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Nitric oxide modulates Na^+, K^+-ATPase activity through cyclic GMP pathway in proximal rat trachea

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Abstract

The present work demonstrated that nitric oxide (NO) modulates Na^+, K^+-ATPase activity in the proximal rat trachea. Sodium nitroprusside induced concentration-dependent (10–100 μM) stimulation in proximal trachea Na^+, K^+-ATPase activity. The effect was specific for Na^+, K^+-ATPase since Mg-ATPase activity was unaffected. This NO-donor changed neither Na^+, K^+-ATPase nor Mg-ATPase activity in the distal segment. The modulatory action on Na^+, K^+-ATPase induced by sodium nitroprusside was linked to an increase in nitrates/nitrites and cyclic GMP levels in proximal segments. Modulation of proximal Na^+, K^+-ATPase activity by sodium nitroprusside was mimicked by *S*-nitroso-*N*-acetylpenicillamine (100 μM) and 8-bromo-cyclic GMP (100 μM). Both sodium nitroprusside and 8-bromo-cyclic GMP effects on Na^+, K^+-ATPase activity of proximal segments of trachea were blocked by 2 μM of KT 5823 (a cyclic GMP-dependent protein kinase inhibitor), but not by 0.5 μM of KT 5720 (a cyclic AMP-dependent protein kinase inhibitor). Both kinase inhibitors decreased proximal Na^+, K^+-ATPase activity, but did not change Mg-ATPase activity. Okadaic acid (1 μM), a phosphatase-1 inhibitor, increased proximal Na^+, K^+-ATPase but not Mg-ATPase activity. The effect of okadaic acid was non-additive with that of 8-bromo-cGMP on Na^+, K^+-ATPase activity. Our results suggest that NO modulates proximal rat trachea Na^+, K^+-ATPase activity through cyclic GMP and cyclic GMP-dependent protein kinase.

Keywords: Nitric oxide (NO); cGMP; Protein kinase G; Na^+, K^+-ATPase; Trachea, rat

1. Introduction

The smooth muscle relaxation induced by nitric oxide (NO)-generating compounds has been considered to be mediated by the increase in guanosine 3′,5′-cyclic monophosphate (cyclic GMP), formed through the NO catalytic action on soluble guanylyl cyclase, which in turn activates cyclic GMP-dependent protein kinase (Katsuki and Murad, 1977; Murad et al., 1978; Buga et al., 1989; Gruetter et al., 1989; Torphy, 1994; Nijkamp and Folkerts, 1994, 1995; Folkerts et al., 1995). Dilation responses of airways smooth muscle of several species elicited by the inhibitory non-cholinergic non-adrenergic system are thought to be mediated by NO also (Katsuki and Murad, 1977; Murad et al., 1978; Buga et al., 1989; Gruetter et al., 1989; Li and Rand, 1991; Belvisi et al., 1992; Ellis and Undem, 1992; Nijkamp and Folkerts, 1994). In addition, NO is involved in airway inflammation and, therefore, it is assumed that NO plays a double role in the regulation of physiological and pathophysiological processes of airways (Barnes and Belvisi, 1993; Belvisi et al., 1995).

We analysed the regulatory role of the Na^+, K^+-ATPase pump in kidney, using agents which increase cyclic GMP and, consequently, activate cyclic GMP-dependent protein kinase. Our data showed a novel intracellular mechanism for Na^+, K^+-ATPase regulation (McKee et al., 1994; Scavone et al., 1995). The results also showed that the

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direction of the effect on the pump depends on the predominant αNa^+, K^+-ATPase isoform present. Accordingly, atrial natriuretic peptide, NO-generating compounds (such as sodium nitroprusside and nitroglycerine), and hormones that increase NO production and cyclic GMP levels induce a decrease in the activity of the α_1-isoform enzyme (McKee et al., 1994; Scavone et al., 1995). Conversely, carbon monoxide (CO) and glutamate, as well as free radicals, induce a long-lasting increase in rat cerebellar $\alpha_3 Na^+$, K^+-ATPase activity (Nathanson et al., 1995).

The anatomical organisation of airways may imply physiologically relevant regional differences that could result in different tonus responses. Thus, Ward et al. (1995) reported that human airways show a heterogeneous distribution of NO-immunoreactive nerves in airways. Besides, proximal and distal isolated segments from tracheal smooth muscle show different degrees of responsiveness to serotonin, histamine, acetylcholine and immunological challenges (Souhrada et al., 1983; Zimmerman et al., 1979; Tavares da Lima and Silva, 1998). It was also found that NO can reverse the cholinergically induced bronchoconstriction in guinea-pig (Dupuy et al., 1992). Moreover, it was demonstrated that NO synthesis inhibitors induce a hyperresponsiveness of guinea-pig airways to histamine in vitro and in vivo which can be reversed by L-arginine (Nijkamp et al., 1993). Furthermore, high concentrations of inhaled NO produce bronchodilation in vivo and protect against cholinergic bronchoconstriction in guinea-pig (Dupuy et al., 1992). Therefore, the different degree of responsiveness to histamine, as well as to acetylcholine, serotonin and immunological challenge in proximal and distal tracheal smooth muscle could be related to a differential NO production or to uneven effects in each of these segments.

