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Stimulation of sodium pump by vasoactive intestinal peptide in guinea-pig isolated trachea: potential contribution to mechanisms underlying relaxation of smooth muscle

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1 Relaxation of airway smooth muscle induced by vasoactive intestinal peptide (VIP) is mediated by adenosine 3′:5′ cyclic monophosphate (cyclic AMP). An interaction between the synthesis of cyclic AMP and enzymic activity of the plasmalemmal sodium pump (Na^+-K^+-ATPase) exists in certain isolated cell systems. This study sought to determine the contribution of Na^+-K^+-ATPase activity to relaxation of airway smooth muscle evoked by VIP.

2 All experiments were performed on isolated strips of guinea-pig trachea from which the epithelium had been removed. VIP was a more potent relaxant in tissues that were contracted with carbachol than those contracted with an equi-effective depolarizing concentration of K^+.

3 Ouabain (0.1 μM – 10 μM) induced contraction of tracheal strips. Contraction to ouabain (5 μM) was abolished following incubation of tissues with K^+-free, or Ca^{2+}-free (+EGTA, 0.1 mM) physiological solutions. The contractile response to ouabain (5 μM) was not influenced significantly by exposure of the tissues to atropine (1 μM), phentolamine (5 μM) and diphenhydramine (1 μM) for 60 min.

4 Tissues were incubated with ouabain (5 μM; 60 min) or K^+-free physiological solution (60 min) to inhibit Na^+-K^+-ATPase activity. These procedures reduced relaxation induced by VIP, peptide histidine isoleucine, forskolin, isoprenaline and sodium nitroprusside.

5 Relaxation to VIP was impaired significantly following exposure of tissues to a low Na^+ solution (30 min) or amiloride (500 μM; 30 min).

6 Ouabain-sensitive uptake of ^{86}Rb was measured in tracheal strips (devoid of epithelium and cartilage) as an index of Na^+-K^+-ATPase activity. VIP (1 μM; 2 min) caused a 4.7 fold stimulation of ouabain-sensitive uptake of ^{86}Rb. This effect was impaired significantly by low Na^+ solution.

7 The results suggest that (i) relaxation of tracheal smooth muscle to VIP is sensitive to procedures that inhibit activity of Na^+-K^+-ATPase and invoke a role for altered sodium pump function in the mechanisms that underlie cyclic AMP-dependent relaxation; and (ii) VIP stimulates ouabain-sensitive uptake of ^{86}Rb in airway smooth muscle in a Na^+-dependent manner.

Keywords: Smooth muscle relaxation; VIP; Na^+-K^+-ATPase activity; ouabain-sensitive uptake of ^{86}Rb

Introduction

A relationship exists between Na^+-K^+-ATPase activity and the status of the contractile apparatus in smooth muscle cells (Scheid *et al.*, 1979; Brock *et al.*, 1982; Lynch *et al.*, 1986; Navran *et al.*, 1988; 1991). Both contractile and relaxant agonists stimulate Na^+-K^+-ATPase activity. Thus, the constrictor agonists, 5-hydroxytryptamine (5-HT) and phenylephrine, stimulate Na^+-K^+-ATPase activity in vascular smooth muscle (Navran *et al.*, 1988; 1991), whereas enhanced Na^+-K^+-ATPase activity contributes to the relaxant response to isoprenaline (non-preferential β-adrenoceptor agonist) in frog isolated stomach smooth muscle cells (Scheid *et al.*, 1979). These changes in Na^+-K^+-ATPase activity may be accounted for by two main mechanisms: (i) stimulation of Na^+-K^+-ATPase activity is secondary to agonist-evoked influx of Na^+. For example, ouabain-sensitive uptake of ^{86}Rb (an index of Na^+-K^+-ATPase activity) that is induced by 5-HT is impaired by inhibition of Na^+-H^+ exchange (Navran *et al.*, 1991). Also, the ability of α-adrenoceptor agonists to stimulate Na^+-K^+-ATPase activity is impaired by exposure of tissues to low Na^+ solution. The underlying mechanism may involve inhibition of Na^+-H^+ exchange (Navran *et al.*, 1988); (ii) receptor occupancy may lead to structural/biochemical modification of the pump itself by specific receptor-generated intracellular second messengers. Stimulation of Na^+-K^+-ATPase activity following occupancy of the 5-HT_2 receptor subtype in vascular smooth muscle occurs, in part, via a process that is dependent on protein kinase C (Navran *et al.*, 1988). Indeed, the α-subunit of the sodium pump serves as a substrate for phosphorylation by protein kinase C *in vivo* (Chibalin *et al.*, 1992; Middleton *et al.*, 1993). Also, the cyclic AMP-protein kinase A signal pathway has been implicted in the stimulation of Na^+-K^+-ATPase activity (Scheid *et al.*, 1979). Protein kinase A phosphorylates directly Na^+-K^+-ATPase α-subunits in microsomes from *Xenopus* oocytes (Chibalin *et al.*, 1992).

