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The spasmogenic effects of vanadate in human isolated bronchus

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1 Inhalation of vanadium compounds, particularly vanadate, is a cause of occupational bronchial asthma. We have now studied the action of vanadate on human isolated bronchus. Vanadate (0.1 μM–3 mM) produced concentration-dependent, well-sustained contraction. Its $-\log EC_{50}$ was 3.74 ± 0.05 (mean $\pm$ s.e.mean) and its maximal effect was equivalent to $97.5 \pm 4.2\%$ of the response to acetylcholine (ACh, 1 mM).

2 Vanadate (200 μM)-induced contraction of human bronchus was epithelium-independent and was not inhibited by indomethacin (2.8 μM), zileuton (10 μM), a mixture of atropine, mepyramine and phentolamine (each at 1 μM), or by mast cell degranulation with compound 48/80.

3 Vanadate (200 μM)-induced contraction was unaltered by tissue exposure to verapamil or nifedipine (each 1 μM) or to a Ca^{2+}-free, EGTA (0.1 mM)-containing physiological salt solution (PSS). However, tissue incubation with ryanodine (10 μM) in Ca^{2+}-free, EGTA (0.1 mM)-containing PSS reduced vanadate-induced contraction. A series of vanadate challenges was made in tissues exposed to Ca^{2+}-free EGTA (0.1 mM)-containing PSS with the object of depleting intracellular Ca^{2+} stores. In such tissues cyclopiazonic acid (CPA; 10 μM) prevented Ca^{2+}-induced recovery of vanadate-induced contraction.

4 Tissue incubation in K^+-rich (80 mM) PSS, K^+-free PSS, or PSS containing ouabain (10 μM) did not alter vanadate (200 μM)-induced contraction. Ouabain (10 μM) abolished the K^+-induced relaxation of human bronchus bathed in K^+-free PSS. This action was not shared by vanadate (200 μM). The tissue content of Na^+ was increased and the tissue content of K^+ was decreased by ouabain (10 μM). In contrast, vanadate (200 μM) did not alter the tissue content of these ions. Tissue incubation in a Na^+-deficient (25 mM) PSS or in PSS containing amiloride (0.1 mM) markedly inhibited the spasmogenic effect of vanadate (200 μM).

5 Vanadate (200 μM)-induced contractions were markedly reduced by tissue treatment with each of the protein kinase C (PKC) inhibitors H-7 (10 μM), staurosporine (1 μM) and calphostin C (1 μM). Genistein (100 μM), an inhibitor of protein tyrosine kinase, also reduced the response to vanadate.

6 Vanadate (0.1–3 mM) and ACh (1 μM–3 mM) each increased inositol phosphate accumulation in bronchus. Such responses were unaffected by a Ca^{2+}-free medium either alone or in combination with ryanodine (10 μM).

7 In human cultured tracheal smooth muscle cells, histamine (100 μM) and vanadate (200 μM) each produced a transient increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

8 Intracellular microelectrode recording showed that the contractile effect of vanadate (200 μM) in human bronchus was associated with cellular depolarization.

9 It is concluded that vanadate acts directly on human bronchial smooth muscle, promoting the release of Ca^{2+} from an intracellular store. The Ca^{2+} release mechanism involves both the production of inositol phosphate second messengers and inhibition of Ca-ATPase. The activation of PKC plays an important role in mediating vanadate-induced contraction at values of $[Ca^{2+}]_i$ that are close to basal.