Considering that the Na^+, K^+-ATPase system appears also to be related to substrates for cyclic GMP-dependent protein kinase in tracheal smooth muscle, which in turn may mediate the relaxing responses of airways via the NO pathway, we now analysed whether proximal and distal segments of rat trachea show a NO modulatory action on Na^+, K^+-ATPase activity through cyclic GMP and cyclic GMP-dependent protein kinase. The effects of two NO-generating compounds (sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine), as well as of a cell-permeable analogue of cyclic GMP (8-bromo-cyclic GMP), on Na^+, K^+-ATPase and Mg-ATPase activities were determined in proximal and distal segments of rat trachea. Also, the effects of sodium nitroprusside or 8-bromo-cyclic GMP in the presence of the inhibitor of either cyclic AMP-dependent protein kinase or cyclic GMP-dependent protein kinase activity on both ATPases activities (Na^+, K^+-ATPase and Mg-ATPase) were determined in the proximal segment of the trachea. Finally, the influence of the inhibition of phosphatase activity by okadaic acid on 8-bromo-cyclic GMP effects on proximal Na^+, K^+-ATPase was determined.

2. Materials and methods

2.1. Tissue preparation

Male Wistar rats (4-months old) were killed with an overdose of chloral hydrate (> 400 mg/kg, i.p.), exsanguinated and the thorax was cut open. The trachea was removed and dissected free of adherent connective tissue, and was divided into two tubular portions (rings), here designated as proximal (corresponding to 3–5 first cartilaginous rings close to the larynx) and distal segments (corresponding to 3-5 last cartilaginous ring close to the carina). The segments of each ring portion (proximal or distal) from 10 animals were pooled and slices ($0.3 \times 0.3 \times 1$ mm) were prepared on a Brinkmann tissue chopper, washed extensively to remove small particles, cooled to 4°C and resuspended (15 mg/ml) in buffer containing (in mmol/l): NaCl, 137; KCl, 5; $MgSO_4$, 0.8; $CaCl_2$, 1.0; HEPES, 10; and NaOH to adjust the pH to 7.4 at 34°C.

2.2. Measurement of Na^+, K^+-ATPase activity

The drugs were added (10 μl) to tubes containing 990 μl aliquots of the tissue preparation (five replicates), incubated for 15 min at 34°C, and then rapidly frozen on dry ice to stop the reaction. The samples were thawed, homogenised, centrifuged at $1700 \times g$ for 5 min and the supernatants were removed, heated at 90°C for 5 min to prevent degradation of cyclic GMP, and kept at −80°C for cyclic GMP assay. A fresh reaction buffer (1.0 ml of ATPase reaction buffer containing in mmol/l: NaCl, 85; KCl, 20; $MgCl_2$, 4; EGTA, 0.2; histidine, 30; and NaOH, approximately 20 μl to adjust to pH 7.2 at 34°C) was added to each tube, followed by the addition of a V_{max} concentration (10 mM) of ATP and 0.3 up to 0.5 μCi of γ-^{32}P-ATP. The tubes were incubated for 60 min at 34°C, and aliquots (100 μl) of reaction buffer were periodically withdrawn from each tube to assay hydrolysed ^{32}P. Hydrolysed ^{32}P was measured by scintillation counting of labelled ATP by addition of five volumes of 5% trichloroacetic acid containing 10% activated charcoal and 1 mM NaH_2PO_4. For determination of the ouabain-sensitive portion of total-ATPase activity, ^{32}P released in complete buffer for each group was compared with the activity of identically treated slices incubated in reaction buffer containing 3 mM ouabain, and in which NaCl and KCl had been replaced by choline hydrochloride, and Tris base was added for pH adjustment. This latter, ouabain-insensitive ATPase (Mg-ATPase) was subtracted from that found earlier (total ATPase) and the activity was corrected for protein content, determined in the homogenised tissue by colorimetric (Biorad, Melville, NY) assay. Under the incubation conditions used for the ATPase reaction, [Na^+], [K^+] and [MgATP] were saturating and basal Na^+, K^+-ATPase was optimised.

2.3. Cyclic GMP measurement

The content of cyclic GMP in the supernatants was determined by radioimmunoassay after acetylation of samples (DuPont-New England Nuclear, Boston, MA). Preliminary experiments indicated that the amount of cyclic GMP released after the permeabilization procedure was equivalent to that released after homogenisation of slices.