Na^+-K^+-ATPase is present in the sarcolemma of airway smooth muscle cells of numerous species (Souhrada *et al.*, 1981; Chideckel *et al.*, 1987; Raeburn & Fedan, 1989), although the contribution of this membrane pump to smooth muscle relaxation has not been determined. Regulation of Na^+-K^+-ATPase activity by smooth muscle relaxants, however, has been inferred from experiments in canine (Gunst & Stropp, 1988) and rabbit trachea (Kanemura *et al.*, 1993).

Vasoactive intestinal peptide (VIP) is one of the most potent endogenous bronchodilators (Palmer *et al.*, 1986). Activation of VIP receptors is associated with increased activity of adenylyl cyclase and production of cyclic AMP (Robberecht *et al.*, 1981; 1982). This in turn mediates the bronchodilator action of the peptide (Frandsen *et al.*, 1978). The role of the sarcolemmal Na^+-K^+-ATPase as a target for the VIP-induced

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cyclic AMP-dependent relaxant signal in airway smooth muscle cells has not been defined. The present study, therefore, sought to determine the contribution of the sarcolemmal Na^+-K^+-ATPase to the mechanisms of relaxation of airway smooth muscle to VIP. VIP-induced relaxation was compared with that to other agents, which act via different mechanisms, in order to gain insight into the specific sites of interaction of the VIP-generated relaxant signal with the sodium pump. The guinea-pig isolated trachea was chosen as the model for study, since the electrogenic sodium pump has been identified and characterized in this tissue (Souhrada *et al.*, 1981).

Methods

Preparation of tracheal strips for measurement of isometric force

Male, Hartley guinea-pigs (350–500 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and killed by exsanguination. The trachea was excised and placed in cold physiological solution of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, calcium disodium edetate 0.026, and glucose 11.1 (control solution). Fat and connective tissue were cleaned from the trachea and rings (2 cartilage rings wide) were prepared. Transverse tracheal strips were then prepared from each ring by making a longitudinal cut along the ventral surface of the cartilage. The epithelium was removed by stroking the epithelial surface gently with a cotton tipped applicator. Successful removal of epithelial cells without damage to the underlying smooth muscle layers was confirmed by histological examination in preliminary experiments (Flavahan *et al.*, 1988). Tracheal strips were suspended between two stainless steel wire hooks, placed through the cartilage, under a resting load of 1.5 g (Farmer & Togo, 1990) in tissue baths containing control solution (15 ml), maintained at 37°C, and gassed with a mixture of 95%O_2: 5%CO_2 (pH 7.4±0.2). Tissues were connected to a strain gauge (Statham Gould UC2) for recording isometric force and allowed to equilibrate for 60 min with regular washing prior to the start of experiments.

Measurement of ouabain-sensitive uptake of ^{86}Rb

Ouabain-sensitive uptake of ^{86}Rb was used as an index of sodium pump activity (e.g. Navran *et al.*, 1991). ^{86}Rb substitutes for K^+ and is taken up by the cell in exchange for Na^+. Strips of tracheal smooth muscle (four from each animal), which were devoid of epithelium and cartilage, were prepared and blotted dry before weighing. Tissues were subjected to three serial incubations in different solutions: (i) tissues were incubated in K^+-free soltuion (2 ml; 37°C; 95% O_2–5% CO_2; 60 min) in order to inhibit activity of the sodium pump and to load the smooth muscle cells with Na^+ (Sasaguri & Watson, 1990); (ii) tissues were incubated in K^+-free solution in the presence or absence of ouabain (1 mM; 15 min); and finally (iii) tissues were bathed in a low K^+ solution (1 mM; 2 ml), containing ^{86}Rb (2–3 μCi ml^{-1}), either in the presence or absence of ouabain (1 mM). In certain experiments the final incubation solution was also deficient in Na^+. At various time intervals following exposure of tissues to this final solution, the tissues were removed from the bathing medium and washed successively three times in ice-cold K^+-free physiological solution (2 ml) (Navran *et al.*, 1991). VIP was present during the final incubation. Indomethacin (3 μM) was present throughout these experiments to negate the potential contribution of tissue-derived prostanoids to the activity of the sodium pump. Tissues were then counted for radioactivity in a gamma counter. Ouabain-sensitive uptake of ^{86}Rb was calculated as the difference between counts obtained from control tissues and ouabain-treated tissues. Ouabain-sensitive uptake of ^{86}Rb was expressed as pmol of Rb per mg wet weight of tissue.