Keywords: Vanadate; human airway smooth muscle; Ca^{2+} influx inhibitors; ryanodine; cyclopiazonic acid; ouabain; inositol phosphates; protein kinase C inhibitors; genistein; membrane potential

Introduction

Occupational inhalation of airborne vanadium compounds, particularly vanadate which is the predominant species in the body fluids (Nechay, 1984), causes bronchial asthma (US Department of Health, Education and Welfare Report, 1977; Musk & Tees, 1982). In the anaesthetized guinea-pig, vanadate produces bronchoconstriction (Nayler & Mitchell, 1987). It has been suggested from clinical (Musk & Tees, 1982) and animal (Nayler & Mitchell, 1987) studies that the pulmonary effects of vanadate involve a direct action on the smooth muscle of airways. Other actions of an immuological (Zenz *et al*., 1962) or a neurogenic (Nayler & Mitchell, 1987) nature may also play a role. Vanadate contracts tracheal smooth muscle isolated from the guinea-pig (Nayler & Sparrow, 1983; Cortijo *et al*., 1993b), dog (Lee *et al*., 1994), and monkey and rabbit (Ueda *et al*., 1985). Vanadate has a variety of actions *in vitro* but the principal mechanism of vanadate-induced contraction of guinea-pig trachea appears to be intracellular Ca^{2+} mobilization linked to inhibition of Ca^{2+}-dependent ATPase activity (Nayler & Sparrow, 1983).

Although the *in vitro* effects of vanadate have been studied in various airway smooth muscle preparations derived from experimental animals, its effects on human isolated airway smooth muscle have not yet been studied. The aim of the present study was to examine the mechanical, biochemical and electrophysiological effects of vanadate in human isolated bronchus. A preliminary account of part of this work has been communicated to the British Pharmacological Society (Morcillo *et al*., 1995).

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Methods

Preparation of human tissues for pharmacomechanical experiments

Lung tissue was obtained from patients who were undergoing surgery for lung carcinoma. None of the patients had a history of asthma. After the resection of one or more lung lobes, a piece of macroscopically normal tissue was excised and immersed in physiological salt solution (PSS, for composition see drugs and solutions) at 4°C for transport to the laboratory. Parts of the bronchus were then dissected free from parenchymal tissue and preparations cut (3–4 mm length × 3–4 mm internal diameter) as previously described (Cortijo *et al*., 1993a). Preparations were stored in PSS, equilibrated with 5% CO_2 in O_2 at 4°C until use. Experiments were routinely completed within 24 h of initial tissue storage.

Bronchial rings were set up in 10 ml organ baths containing PSS, gassed with 5% CO_2 in O_2 at 37°C (pH 7.4). Each preparation was connected to a force displacement transducer (Grass FTO3) and isometric tension changes were recorded on a Grass polygraph (model 7P). The preparations were equilibrated for 60–90 min (with changes in bath fluid every 20 min) before any pharmacological intervention occurred. Imposed tension of 2 g was maintained throughout the equilibration period and a stable resting level of tone was present at the end of this period. In all experiments, strips of human bronchus were initially challenged with acetylcholine (ACh, 1 mM) in order to determine the maximal contractile response of the tissue.

Assessment of the effect of vanadate on spontaneous bronchial tone and its modification by drugs and other interventions

A cumulative concentration-response curve for vanadate (0.1 μM–3 mM) was constructed. In some tissues, a second concentration-response curve for vanadate was obtained following a wash out period of 30 min. A concentration of vanadate (200 μM) close to its EC_{50} was selected for further experiments. The response to this concentration of vanadate was monitored for 60 min both in time-matched control tissues and in test tissues. Test tissues were subjected to epithelial removal (Iriarte *et al*., 1990) or were treated with an agent such as indomethacin (2.8 μM), zileuton (10 μM), compound 48/80 (two consecutive 30 min challenges each at a concentration of 100 μg ml^{-1}), a mixture of atropine, phentolamine and mepyramine (each at 1 μM), verapamil (1 μM), nifedipine (1 μM), ouabain (10 μM), amiloride (100 μM), H-7 (10 μM), staurosporine (1 μM), calphostin C (1 μM), or genistein (100 μM). With the exception of compound 48/80, the response of test tissues to vanadate was obtained after 30 min preincubation with, and in the presence of, the relevant drug treatment.