2.4. Determination of nitrite / nitrate

The drugs were added (10 μl) to tubes containing 990 μl aliquots of the tissue preparation (five replicates), incubated for 15 min at 34°C, and then rapidly frozen on dry ice to stop the reaction. Samples were thawed, homogenised, centrifuged at $1700 \times g$ for 5 min and the supernatants were removed. NO production was determined indirectly by quantification of the nitrates/nitrites levels, as described by Green et al. (1982), in the supernatant obtained from proximal segments of trachea treated with sodium nitroprusside (1 and 100 μM). In brief, samples (50 μl) were incubated with the same volume of Griess reagent at room temperature for 10 min. Absorbance was determined with an ELISA Multiskan microplates reader at 550 nm. The results were plotted on a standard curve generated by analysing standards to which nitrate and nitrite had been added in known ratios.

2.5. Reagents

Routine reagents, sodium nitroprusside, 8-bromo-cyclic GMP, alamethicin, ouabain, KT 5720 and KT 5823 were obtained from Sigma (St. Louis, MO). ATP tetra-(triethylamonium) salt (G-^{32}P) (6000 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). *S*-nitroso-*N*-acetylpenicillamine and okadaic acid were obtained from Research Biochemicals International (Natick, MA).

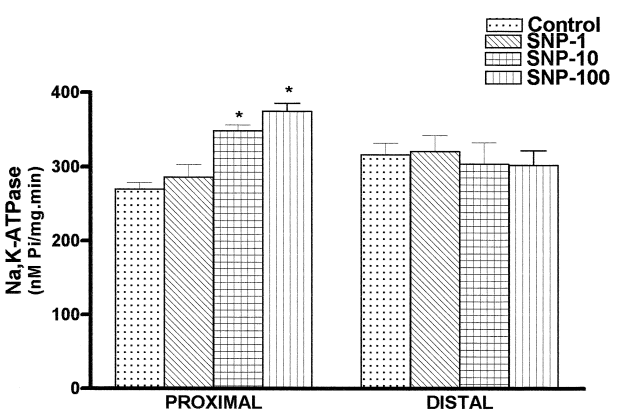

Fig. 1. Effects of sodium nitroprusside (1–100 μM) on Na^+, K^+-ATPase activity in proximal and distal rat trachea. Proximal or distal homogenates were incubated with each drug for 15 min at 34°C. Following incubation, the drug was removed and ATPase was assayed. Mg-ATPase activity was measured in the presence of 3 mM ouabain. Na^+, K^+-ATPase was determined from the difference between total ATPase and Mg-ATPase activity. Statistical analysis: Na^+, K^+-ATPase proximal-ANOVA, $F(3,8) = 18.2$, $P < 0.001$, Student–Newman–Keuls: control = sodium nitroprusside 1 μM < sodium nitroprusside 10 μM = sodium nitroprusside 100 μM, $^*P < 0.01$. Na^+, K^+-ATPase distal-ANOVA, $F(3,8) = 0.9$, NS.

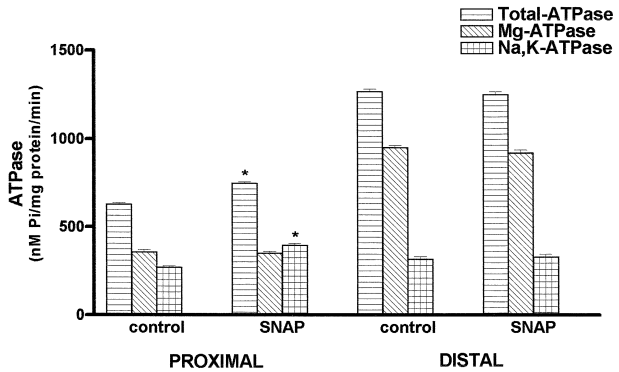

Fig. 2. Effects of *S*-nitroso-*N*-acetylpenicillamine (100 μM) on total ATPase, Mg-ATPase and Na^+, K^+-ATPase activity in proximal and distal rat trachea. Proximal or distal homogenates were incubated with each drug for 15 min at 34°C. Following incubation the drug was removed and ATPase was assayed. Mg-ATPase activity was measured in the presence of 3 mM ouabain. Na^+, K^+-ATPase was determined from the difference between total ATPase and Mg-ATPase activity. Statistical analysis: Na^+, K^+-ATPase-ANOVA, $F(3,8) = 15.1$, $P < 0.001$ and Total-ATPase ANOVA, $F(3,8) = 611,4$, $P < 0.001$; Na,K-ATPase and Total-ATPase-Student–Newman–Keuls: *S*-nitroso-*N*-acetylpenicillamine proximal > control proximal, $^*P < 0.001$, *S*-nitroso-*N*-acetylpenicillamine distal = control distal. Mg-ATPase-ANOVA, $F(3,8) = 635,3$, $P < 0.001$, Student–Newman–Keuls: *S*-nitroso-*N*-acetylpenicillamine proximal = control proximal < *S*-nitroso-*N*-acetylpenicillamine distal = control distal.