Chemicals

The following drugs were used: acetylcholine hydrochloride; amiloride; atropine sulphate; carbamylcholine chloride (carbachol); cocaine hydrochloride; diphenhydramine hydrochloride; forskolin; hydrocortisone hemisuccinate; indomethacin; (−)-isoprenaline (+)-bitartrate; ouabain octahydrate; papaverine hydrochloride; peptide histidine isoleucine (PHI); sodium nitroprusside (SNP) (all from Sigma Chemical Co. St. Louis, MO, U.S.A.); phentolamine mesylate (Research Biochemicals International, Natick, MA, U.S.A.); vasoactive intestinal peptide (Calbiochem, San Diego, CA, U.S.A.). Most drugs were prepared daily in distilled water, kept on ice and added to tissue baths in volumes not exceeding 150 μl. A stock solution of forskolin was prepared in dimethyl sulphoxide, divided into aliquots and frozen until required. Dilutions were made in distilled water. Indomethacin was prepared with an equimolar concentration of Na_2CO_3. Concentrations of drugs are expressed as the final organ chamber concentrations (M). ^{86}Rb (1 mCi; 0.2–0.5 mg Rb ml^{-1}) was obtained from Amersham (Arlington Heights, IL).

Solutions

K^+-free solution was prepared as for control solution except that KCl and KH_2PO_4 were substituted with molar equivalents of NaCl and NaH_2PO_4, respectively. Low Na^+ solution was prepared by omitting NaCl from the control solution. Na^+ ions were still present in the solution in the form of Na_2CO_3 (25 mM). Osmolality of the solution was maintained by replacing NaCl with N-methyl disodium glutamate.

Experimental protocol

All experiments were performed in the presence of indomethacin (3 μM).

Measurement of isometric force in tissue baths

Each tissue was contracted with acetylcholine (100 μM). This response was used as a reference contraction to establish the concentration of contractile agent that induced 70% response. Following a recovery period of 30 min during which time the tissues were washed with control solution, the tracheal strips were incubated with either ouabain (10 nM–5 μM; 15–60 min), K^+-free solution (15–60 min), amiloride (50–500 μM; 30 min) or distilled water (vehicle control; 15–60 min), and then contracted with equi-effective concentrations of carbachol (to give a final contraction level of 70% of that to acetylcholine 100 μM). When the force developed in response to carbachol had stabilized at the desired level, concentration-response curves to VIP (1–100 nM), PHI (0.1–3 μM), isoprenaline (1 nM–1 μM), forskolin (1 nM–1 μM) or SNP (1 nM–10 μM) were obtained. Relaxation to isoprenaline was measured in the presence of cocaine (30 μM), hydrocortisone (3 μM) and phentolamine (5 μM) to inhibit neuronal and extraneuronal uptake of the catecholamine, and non-specific activation of α-adrenoceptors, respectively. Papaverine (300 μM) was added to each tissue bath at the end of the experiment to induce maximum relaxation of the tissue. Relaxation to each of the test agents is expressed as a percentage of the maximum response to papaverine (300 μM). None of the treatments affected significantly the relaxation to papaverine (300 μM) in absolute terms (g mg^{-1} tissue weight). In certain experiments, tissues were contracted with K^+ (concentration giving 70% of maximum response to acetylcholine 100 μM). In other experiments, the effect of low sodium solution on the relaxant ability of VIP was studied. Exposure of tissues to low Na^+ solution induced a marked contractile response which stabilized within 1–2 min of adminstration. A comparable contraction to that elicited by low Na^+ (relative to maximum response to acetylcholine 100 μM) was evoked by carbachol in parallel tissues to serve as a time-matched control prior to obtaining the concentration-relaxation curve to VIP (1–100 nM).

Statistical analysis

Results are expressed as mean ± s.e.mean. All n values refer to the number of animals. N values refer to the total number of tissues used in each experiment. Student's paired t test was used for single comparisons between groups. One-way analysis of variance was used, and where significance is indicated, Scheffe's test was applied (Scheffe, 1959) for multiple comparisons between groups. Statistical significance was accepted at P values of less than 0.05.

Results

VIP (1 – 100 nM) induced concentration-dependent relaxation of tracheal smooth muscle preparations that were contracted with equi-effective concentrations of either K^+ (60 ± 4.2 mM) or carbachol (−log M: 6.5 ± 0.22; 70% of maximal response to acetylcholine 100 μM) (Figure 1). The maximum relaxation to VIP was significantly reduced in tissues that were contracted with depolarizing K^+ solution compared to tissues that were contracted with carbachol (carbachol, 96.2 ± 2.8%; K^+, 20.7 ± 2.2%; $P<0.01$).