The response of test tissues to vanadate was also measured in preparations bathed in modified solutions: Ca^{2+}-free PSS containing EGTA (0.1 mM), K^+-rich (80 mM) PSS, K^+-free PSS, and Na^+-deficient (25 mM) PSS (for composition see drugs and solutions). In general, test tissues were exposed to the modified solutions for 30 min before and throughout the vanadate challenge. In the case of the Na^+-deficient PSS, the preincubation time was increased to 60 min. To assess the effect of vanadate on K^+-induced relaxation, the tissues were incubated for 30 min in K^+-free PSS then, for test tissues, the bath fluid was exchanged for K^+-free PSS containing vanadate (200 μM) or ouabain (10 μM). Fifteen minutes later test tissues were challenged with KCl (30 mM). Time-matched control tissues were subjected to the same protocol but were not exposed to vanadate or ouabain.

In further experiments, the response of test tissues to vanadate (200 μM) was measured after 30 min preincubation with, and in the presence of, Ca^{2+}-free PSS containing both EGTA (0.1 mM) and ryanodine (10 μM). In experiments involving the depletion and refilling of internal Ca^{2+} stores, three consecutive challenges with vanadate (200 μM, applied for 15 min) were made at 30 min intervals in the presence of Ca^{2+}-free EGTA (1 mM) PSS. In the case of test tissues, cyclopiazonic acid (CPA; 10 μM) was then added to the medium and remained present for the duration of the experiment. Twenty minutes later, the medium was changed for a K^+-rich (80 mM), Ca^{2+} (2.5 mM)-containing PSS and 50 min later, the medium was changed back to the Ca^{2+}-free EGTA (1 mM)-containing PSS and a fourth vanadate challenge was made. Control tissues were treated identically to test tissues except that they were not exposed to CPA. In further experiments the mechanical effects of CPA (10 μM) were studied by its addition to strips of bronchus bathed by normal PSS.

Assessment of tissue ion content

Tissue Na^+ and K^+ contents were determined by a modified lithium method, essentially as described for rabbit and monkey trachea by Ueda *et al*. (1985). After 60 min incubation with PSS (control tissues), ouabain (10 μM) or vanadate (200 μM), bronchial strips (wet weight approximately 25 mg) were washed for 30 min with cold Li^+ solution (composition in mM: LiCl 142.2, $CaCl_2$ 2.5, $MgCl_2$ 1.0, $LiHCO_3$ 11.9 and glucose 5.5). Tissues were then blotted on filter paper, weighed, digested overnight with 0.5 ml of a 1 : 1 mixture of $HClO_4$ (60% w/v) and HNO_3 (61% w/v) at 180°C. The residue was dissolved in 0.01 N HCl. The Na^+ and K^+ contents of this solution were determined by atomic absorption spectrophotometry (Perkin-Elmer 2380).

Effects of ACh and vanadate on inositol phosphate accumulation

Total inositol phosphate accumulation was determined as previously outlined (Chulia *et al*., 1996). In short, cryostored human bronchi (−80°C, in foetal calf serum containing 1.8 M dimethylsulphoxide (DMSO) Sarria *et al*., 1995) from 2 to 5 patients were rapidly thawed in a 37°C water bath and rinsed in a large volume of PSS to eliminate the DMSO. They were cut into small fragments (about 1 mm^2) and the pool, weighing a total of ~10 g was washed in PSS and incubated in 25 ml PSS containing 50 µCi of *myo*-[^{3}H]-inositol for 4 h at 37°C. After this incubation, the tissue was washed twice with PSS. Aliquots of washed tissue (1–1.5 g) were placed in a 2 ml final volume of PSS and preincubated at 37°C for 30 min (for Ca^{2+}-free experiments, the tissue was rinsed and preincubated for 30 min in Ca^{2+}-free EGTA 0.1 mM PSS with or without ryanodine). Just before stimulation, 20 µl of LiCl was added (final concentration 10 mM). The samples were then stimulated with 20 µl PSS (control), acetylcholine (1 μM–3 mM; values for 3 mM served as reference values of 100%) or vanadate (0.1, 0.3, 1 or 3 mM) for 30 min at 37°C. Stimulation was stopped by addition of 3 ml of ice-cold mixture of chloroform/methanol/HCl 12 M (100 : 200 : 4, v/v/v) and vigorous shaking. The samples were centrifuged (4000 *g*) for 10 min at 4°C and the aqueous phases were brought to pH 4 and stored at −20°C until analysis. The separation of inositol phosphates was performed by a high performance liquid chromatography (h.p.l.c.) ion-exchange system (linear gradient of potassium phosphate 1 M, pH 3.7), and the radioactivity measured in a Flow-One on-line radioactivity detector (Packard, Meriden, U.S.A.) as described by Chulia *et al*. (1996).