2.6. Statistics

The data are from experiments performed in triplicate. Each data point represents the mean and S.E.M. for quintuplicate samples (five in the absence of ouabain and five in the presence of ouabain). Statistical comparisons were performed by One-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls significance of differences test for comparison of means (Snedecor and Cochran, 1967). All P values < 0.05 were considered to reflect a statistically significant difference.

3. Results

3.1. Effects of sodium nitroprusside and S-nitroso-N-acetylpenicillamine on the ATPase activity of proximal and distal rat trachea

Fig. 1 shows the effects of sodium nitroprusside (1–100 μM) on Na^+, K^+-ATPase activity on proximal and distal segments of rat trachea. Sodium nitroprusside, 1 μM, did not change Na^+ pump activity in either segment of rat trachea. However, in proximal segments, but not in distal

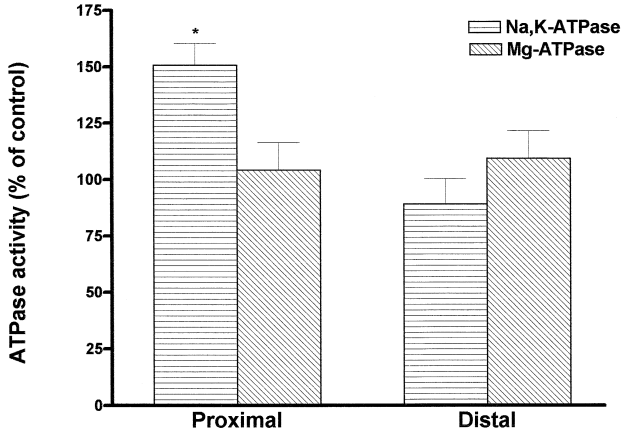

Fig. 3. Effects of 8-bromo-cyclic GMP (100 μM) on Mg-ATPase and Na^+, K^+-ATPase activity in proximal and distal rat trachea. Proximal and distal homogenates were incubated with 8-bromo-cyclic GMP (100 μM) for 15 min at 34°C. Following incubation the drug was removed and ATPase was assayed. Mg-ATPase activity was measured in the presence of 3 mM ouabain. Na^+, K^+-ATPase was determined from the difference between total ATPase and Mg-ATPase activity. Statistical analysis: Na^+, K^+-ATPase-ANOVA, $F(3,8) = 12.6$, $P < 0.001$, Student–Newman–Keuls: 8-bromo-cyclic GMP proximal > control proximal = control distal = 8-bromo-cyclic GMP distal, $* P < 0.01$. Mg-ATPase-ANOVA, $F(3,8) = 125,9$, $P < 0.0001$, Student–Newman–Keuls: 8-bromo-cyclic GMP proximal = control proximal < control distal = 8-bromo-cyclic GMP distal, $^* P < 0.01$.

segments, sodium nitroprusside, 10 μM and 100 μM, significantly increased Na^+, K^+-ATPase activity. None of the concentrations of sodium nitroprusside affected Mg-ATPase activity in either proximal or distal segments (data not shown).

The incubation of proximal segments with *S*-nitroso-*N*-acetylpenicillamine (100 μM) showed that this NO-donor did not affect Mg-ATPase, whereas Na^+, K^+-ATPase activity was significantly increased (Fig. 2). This figure also shows that *S*-nitroso-*N*-acetylpenicillamine, 100 μM, was not able to modulate either Na^+, K^+-ATPase or Mg-ATPase activity in distal segments of rat trachea. These data suggest that Na^+, K^+-ATPase activity in proximal, but not in distal rat trachea, is regulated by NO-donor compounds.

3.2. The cyclic GMP pathway and Na^+, K^+-ATPase activity of proximal and distal rat trachea

In another series of experiments we analysed the effects of the cyclic GMP level on Na^+, K^+-ATPase activity. We measured Na^+, K^+-ATPase in proximal and distal segments of trachea stimulated with 8-bromo-cyclic GMP (100 μM). The data showed that 8-bromo-cyclic GMP increased Na^+, K^+-ATPase activity in proximal segments but not in distal segments of rat trachea. On the other hand, 8-bromo-cylic GMP induced no change in Mg-ATPase activity in either proximal or distal segments of rat trachea (Fig. 3).