Ouabain (0.1 – 10 μM) induced concentration-dependent contraction of tracheal strips (Figure 2). The maximum contractile response to ouabain (5 μM) was 1.1 ± 0.18 g [39.0 ± 2.7% of the maximum response to ACh (100 μM); $n=3$] (Figure 2a). Ouabain (5 μM) did not induce contraction of tissues which had been incubated with K^+-free physiological solution (Figure 2b). Contraction to ouabain (5 μM) was not significantly affected by exposure of the tissues to atropine (1 μM; non-preferential muscarinic receptor antagonist), phentolamine (5 μM; non-preferential α-adrenoceptor antagonist) and diphenhydramine (1 μM; H_1-histamine receptor antagonist) for 60 min (Figure 2c) [0.7 ± 0.2 g versus 1.1 ± 0.18 g; $n=3$; $P>0.05$]. Ouabain (5 μM) did not induce contraction of tissues which had been incubated with Ca^{2+}-free (+0.1 mM EGTA) physiological solution for 60 min (Figure 2d). Upon washing the tissues with control solution for 60 min, however, subsequent exposure to ouabain (5 μM) evoked a contractile response (Figure 2d).

Under control conditions, VIP induced almost complete relaxation of guinea-pig tracheal strips that were contracted with carbachol (97.2 ± 1.3% of maximal response to papaverine, 300 μM) (Figure 3a; Table 1). Relaxation to VIP was impaired significantly by procedures which inhibit Na^+-K^+-ATPase activity (Figure 3a; Table 1). The effects of ouabain

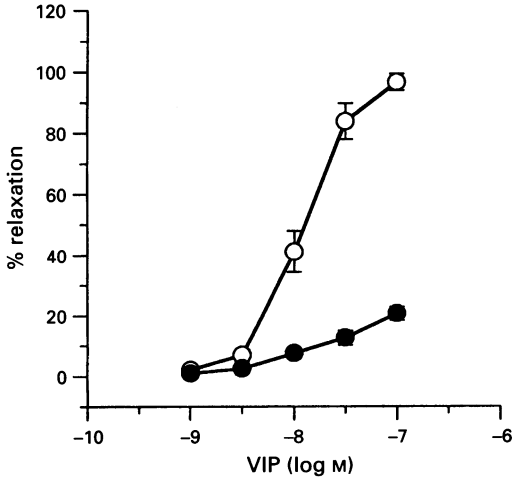

Figure 1 Concentration-relaxation curves to VIP in guinea-pig isolated tracheal strips. Tissues were contracted with equi-effective concentrations of either carbachol (○) or KCl (●) (70% of maximum response to ACh, 100 μM: 1.54 ± 0.14 g). Relaxation is expressed as a percentage of the maximum response to papaverine (300 μM). Data are presented as mean ± s.e.mean. $n=5$.

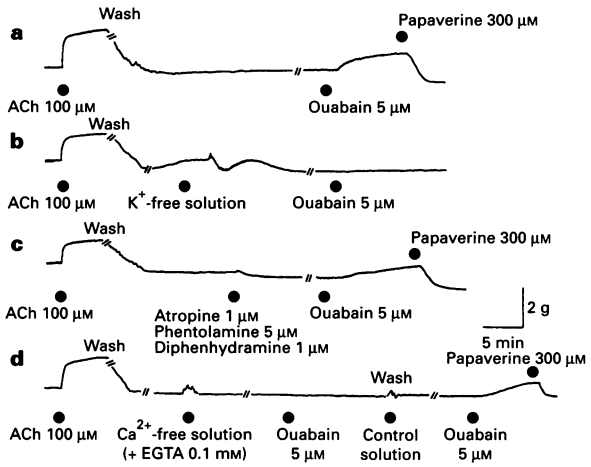

Figure 2 Contraction of guinea-pig isolated trachea to ouabain. Representative traces are shown. $n=3$.

were concentration- and time-dependent (0.1 nM – 10 μM; 15 – 60 min) and the effects of K^+-free solution were time-dependent (15 – 60 min) (data not shown).

Relaxation to PHI, which shares close structural homology with VIP, was inhibited by ouabain (5 μM) and K^+-free solution (Figure 3b and Table 1).

