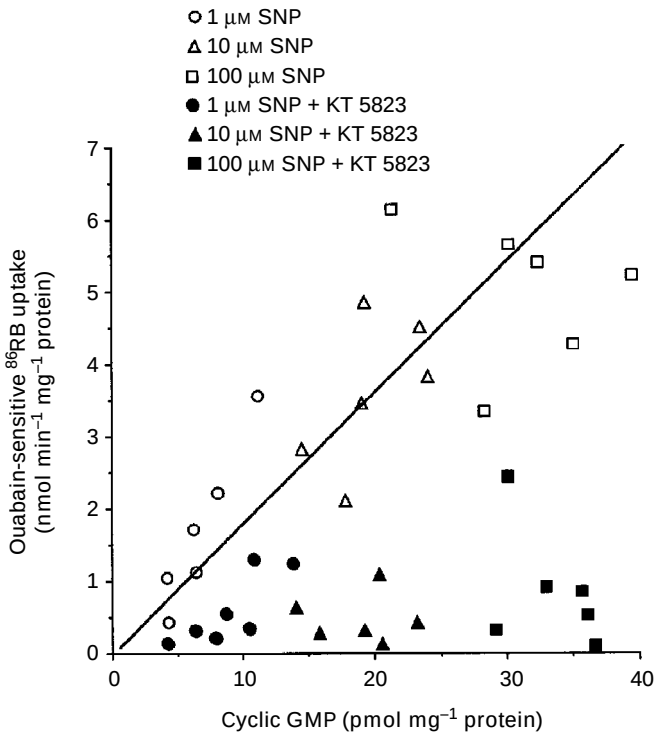

Figure 3 Effect of sodium nitroprusside (SNP) on intracellular cyclic GMP levels and ouabain-sensitive ^{86}Rb in canine pulmonary artery smooth muscle cells. The cells were incubated for 15 min with various concentrations of SNP (1, 10 and 100 μM) in the absence and presence of KT 5823 (2 μM). The values for cyclic GMP and ouabain-sensitive ^{86}Rb uptake without KT 5823 treatment were significantly correlated ($P<0.05$, $n=18$).

total ^{86}Rb uptake, was also increased by sodium nitroprusside and this effect was greatly attenuated by KT 5823. In the cells without KT 5823 treatment, the values for cyclic GMP levels and ouabain-sensitive ^{86}Rb uptake were significantly correlated ($P<0.05$, $n=18$; Figure 3).

Discussion

This study demonstrated that vascular smooth muscle cells isolated from the canine pulmonary artery respond to the smooth muscle depolarizing agent KCl, the nitrovasodilator sodium nitroprusside, and the cell-permeable cyclic GMP analogue 8-bromo-cyclic GMP, in a similar way to intact tissues. Addition of KCl shortened the smooth muscle cells with a time course similar to that of smooth muscle cells from cat pulmonary and cerebral arteries from cats (Madden *et al.*, 1992), and the magnitude of this contraction was concentration-dependently reduced by sodium nitroprusside and 8-bromo-cyclic GMP. Furthermore, these responses did not require the presence of endothelial cells or extravascular tissues. Although other studies have found that smooth muscle cells maintained in culture may exhibit phenotypic changes (Chamley *et al.*, 1977) or uncharacteristic responses to vasoactive agents (Mauger *et al.*, 1975; Chamley-Campbell *et al.*, 1979), the freshly-isolated, non-dedifferentiated smooth muscle cells used in the present experiments were viable and retained *in situ* contractile and relaxant properties.

Vascular relaxation in response to nitrovasodilators involves signal transduction from nitric oxide to cyclic GMP synthesis via activation of the soluble guanylyl cyclase. Once stimulated, guanylyl cyclase generates cyclic GMP, which in turn causes smooth muscle relaxation (Bohme *et al*., 1978). In the present study, the relaxant responses of pulmonary artery smooth muscle cells to sodium nitroprusside and 8-bromo-cyclic GMP were abolished by KT 5823, a specific cyclic GMP-dependent protein kinase inhibitor (Kase *et al*., 1987). Thus the observed vasodilatation was accomplished by the activation of cyclic GMP-dependent protein kinase.