The effect of vanadate on $[Ca^{2+}]_i$

Primary cultures of human airway smooth muscle cells were prepared from explants of trachealis muscle obtained within 8 h of death from individuals without respiratory disease. The trachealis muscle was dissected under sterile conditions in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine, penicillin (100 u ml^{-1}), streptomycin (100 µg ml^{-1}) and amphotericin B (2.5 µg ml^{-1}); 1–2 g of wet tissue was minced and washed in

supplemented DMEM. The tissue slurry was centrifuged at 150 *g* for 5 min at 4°C. The pellet was resuspended in 10 ml of DMEM containing 640 u ml^{-1} collagenase (type IV, Sigma) and 10 u ml^{-1} elastase (type I, Sigma), and digested for 90 min in a shaking bath at 37°C. The cell suspension was filtered and centrifuged at 150 *g* for 10 min, resuspended in DMEM/Ham's F12 medium supplemented as indicated above, counted in a haemocytometer, and viability assessed by trypan blue exclusion (>95%). Cell suspensions were plated in 25 cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2 in air, with fresh medium exchanged at 48–72 h intervals. Cells were subcultured after reaching confluency (~21 days). Subculturing was performed for up to 4–5 passages. Cells in subcultures were examined with a monoclonal antibody against smooth muscle actin (1:100 dilution; Sigma) using the avidin-biotin-peroxidase method (Hsu *et al*., 1981); >95% of the cells were positively stained. Measurements of $[Ca^{2+}]_i$ were performed on cell suspensions essentially as described previously (Cortijo *et al*., 1996). Briefly, human tracheal smooth muscle cells cultured in 25 cm^2 flasks were incubated with 3 μM fura-2/AM (3 mM stock solution in dimethylsulphoxide), at 37°C for 45 min in HEPES buffer (composition, mM: NaCl 145, KCl 5, $MgSO_4$ 1, HEPES 10, $CaCl_2$ 2, glucose 10, pH 7.45). The cells were then washed with this buffer and incubated for 15 min in the absence of fura-2. The cells were detached with trypsin (2 min at 37°C) and washed with HEPES buffer. Fura-2 loaded cells were resuspended at 10^6 cells ml^{-1} and placed in 1 cm quartz cuvettes. Cells in cuvettes were pre-incubated for 2 min at 37°C, with gentle stirring, in a temperature-controlled cuvette holder before fluorescence intensities were measured with a Perkin-Elmer LS-50 spectrofluorimeter. Vanadate (200 μM) or histamine (100 μM) was added in a volume of 20 μl to 1 ml cell suspension and the changes were monitored for 30 min (vanadate) or 4 min (histamine). $[Ca^{2+}]_i$ was calculated from the fluorescence intensities measured at 510 nm after excitation at 340 and 380 nm as described by Grynkiewicz *et al*. (1985). Maximum and minimum fluorescence intensities were obtained with 0.1% Triton X-100 and 10 mM EGTA in 2 M Tris-HCl, pH 8.5, respectively.