Isoprenaline (β-adrenoceptor agonist which elicits relaxation of smooth muscle via production of cyclic AMP) induced complete relaxation of tracheal strips under control conditions (Figure 3c; Table 1). Ouabain (5 μM; 60 min) and K^+-free solution (60 min) impaired the relaxation to isoprenaline significantly (Figure 3c; Table 1). Forskolin (activator of adenylyl cyclase) induced complete relaxation of tracheal strips under control conditions (Figure 3d; Table 1). Ouabain (5 μM; 60 min) and K^+-free solution (60 min) impaired relaxation to forskolin significantly (Figure 3d).

SNP (nitro-bronchodilator which elicits smooth muscle relaxation via production of cyclic GMP) induced complete relaxation of tracheal strips (Figure 3e; Table 1). Ouabain (5 μM; 60 min) and K^+-free solution (60 min) impaired relaxation to SNP significantly (Figure 3e; Table 1).

Relaxation to VIP was impaired significantly following incubation of tissues with low Na^+ solution (30 min) (Figure 4a); maximum response: control, 93.1 ± 4.1% low Na^+, 39.3 ± 5.8%; $P<0.001$. Amiloride (500 μM; 30 min) inhibited relaxation induced by VIP (Figure 4b); maximum response: control, 96.4 ± 1.5%; amiloride, 60.3 ± 6.9%; $P<0.001$.

Basal ouabain-sensitive uptake of ^{86}Rb proceeded in a linear fashion over 10 min (data not shown). Exposure of tracheal smooth muscle strips to VIP (1 μM; 2 min) caused a 4.7 fold increase in ouabain-sensitive uptake of ^{86}Rb (Figure 5). Incubation of tissues with VIP (1 μM) for a shorter time interval (10 s) did not stimulate ouabain-sensitive uptake of ^{86}Rb (data not shown). The effect of VIP (1 μM; 2 min) on ouabain-sensitive uptake of ^{86}Rb was impaired significantly by low Na^+ solution (Figure 5).

Discussion

This study used pharmacological assays to establish the contribution of the sarcolemmal Na^+-K^+-ATPase to the relaxant response evoked by VIP in guinea-pig tracheal smooth muscle. The data demonstrate that ouabain and K^+-free solution impair the ability of VIP and other cyclic AMP-dependent dilators to relax isolated airway smooth muscle, and suggest an involvement of Na^+-K^+-ATPase activity in this response. This interpretation is supported by the observation in separate experiments that VIP stimulates activity of Na^+-K^+-ATPase in tracheal smooth muscle, as measured by ouabain-sensitive uptake of ^{86}Rb.