3.3. Effects of sodium nitroprusside on cyclic GMP and nitrates / nitrites levels in proximal rat trachea

The effect of sodium nitroprusside (1 μM and 100 μM) on cyclic GMP and nitrites/nitrates levels of the proximal segments of rat trachea are presented in Table 1. The data indicate that sodium nitroprusside, 10 μM and 100 μM, but not 1 μM, increased both cyclic GMP and nitrates/nitrites levels in proximal segments when compared to the control group.

3.4. Effects of cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase inhibitors on sodium nitroprusside or 8-bromo-cyclic GMP induced increase in Na^+, K^+-ATPase activity of the proximal segments of rat trachea

In order to evaluate the role of cyclic GMP-dependent protein kinase and cyclic AMP-dependent protein kinase in

Table 1
Effects of sodium nitroprusside (1–100 μM) on cyclic GMP and nitrites/nitrates levels in proximal rat trachea

Drug (μM)	Increase in cyclic GMP or nitrites/nitrates over control	
	Cyclic GMP %	Absolute (fmol/mg)
A		
SNP (1 μM)	102.2	5.38 ± 0.21
SNP (10 μM)	139.5*	7.34 ± 0.12
SNP (100 μM)	170.7*	8.98 ± 0.16
	Nitrites/nitrates %	Absolute (μM/mg)
B		
SNP (1 μM)	109.1	136,6 ± 8.6
SNP (10 μM)	139.5*	174.7 ± 1.1
SNP (100 μM)	154.5*	193.5 ± 3.4

For the experiment, drugs at left were added to proximal segments and cyclic GMP (A) or nitrites/nitrates (B) levels determined.
Values shown are mean ± range of triplicate samples, each assayed for cyclic GMP and nitrites/nitrates in duplicate.
* Statistical analysis: cyclic GMP-ANOVA, $F(3,8) = 122.1$, $P < 0.0001$, Student–Newman–Keuls: = control = SNP 1 μM < SNP 10 μM < SNP 100 μM, $P < 0.001$, nitrites/nitrates-ANOVA, $F(3,8) = 34.6$, $P = 0.0001$, Student–Newman–Keuls: = control = SNP 1 μM < SNP 10 μM = SNP 100 μM, $P < 0.001$.

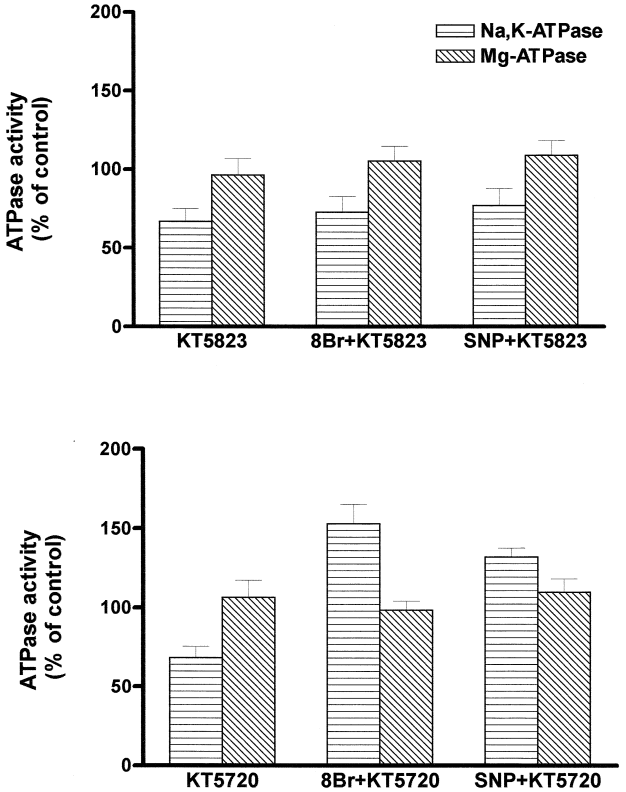

Fig. 4. Effects of (a) KT 5823 (cyclic GMP-dependent protein kinase inhibitor) and (b) KT 5720 (cyclic AMP-dependent protein kinase inhibitor) on sodium nitroprusside (100 μM) or 8 bromo-cyclic GMP (100 μM) effects on proximal Na^+, K^+-ATPase and Mg-ATPase activity in rat trachea. KT 5823 (2.0 μM) was dissolved in dimethyl sulfoxide (DMSO < 0.5%) and KT 5720 (0.5 μM) was dissolved in water. This concentration of DMSO does not influence Na^+, K^+-ATPase activity according to Satoh et al. (1993). Samples were preincubated (3 min at 34°C) with each inhibitor alone, followed by incubation (15 min at 34°C) with each inhibitor and sodium nitroprusside (KT 5720 plus sodium nitroprusside and KT 5823 plus sodium nitroprusside), or 8 bromo-cyclic GMP (KT 5720 plus 8-bromo-cyclic GMP and KT 5823 plus 8-bromo-cyclic GMP), or water (KT 5720, KT 5823). The control group was not pre-incubated before incubation. Mg-ATPase activity was measured in presence of 3 mM ouabain. Statistical analysis: Na^+, K^+-ATPase-ANOVA, $F(8,18) = 50.3$, $P < 0.0001$, Student–Newman–Keuls: 8-bromo-cyclic GMP = sodium nitroprusside = KT 5720 + sodium nitroprusside = KT 5720 + 8-bromo-cyclic GMP > control < KT 5720 = KT 5823 = KT 5823 + sodium nitroprusside = KT 5823 + 8-bromo-cyclic GMP, $P < 0.01$), Mg-ATPase-ANOVA, $F(8,18) = 0.7$, $P = 0.7$).