Electrophysiological studies

The tissue bath used and other technical aspects have been described previously (Small & Weston, 1979; Dixon & Small, 1983). The mucosal membrane covering the luminal surface of the bronchus was carefully removed with a cotton bud and the tissue was set up for the simultaneous recording of intracellular electrical activity and mechanical changes. Microelectrodes (30–50 MΩ: tip potentials of less than 5 mV) were filled with 3 M KCl and were used in conjunction with a WPI Intra 767 amplifier. One or two preparations from each patient (a total of 15 preparations from 8 patients) were examined. In 5 preparations from 4 patients, vanadate (200 μM) was added to the superfusing PSS and changes in membrane potential and tension were monitored for 10 min.

Drugs and solutions; statistical analysis of results

Drug concentrations are expressed as final bath concentrations of the active species. The following drugs were used: acetylcholine chloride, atropine sulphate, calphostin C, cyclopiazonic acid (CPA), ethyleneglycol-bis-(β-amino-ethyl-ether)-N-N′-tetraacetic acid (EGTA), genistein, H-7 (1-(5-isoquinolinylsulphonyl)-2-methyl-piperazine), histamine hydrochloride, indomethacin, mepyramine maleate, ouabain, phentolamine hydrochloride, staurosporine, sodium metavanadate (each from Sigma), amiloride hydrochloride (Merck, Sharp & Dohme), (±)-verapamil hydrochloride (Biosedra-Knoll), ryanodine (Calbiochem) and zileuton (gift from Dr S.A. Esteve). Other chemicals used were of analytical grade (E. Merck; Panreac). Stock solutions of calphostin C, CPA and genistein were prepared in dimethyl sulphoxide (DMSO). Stock solutions of indomethacin, ryanodine, nifedipine and zileuton were prepared in absolute ethanol. Final bath concentrations of the solvents did not themselves affect the mechanical activity of the tissue. Calphostin C, nifedipine and staurosporine were protected from light exposure. Other drugs were dissolved in PSS just before use. The composition (mM) of the physiological salt solution (PSS) was: NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.1. The Ca^{2+}-free PSS was prepared by substituting EGTA (0.1 mM) for $CaCl_2$. The K^+-free PSS was prepared by removing KCl from the PSS and replacing KH_2PO_4 with NaH_2PO_4. The K^+-rich (80 mM) PSS was prepared by raising the KCl concentration by 78.8 mM and reducing the concentration of NaCl to preserve isomolality. The Na^+-deficient (25 mM) PSS was prepared by iso-osmotic replacement of NaCl by sucrose.

Contractile responses are expressed in absolute values (g or mg) and/or as a percentage of the response to ACh (1 mM). The EC_{50} of spasmogens was derived by interpolation in each concentration-effect curve. Data are presented as means ± s.e.mean. The number of experiments is expressed as '*n*/p' where *n* represents the number of preparations examined, and p the number of patients from which those tissues were derived. Statistical analysis of the results was performed by analysis of variance followed by Bonferroni multiple comparison test or by Student's *t* test as appropriate. Differences were considered significant when $P<0.05$.

Results

Contraction of human bronchus induced by vanadate

Vanadate (0.1 μM–3 mM) applied cumulatively to human isolated bronchus caused concentration-dependent tension development (Figure 1). The maximal effect of vanadate was 1.69 ± 0.27 g tension (mean ± s.e.mean) which was equivalent to 97.5 ± 4.2% (*n*/p = 7/4) of the contraction elicited by ACh (1 mM). The $-\log EC_{50}$ for vanadate was 3.74 ± 0.05 (*n*/p = 7/4). A concentration of vanadate (200 μM) close to its EC_{50} was selected for further experiments. The onset of tension development in response to vanadate (200 μM) occurred within 1 min of challenge and maximum tension was achieved after 25–45 min (Figure 2). This tension was maintained for periods in excess of 60 min. Responses to vanadate were reversible following washout and reproducible contractions could be elicited at 30 min intervals (data not shown).