There is ample evidence that sarcolemmal Na^+-K^+ ATPase, which contributes to the maintenance of an electrochemical gradient of Na^+ and K^+ across the cell membranes, plays an important role in the regulation of vascular smooth muscle tone (Clausen & Nielsen, 1994). An increase in Na^+-K^+ ATPase activity may induce smooth muscle relaxation by increasing Na^+/Ca^{2+} exchange and reducing the Ca^{2+} influx through membrane potential-dependent calcium channels (Clausen & Nielsen, 1994). In the present study, incubation of the pulmonary artery cells with the Na^+-K^+ ATPase inhibitor ouabain did not significantly alter the contractile responses to KCl, but substantially attenuated the subsequent relaxations induced by sodium nitroprusside and 8-bromo-cyclic GMP.

The sensitivity of Na^+-K^+ ATPase to ouabain is variable depending on tissues and animal species (Akera & Brody, 1978), and it is uncertain whether the concentration of ouabain used in our contraction studies (0.3 μM) was sufficient to inhibit this enzyme completely in the canine pulmonary artery smooth muscle cells. However, we chose this concentration based on previous studies showing that K^+ loss and Na^+ accumulation were produced by 50 nM ouabain in the guinea-pig atria (Godfraind & Ghysel-Burton, 1977) and that approximately 40% of Na^+-K^+ ATPase activity in the vas deferens was inhibitied by 0.3 μM ouabain (Gerthoffer *et al*., 1979). Our results indicated that cyclic GMP produced vasodilatation, at least in part, through stimulation of Na^+-K^+ ATPase activity.

In support of this view, we found that sodium nitroprusside produced a concentration-dependent increase in ouabain-sensitive ^{86}Rb uptake by cultured cells, an index of Na^+-K^+ ATPase activity (Akera *et al*., 1981), and that this increase was not observed in the presence of KT 5823. Furthermore, the sodium nitroprusside-induced increases in cyclic GMP levels and ouabain-sensitive ^{86}Rb uptake were correlated in cells which had not been treated with KT 5823. These findings suggest that the nitric oxide donor sodium nitroprusside may act, at least in part, by stimulation of smooth muscle Na^+-K^+ ATPase activity via cyclic GMP-dependent protein kinase.

In contrast to the findings of the present study, Gupta and coworkers (1995) have recently shown that nitric oxide stimulates Na^+-K^+ ATPase activity independently of its ability to increase intracellular cyclic GMP in human corpus cavernosum. The reason for the discrepancy between their results and ours is unknown, but could be due to the difference in species and/or cells. Moreover, because our cell culture contained fibroblasts and because Na^+-K^+ ATPase activity is present in these cells (Bhutada & Ismail-Beigi, 1991), the observed effects of sodium nitroprusside may not be attributed solely to smooth muscle cells.

Studies on rat aorta (Rapoport *et al*., 1985) and on canine femoral artery (DeMey & Vanhoutte, 1980) have shown that cyclic GMP stimulates Na^+-K^+ ATPase activity in vascular tissues and that Na^+-K^+ ATPase plays a part in endothelium-dependent vasodilatation. However, any additional role for smooth muscle Na^+-K^+ ATPase in vasodilatation was not established in those studies. It is thus possible that increasing endothelial Na^+-K^+ ATPase activity enhances the release of the endothelium-derived relaxing factor, nitric oxide, and that this increases cyclic GMP levels in vascular smooth muscle cells, which in turn causes vascular relaxation through a mechanism independent of smooth muscle Na^+-K^+ ATPase (Rapoport *et al*., 1985; Boulanger *et al*., 1989; Alonso *et al*., 1993). However, our present experiments in isolated smooth muscle cells excluded any modulation by endothelial cells and showed that cyclic GMP activated vascular smooth muscle Na^+-K^+ ATPase. Although the actual mechanism by which cyclic GMP stimulates smooth muscle Na^+-K^+ ATPase activity remains to be determined, it appears to involve cyclic GMP-dependent protein kinase.

In conclusion, this study provides the first demonstration of a link between the intracellular cyclic GMP concentration and sarcolemmal Na^+-K^+ ATPase activity in freshly-isolated pulmonary artery smooth muscle cells. It seems likely that this link may play a part in the sodium nitroprusside-induced pulmonary vasodilatation.

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