the modulatory effects on Na^+, K^+-ATPase induced by cyclic GMP-donor compounds (8-bromo-cyclic GMP-100 μM) or sodium nitroprusside (100 μM), we used two specific inhibitors of cyclic GMP-dependent protein kinase and cyclic AMP-dependent-protein kinase, KT 5823 and KT 5720, respectively. The concentration of each inhibitor used (2.0 μM KT-5823 and 0.5 μM KT-5720) had approximately 8-fold selectivity for inhibiting their respective kinase (Kase et al., 1987). Fig. 4 shows that inhibition of either cyclic GMP-dependent protein kinase (top panel) or cyclic AMP-dependent protein kinase (lower panel) activity significantly reduced Na^+, K^+-ATPase activity. Neither KT-5823 nor KT-5720 had any effect on Mg-ATPase. Pretreatment with KT-5823 and the cyclic GMP-analogue (8 bromo-cyclic GMP) or sodium nitroprusside also significantly reduced Na^+, K^+-ATPase activity (top panel). However, treatment with the cyclic AMP-dependent protein kinase inhibitor, KT-5720, in presence of either 8 bromo-cGMP or sodium nitroprusside did not change the increase in Na^+, K^+-ATPase activity induced by these compounds (lower panel). Neither KT-5823 nor KT-5720 plus 8 bromo-cGMP or sodium nitroprusside induced a change in Mg-ATPase. These data suggest that in the proximal segment of rat trachea, NO-donor compounds produce a significant increase of Na^+, K^+-ATPase activity by mechanism(s) involving an increase in cyclic GMP and activation of cyclic GMP-dependent protein kinase.

3.5. Effects of okadaic acid on 8-bromo-cyclic GMP induced stimulation of Na^+, K^+-ATPase activity of proximal segment of rat trachea

To evaluate the involvement of phosphorylation mechanisms in the cyclic GMP modulation of Na^+, K^+-ATPase activity of proximal rat trachea we used okadaic acid, an inhibitor of protein phosphatase 1 and 2A activities (Cohen et al., 1990). Fig. 5 shows that incubation of the proximal rat trachea slices with okadaic acid (1 μM) mimicked the action of sodium nitroprusside, *S*-nitroso-*N*-acetylpenicillamine and 8-bromo-cyclic GMP to stimulate proximal total ATPase and Na^+, K^+-ATPase activity. In addition, the effect of 8-bromo-cyclic GMP in the presence of okadaic acid was not additive, which suggests a similar

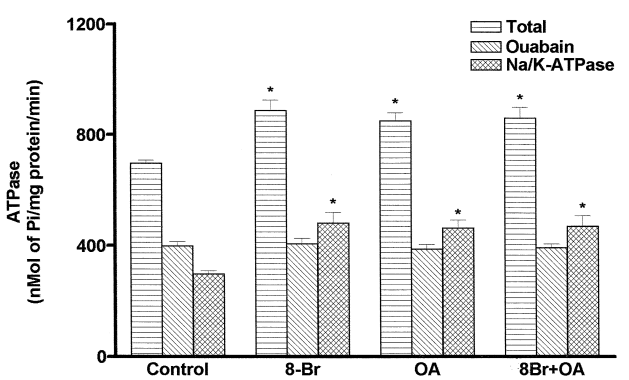

Fig. 5. Effects of okadaic acid (1 μM) on 8 bromo-cyclic GMP (100 μM)-induced stimulation of proximal Na/K-ATPase activity in rat TSM. OA was dissolved in DMSO (0.1%) and samples were preincubated (3 min at 34°C) followed by 8 bromo-cyclic GMP (100 μM) treatment (15 min at 34°C). The control group was no pre-incubated. Mg-ATPase activity was measured in presence of 3 mM ouabain. * Statistical analysis: (Na^+, K^+-ATPase-ANOVA, $F(3,8) = 7.5$, $P < 0.01$, Student–Newman–Keuls: 8-bromo-cyclic GMP = okadaic acid = okadaic acid + 8-bromo-cyclic GMP > control, $P < 0.05$), (Mg-ATPase-ANOVA, $F(3,8) = 0.3$, $P = 0.8$).

site of action. Okadaic acid alone or plus 8-bromo-cyclic GMP did not alter Mg-ATPase activity.