Role of epithelium and endogenous mediators in vanadate-induced contractions

Removal of the epithelium did not alter the response of the tissue strips to vanadate (200 μM) (Table 1). Tissue incubation with either indomethacin (2.8 μM) or zileuton (10 μM) for 30 min had no effects on basal tone. Furthermore, neither indomethacin nor zileuton modified the contractile response to vanadate (200 μM) (Table 1). The initial exposure of bronchial strips to compound 48/80 (100 μg ml^{-1}) induced a contraction equivalent to 53.8 ± 6.3% of that evoked by ACh (1 mM; *n*/p = 6/4), but the response to the second challenge with 48/80 was only 12.3 ± 5.2% (*n*/p = 6/4) of the response evoked by ACh. The contractile response to vanadate in tissues twice-challenged with compound 48/80 did not differ from that seen in time-matched control tissues (Table 1). Similarly, the contractile response to vanadate was unaltered by tissue treatment with a mixture of atropine (1 μM), phentolamine (1 μM) and mepyramine (1 μM) (Table 1).

Effect of extracellular Ca^{2+}, Ca^{2+} channel blockers, ryanodine and cyclopiazonic acid on vanadate-induced contraction

Verapamil (1 μM) and nifedipine (1 μM) reduced the spontaneous tone of the tissues by 378 ± 82 mg and 314 ± 122 mg respectively (*n*/p = 6/4). However, tissue treatment with either

of these inhibitors of Ca^{2+} influx failed to reduce the contractile effect of vanadate (200 μM) (Table 1). Baseline tension fell (by 842 ± 146 mg; $n/p = 9/5$) when PSS was changed to a Ca^{2+}-free PSS containing EGTA (0.1 mM). Vanadate (200 μM) contractions elicited after 60 min incubation in the Ca^{2+}-free, EGTA-containing PSS were of amplitude and time course similar to those observed in time-matched control tissues bathed by normal PSS (Figure 2a). In some tissues the addition of ryanodine (10 μM) to the Ca^{2+}-free, EGTA-containing PSS caused a small, transient contraction. However, ryanodine did not otherwise modify the tissue tone loss induced by the Ca^{2+}-free medium. The addition of ryanodine to the Ca^{2+}-free medium significantly reduced the contractile response to vanadate (200 μM) (Figure 2a and Table 1). In experiments involving the depletion and refilling of intracellular Ca^{2+} stores, the response to the third vanadate challenge in the Ca^{2+}-free, EGTA (1 mM)-containing PSS was significantly smaller than the response to the initial challenge (Table 2). Exposure of control tissues to the Ca^{2+} (2.5 mM)-containing, K^{+}-rich (80 mM) PSS restored the response to vanadate, the response to the fourth vanadate challenge (made in the Ca^{2+}-free EGTA-containing medium) did not differ from the response to the initial challenge. In test tissues, the third response to vanadate was similarly reduced compared with the response to the initial challenge. However, the presence of CPA (10 μM) during the Ca^{2+} store refilling process blocked the recovery of the response to vanadate. The test tissue response to the fourth vanadate challenge was significantly smaller than the response to the initial challenge. It was also significantly smaller than the response to the fourth challenge in the control tissues (Table 2). Added to bronchial strips bathed by normal PSS, CPA (10 μM) evoked tension development. Peak tension was reached after approximately 10 min and was equivalent to $83.9 \pm 5.3\%$ of the ACh maximum ($n/p = 3/2$).