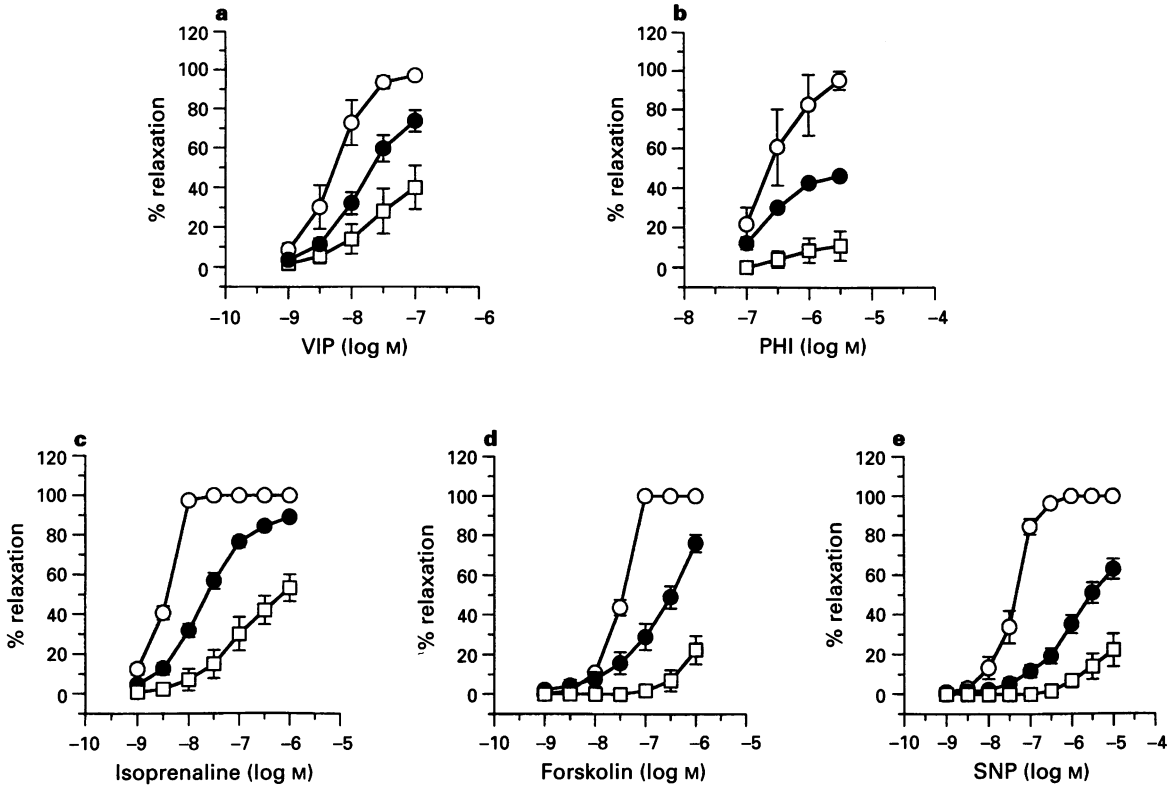

Figure 3 Effects of ouabain (5 μM; 60 min; ●) and K^+-free solution (60 min; □) on concentration-relaxation curves to VIP (a; $n=5$), PHI (b; $n=3$), isoprenaline (c; $n=7$), forskolin (d; $n=6$) and SNP (e; $n=5$) in guinea-pig isolated trachea. Control responses (○). Tissues were contracted with carbachol (70% of maximum response to ACh, 100 μM: a, 1.06±0.05 g; b, 1.79±0.13 g; c, 1.23±0.07 g; d, 0.94±0.08 g; e, 1.13±0.09 g; $P>0.05$). Relaxation is expressed as a percentage of the maximum response to papaverine (300 μM). Data are presented as mean±s.e.mean.

Table 1 Effects of ouabain and K^+-free solution on relaxation of guinea-pig trachea

Agonist	*Treatment*	n	*EC_{50} value* (–log M)	*Maximum response*[a]	*Area under curve*[b]
VIP	Control	5	8.4±0.12	97.2±1.3	279.2±22.3
	Ouabain 5 μM	5	7.8±0.06[c]	70.1±7.0	126.8±20.9[c]
	K^+-free solution	5	7.7±0.14[d]	40.1±10.9[d]	68.7±28.5[d]
PHI	Control	3	6.6±0.22	95.2±4.8	202.2±41.3
	Ouabain 5 μM	3	6.7±0.05	46.4±0.3[c]	102.4±1.4[c]
	K^+-free solution	3	6.3±0.2	11.1±7.3[c]	18.4±13.9[c]
Forskolin	Control	6	7.5±0.03	100.0	306.1±6.1
	Ouabain 5 μM	6	6.8±0.1[e]	76.1±4.4[c]	144.2±25.3[d]
	K^+-free solution	6	6.3±0.06[e]	22.4±7.1[e]	20.1±10.6[e]
Isoprenaline	Control	7	8.4±0.03	100.0	558.7±63.5
	Ouabain 5 μM	7	7.7±0.07[d]	89.0±1.2	308.9±15.8[c]
	K^+-free solution	7	7.1±0.19[e]	53.2±6.8[e]	123.3±32.3[e]
SNP	Control	5	7.4±0.1	100.0	481.6±21.1
	Ouabain 5 μM	5	6.1±0.06[e]	63.1±5.2[c]	158.1±14.5[e]
	K^+-free solution	5	5.6±0.14[e]	22.2±8.3[e]	33.5±14.5[e]

[a]Maximum response is expressed as percentage of relaxation to papaverine (300 μM). [b]Area under curve is expressed in arbitrary units (calculated by Lotus 123 analytical software). [c]$P<0.05$; [d]$P<0.01$; [e]$P<0.001$.

Relaxation of guinea-pig isolated tracheal strips to VIP is reduced significantly in tissues that are contracted with a depolarizing concentration of K^+. The contribution of Na^+-K^+-ATPase activity to VIP-evoked smooth muscle relaxation was evaluated by studying the effects of the cardiac glycoside, ouabain and K^+-free solution. Ouabain inhibits Na^+-K^+-ATPase activity in electrophysiological experiments in guinea-pig isolated trachea under the conditions used in this study (Souhrada *et al.*, 1981). Ouabain, however, possesses other non-specific effects. In particular, ouabain contracts the isolated trachea of the guinea-pig (Raeburn & Fedan, 1989). Preliminary experiments were conducted, therefore, to establish the mechanisms by which this contractile response is evoked. Ouabain-induced contraction was abolished following inhibition of Na^+-K^+-ATPase by exposure of tissues to K^+-free solution. Further, ouabain did not evoke contraction of the tracheal preparations in the presence of Ca^{2+}-free solution. These data are consistent with the notion that ouabain-induced contraction is mediated via inhibition of Na^+-K^+-ATPase activity and subsequent activation of Na^+-Ca^{2+} exchange (Knox *et al.*, 1990). The Na^+-K^+-ATPase activity involved must be localized to smooth muscle cells rather than epithelial