4. Discussion

In this study we demonstrated a differential response of Na^+, K^+-ATPase activity of the proximal and the distal segments of rat trachea. Our results confirm previous evidence suggesting the existence of differences among the physiological responses of proximal and distal segments from tracheal smooth muscle. In fact, Souhrada et al. (1983) had already shown that the contractile responses of isolated distal canine segments to histamine and serotonin, as well as to electric field stimulation are greater than those of proximal segments.

We now showed that sodium nitroprusside and S-nitroso-N-acetylpenicillamine induced a significant increase in Na^+, K^+-ATPase activity in proximal but not in distal segments of rat trachea, and this without affecting Mg-ATPase activity. The stimulation induced by either sodium nitroprusside or *S*-nitroso-*N*-acetylpenicillamine was long-lasting, as indicated by the linear time course of the stimulated activity observed for at least 60 min (longer periods were not evaluated due to the loss of assay linearity under basal conditions beyond 60 min). Because control Na^+, K^+-ATPase had been improved using saturating $[Na^+]$, $[K^+]$ and [MgATP], our for the sodium nitroprusside or *S*-nitroso-*N*-acetylpenicillamine induced changes in proximal segments of rat tracheas may represent an increase in enzyme V_{max}.

This effect was linked to changes in cyclic GMP and nitrites/nitrates levels in the proximal segment in response to sodium nitroprusside, 10 μM and 100 μM. However, this compound at 1 μM changed neither cyclic GMP nor nitrites/nitrates levels in proximal segments. In fact, sodium nitroprusside at 1 μM tended to stimulate Na^+ pump activity in proximal segments.

Data obtained with 8-bromo-cyclic GMP indicate that the NO-cyclic GMP pathway may modulate Na^+, K^+-ATPase activity in the proximal, but not in the distal segment, since this compound induced an increase in Na^+, K^+-ATPase activity of proximal rat trachea, without affecting Na^+, K^+-ATPase activity from distal segments. Besides, 8-bromo-cyclic GMP was not able to change Mg-ATPase activity in either proximal or distal segments.

Na^+, K^+-ATPase activity in distal tracheal segment represents 25% of the total ATPase activity, while in the proximal segment Na pump activity represents 43% of total activity. Therefore, the distal segments could be less sensitive to a NO modulatory action than the proximal segments. This would explain why NO-donors or 8-bromo-cyclic GMP produced na effect on proximal but not on distal segments. However, we believe that this is unlikely because 8-bromo-cyclic AMP (100 μM) stimulated Na^+, K^+-ATPase activity in proximal and distal segments (data not shown). Therefore, if the stimulation of Na^+, K^+-ATPase induced by NO-donors is linked to cyclic GMP production in the proximal segment, we suggest that sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine effects are linked to the existence of a selective interaction between NO-cyclic GMP and Na^+, K^+-ATPase activity in the proximal, but not in the distal tracheal segment.

Since cyclic GMP can cross-activate cyclic AMP-dependent protein kinase (Jiang et al., 1992; Francis and Corbin, 1994), which also has been reported to modulate Na^+, K^+-ATPase activity (Meister et al., 1989; Aperia et al., 1991; Bertorello et al., 1991; McKee et al., 1994), we decided to verify whether the effects of sodium nitroprusside and 8-bromo-cyclic-GMP on Na^+, K^+-ATPase activity of proximal segment of rat trachea were only dependent on cyclic GMP-dependent protein kinase activity, or could also involve stimulation of cyclic AMP-dependent protein kinase. Our results showed that both kinase inhibitors produced a reduction in Na^+, K^+-ATPase activity of the proximal segment of rat trachea. However, the cyclic AMP-dependent protein kinase inhibitor (KT 5720) was not able to abolish the effects of either compound, 8-bromo-cyclic GMP and sodium nitroprusside, on Na^+, K^+-ATPase activity in this segment. Conversely, the inhibition of cyclic GMP-dependent protein kinase in presence of 8-bromo-cyclic GMP or sodium nitroprusside induced a similar decrease (compared with the enzyme activity measured in the presence of the inhibitor alone) of Na^+, K^+-ATPase activity. Neither inhibitor (KT-5823 or KT-5720), alone or in presence of 8-bromo-cyclic GMP or sodium nitroprusside had any effect on Mg-ATPase activity. Therefore, we speculated that a NO modulatory action on Na^+, K^+-ATPase activity could involve a cyclic GMP/cyclic GMP-dependent protein kinase pathway in proximal segments of rat trachea.