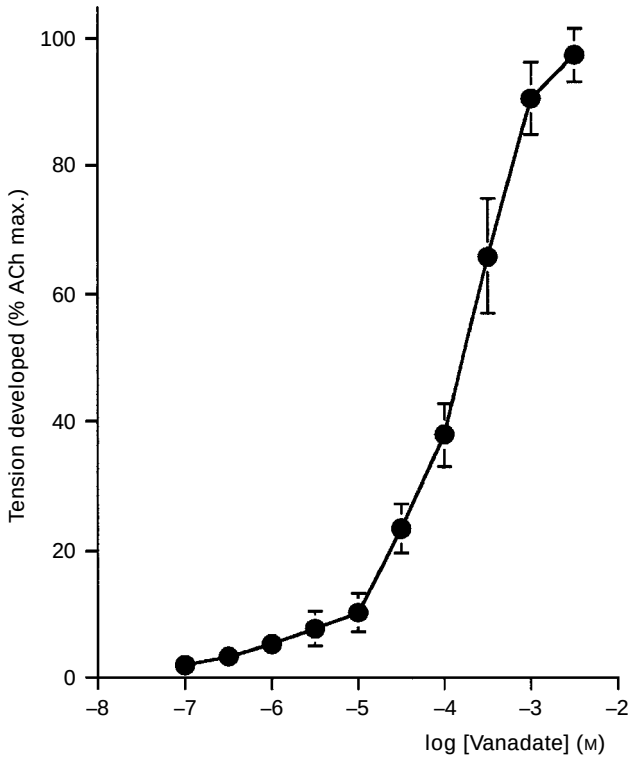

Figure 1 Log concentration-response curve for the contractile action of vanadate in human isolated bronchus. Abscissa scale: molar concentration of vanadate on a log scale. Ordinate scale: tension developed expressed as a percentage of that evoked by ACh (1 mM). Data points indicate means of values from 7 preparations taken from 4 patients. Vertical lines indicate s.e.mean.