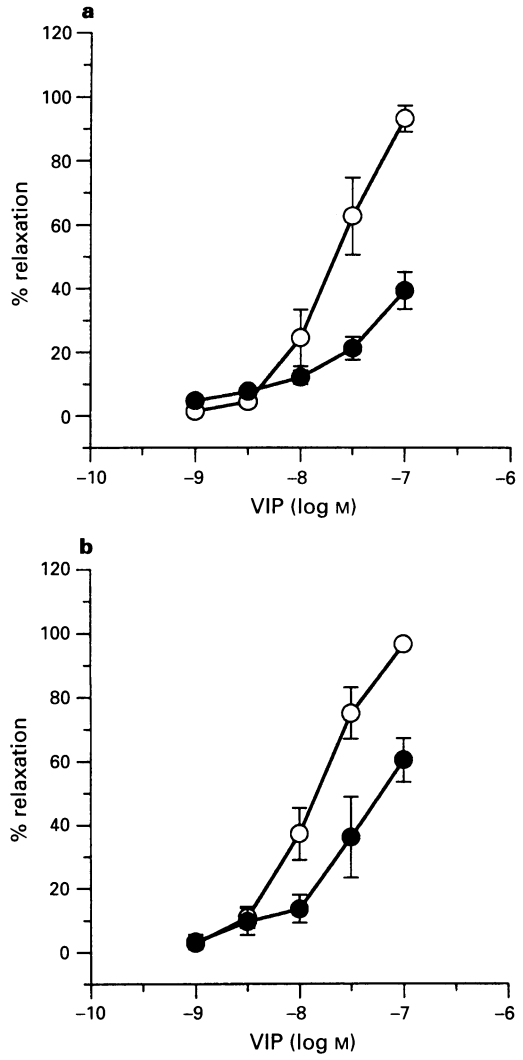

Figure 4 Effects of low Na^+ solution (30 min; a) and amiloride (500 μM, 30 min; b) on concentration-relaxation curves to VIP in guinea-pig isolated trachea. Control responses (○); low Na^+ (●; a); amiloride (●; b). (a) Control contraction to carbachol 1.6±0.27 g, contraction to low Na^+ solution 2.1±0.29 g; (b) tissues were contracted with carbachol (70% of maximum response to ACh, 100 μM: 1.21±0.11 g). Relaxation is expressed as a percentage of the maximum response to papaverine (300 μM). Data are presented as mean±s.e.mean. $n=5$.

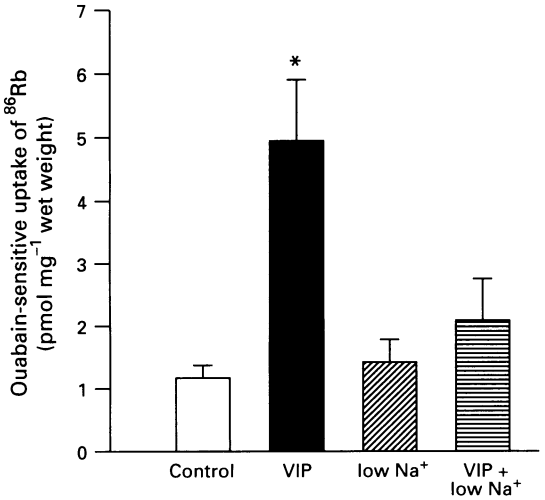

Figure 5 Effects of VIP on ouabain-sensitive uptake of ^{86}Rb in guinea-pig isolated tracheal smooth muscle under control conditions and in the presence of low Na^+. *$P<0.05$. Mean weight of tissues 2.82±0.2 mg ($n=3$; $n=18$).

cells since the epithelial layer was removed by mechanical means from all tracheal strips that were used. Also, contraction to ouabain did not appear to be mediated by the action of neurotransmitters that were released from nerve endings in the tissue preparation because blockade of neurotransmitter receptors with atropine, phentolamine and diphenhydramine, failed to influence contraction to the cardiac glycoside.