The data presented may shed some light on the mechanism by which NO modulates Na^+, K^+-ATPase activity, at least in proximal rat trachea. Thus, we assumed that cyclic GMP-dependent protein kinase modulates Na^+, K^+-ATPase activity through phosphorylation of the protein phosphatase inhibitors, Inhibitor 1 and Dopamine-cAMP-related-phosphoprotein-32, as was shown to occur in the kidney (Aperia et al., 1991; McKee et al., 1994; Scavone et al., 1995; Wang and Robinson, 1997). Further support for this hypothesis came from experiments with okadaic acid, a protein phosphatase inhibitor that enhances the net phosphorylated state of several intracellular proteins (Walaas and Greengard, 1991). The stimulation of Na^+, K^+-ATPase induced by okadaic acid alone was similar and non-additive to that with 8-bromo-cGMP. Therefore, these results suggest that okadaic acid acts at a common site of action of a NO-cyclic GMP-cyclic GMP-dependent protein kinase downstream pathway.

However, the results do not rule out the possibility that the modulatory effects of NO on Na^+, K^+-ATPase could also be a compensatory action due to a primary action of

the cyclic GMP-cyclic GMP-dependent protein kinase on several functionally relevant proteins similar to the cyclic AMP-cyclic AMP-dependent protein kinase phosphorylation cascade (Kume et al., 1994; Torphy, 1994; Alioua et al., 1995).

The present work demonstrated that NO, through generation of cyclic GMP and stimulation of cyclic GMP-dependent protein kinase, plays an important role in the modulation of Na^+, K^+-ATPase activity in the proximal but not in the distal segment of rat trachea. In addition, since the cyclic AMP-dependent protein kinase inhibitor produced a reduction in proximal Na^+, K^+-ATPase activity similar to that by the cyclic GMP-dependent protein kinase inhibitor, the results also support the more general dual or multiple signal hypothesis of regulation of Na^+, K^+-ATPase activity. In fact, considering that cyclic AMP-cyclic AMP-dependent protein kinase and cyclic GMP-cyclic GMP-dependent protein kinase modulatory actions on Na^+, K^+-ATPase are not restricted to segments of rat trachea, this mechanism seems to be a part of more widespread complex regulatory mechanisms.

Taken together, our results demonstrated that the action of NO on the Na^+, K^+-ATPase activity of the proximal segment of rat trachea involves cyclic GMP-mediated changes in protein phosphorylation via a cyclic GMP-dependent protein kinase mechanism.

We did not analyse the physiological or physiopathological relevance of these findings in the present study. However, there is evidence suggesting that sodium nitroprusside can relax smooth muscle through both cyclic GMP-dependent and independent mechanisms (Murad et al., 1978; Buga et al., 1989: Diamond, 1993; Gaston et al., 1994; Sadeghi-Hashjin et al., 1996). In addition, the bradykinin induced relaxation of isolated guinea-pig trachea seems to be mediated by NO and prostanoids. Moreover, the relaxation induced by sodium nitroprusside (1 μM) is not affected by inhibition of nitric oxide synthase (Schlemper and Calixto, 1994). In fact, the sodium nitroprusside stimulatory action on Na^+, K^+-ATPase at 10 μM and 100 μM was linked to an increase in nitrites/nitrates and cyclic GMP production, but we found no changes in nitrites/nitrates or cyclic GMP levels with sodium nitroprusside (1 μM). It is important to note that due to the conditions of our study, we do not know whether sodium nitroprusside induced relaxation in rat proximal or distal segments.

Several reports have also suggested that *N*-methyl-D-aspartate receptors are present in the airways, as well as in other peripheral tissues (Shannon and Sawyer, 1989; Shigemoto et al., 1992; Inagaki et al., 1995; Said et al., 1996). Glutamate production during a pathological process has been considered a defence mechanism. According to some authors, the stimulation of Na^+, K^+-ATPase activity by glutamate in the brain occurs through a NO/CO-cyclic GMP and cyclic GMP-dependent protein phosphorylation cascade (Nathanson et al., 1995). It is interesting to note that recent data showed that, in guinea-pig, CO can cause bronchodilatation in vivo via the cyclic GMP pathway (Cardell et al., 1998). Thus, if this system could be detected in peripheral airways tissues, then it is conceivable that the convergence of several different pathways (such as: cyclic AMP/cyclic AMP-dependent protein kinase and cyclic GMP/cyclic GMP-dependent protein kinase) onto Na^+, K^+-ATPase activity would represent integrated effects of the different inputs, to allow a more sophisticated control of cellular function in the pathogenesis of allergic diseases.

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