Na^+-K^+-ATPase activity and relaxation of tracheal smooth muscle

Smooth muscle relaxation to VIP and its structural homologue, PHI, is mediated by cyclic AMP (Frandsen *et al.*, 1978; Tatemoto, 1984). Relaxation of guinea-pig tracheal strips to these structurally-related peptides was impaired by both ouabain and K^+-free solution, which extends observations in rabbit trachea (Kanemura *et al.*, 1993). If these procedures act specifically at the sodium pump, then this membrane pump must participate in the mechanisms of relaxation to VIP and PHI. Alternatively, the inhibitory actions of ouabain and K^+-free solution on the relaxation to VIP and PHI may reflect a generalized opposition to relaxation since both of these procedures induce contraction of the tissues. Concentration-relaxation curves to each agent, however, were obtained in tissues that were contracted to the same degree of force which was relative to the individual maximum response to acetylcholine in each tissue. Further, relaxation to papaverine was not influenced by either inhibitory procedure. This allowed relaxation to other agents to be expressed as a percentage of the maximal response to papaverine.

The inhibitory effects of ouabain and K^+-free solution on relaxation were not confined to events initiated by activation of VIP/PHI receptors. Thus, the response to stimulation of β-adrenoceptors was also sensitive to inhibition of sodium pump activity. Since relaxation to isoprenaline is a cyclic AMP-dependent process, the inhibitory actions of ouabain and K^+-free solution are not directed preferentially at VIP/PHI receptors. These data suggest that events distal to the agonist-receptor interaction may be targeted by ouabain and K^+-free solution. Relaxation to forskolin, which stimulates adenylyl cyclase directly, was impaired by both ouabain and K^+-free solution. It is clear, therefore, that cyclic AMP-dependent processes are sensitive to ouabain and K^+-free solution. Furthermore, these data implicate the sodium pump as a target for cyclic AMP-dependent signals during relaxation of smooth muscle. The ability of ouabain and K^+-free solution to impair tracheal relaxation was not confined to cyclic AMP-dependent processes. Relaxation to SNP was sensitive to ouabain and K^+-free solution. This nitrodilator exerts its effect via the release of nitric oxide, activation of guanylyl cyclase and subsequent formation of guanosine 3′;5′ cyclic monophosphate (cyclic GMP) (Murad *et al.*, 1978). This is consistent with data obtained in vascular smooth muscle (Rapaport & Murad, 1983), but contrasts with observations in canine trachea (Gunst & Stropp, 1988). The relaxant effect of SNP, however, may not be dependent on cyclic GMP (Morrison & Pollock, 1988). Nitric oxide stimulates Na^+-K^+-ATPase activity via a cyclic GMP-*independent* mechanism in vascular smooth muscle cells (Gupta *et al.*, 1994). Thus the use of other nitric oxide-donor compounds may not clarify interpretation of this observation.

A consistent observation throughout this study is the greater ability of K^+-free solution than ouabain to impair relaxation. This effect may be attributed to different mechanisms of inhibition of the sodium pump. Cardiac glycosides exert their inhibitory action by binding to the α-subunits of the pump and preventing binding of Na^+ and K^+ at the intra- and extra-cellular membrane surfaces, respectively (Fleming, 1980). In contrast, K^+-free solution inhibits pump activity by simply depriving the sodium pump of K^+ at the extracellular site (Fleming, 1980). K^+-free solution may produce a more efficient inhibition of sodium pump activity.

It may be anticipated that, if VIP and other cyclic AMP-

dependent relaxants induce relaxation via activation of Na^+-K^+-ATPase, blockade of cellular influx of Na^+ would impede the relaxant processes. Lowering the Na^+ content of the bathing medium impaired significantly the relaxation of tracheal strips to VIP. Also, amiloride, an inhibitor of Na^+-H^+ exchange, inhibited VIP-induced relaxation. These data are consistent with findings in vascular tissues (Navran *et al.*, 1988; 1991). Receptor-mediated activation of Na^+-K^+-ATPase may be effected via direct phosphorylation of the α-subunits and is independent of cellular Na^+ content. This may account for the residual relaxation that is observed in the presence of a low Na^+ medium and amiloride.

Ouabain-sensitive uptake of ^{86}Rb

Impairment of smooth muscle relaxation *in vitro* by ouabain and K^+-free solution does not, necessarily, involve reduced activity of Na^+-K^+-ATPase (Navran *et al.*, 1988). In order to establish the activity of Na^+-K^+-ATPase in this tissue preparation, ouabain-sensitive uptake of ^{86}Rb was measured in response to VIP (e.g. Navran *et al.*, 1988; Sasaguri & Watson, 1990). Exposure of Na^+-loaded tissues to VIP for 2 min, at a concentration that is maximal for relaxation, resulted in a significant increase in sodium pump function. These data taken together with the inhibitory effects of ouabain and K^+-free solution on relaxation to VIP are consistent with a role for Na^+-K^+-ATPase activity in cyclic AMP-dependent smooth muscle relaxation.

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