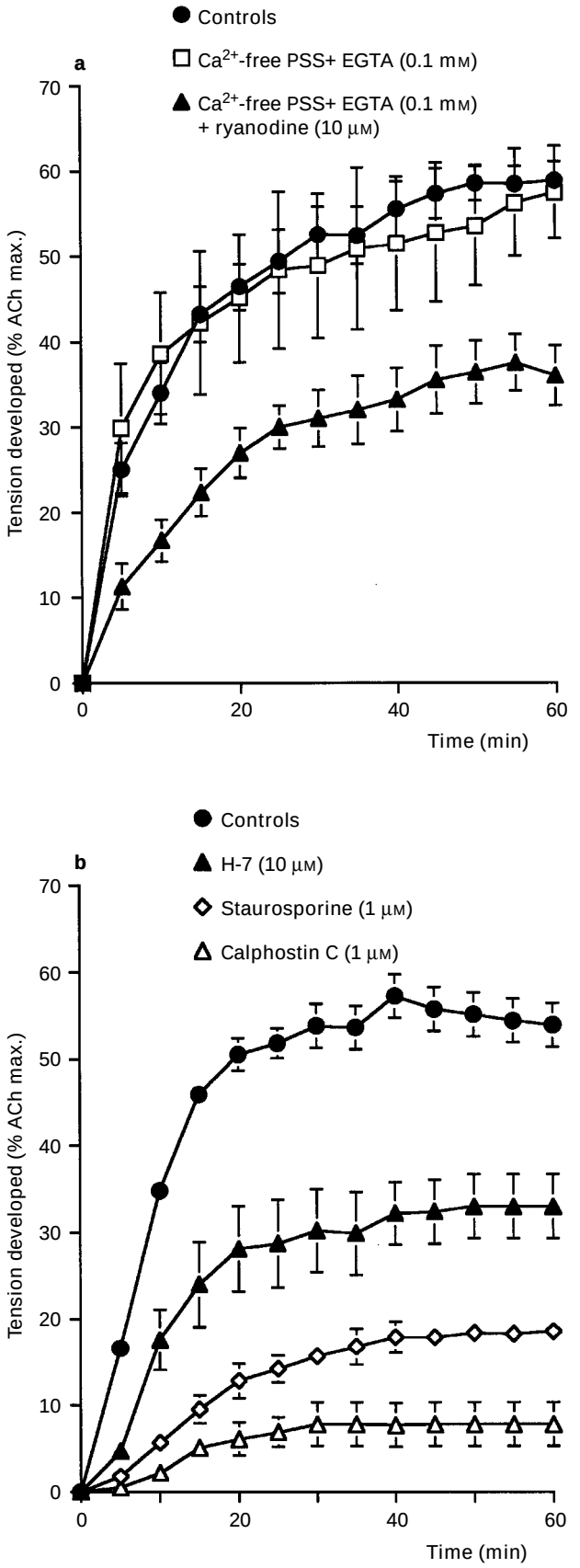

Figure 2 The spasmogenic effect of vanadate (200 μM) in human isolated bronchus: its time course and the effects of some drugs and modified physiological salt solutions. Abscissa scales: time after administration of vanadate. Ordinate scales: tension developed expressed as a percentage of that evoked by ACh (1 mM). In both panels (a) and (b) data points indicate means and vertical lines show s.e.mean. At all time points (except time zero) responses to vanadate obtained in the presence of the Ca^{2+}-free, ryanodine-containing PSS, H-7, staurosporine or calphostin C were significantly ($P < 0.05$) smaller than those observed in the control tissues.

Table 1 Analysis of the contractile action of vanadate (200 μM) in human isolated bronchus

Procedure or agent	*Control tissues*	n/*p*	*Test tissues*	n/*p*
Epithelial removal	67.3±4.4	4/3	61.6±5.4	4/3
Indomethacin (2.8 μM)	65.6±3.5	5/4	68.7±3.2	5/4
Zileuton (10 μM)	59.5±4.6	5/3	63.3±5.7	5/3
Compound 48/80	62.8±2.9	6/3	57.2±5.7	6/4
Atropine (1 μM)+phentolamine (1 μM)+mepyramine (1 μM)	65.3±2.7	5/4	67.0±1.4	5/4
Verapamil (1 μM)	59.6±5.4	6/3	66.7±8.0	6/3
Nifedipine (1 μM)	53.6±4.5	6/3	42.4±6.6	6/3
Ca^{2+}-free, EGTA (0.1 mM)-containing PSS	58.9±2.2	6/3	57.5±5.4	9/5
Ryanodine (10 μM)	57.4±5.3	4/3	36.0±3.5*	4/3
K+ (80 mM)-rich PSS	62.2±2.0	6/4	64.8±8.3	6/4
K+-free PSS	53.4±6.3	4/3	39.6±6.3	4/3
Na+-deficient (25 mM) PSS	58.9±2.2	6/3	9.4±9.3*	7/3
Amiloride (100 μM)	58.9±2.2	6/3	7.0±4.1*	6/3
Ouabain (10 μM)	51.8±4.9	5/4	49.2±5.9	5/4
Genistein (100 μM)	60.4±6.7	8/4	41.8±5.0*	8/4

Data represent responses (mean±s.e.mean) to vandate expressed as a percentage of the contractile response to ACh (1 mM). *n*=number of bronchial strips and p=number of patients from which the bronchial strips were derived. The effects of ryanodine (10 μM) were examined in experiments where both the test and time-matched control tissues were bathed by Ca^{2+}-free, EGTA (0.1 mM)-containing PSS. The experiments with a Ca^{2}-free medium, a Na^{+}-deficient medium and an amiloride-containing medium were performed concurrently and hence used a common group of control tissues. *Significant ($P<0.05$) difference from the control value.

Table 2 Human bronchus: the effects of CPA (10 μM) on Ca^{2+}-induced recovery of vandate responses following depletion of intracellular Ca^{2+} stores

	Response to 1st vanadate challenge (%)	*Response to 3rd vanadate challenge* (%)	*Response to 4th vanadate challenge* (%)
Control tissues	63.9±1	48.3±1.1*	59.6±1.6
Test tissues	69.0±3.5	46.1±4.2*	44.1±2.3*,†

All responses are expressed in terms (%) of the maximal response to ACh. *Significant ($P<0.05$) difference from the initial response to vanadate in the series. †Significant ($P<0.01$) difference from the corresponding response in the control tissues. For both tissue groups *n*/p=4/3.

Effect of inhibitors of protein kinase C and protein tyrosine kinase on vanadate-induced contraction

Staurosporine (1 μM) did not affect the spontaneous tone of human bronchus (*n*/p=6/3). However, H-7 (10 μM) and calphostin C (1 μM) reduced tone by 569±106 mg (*n*/p=6/3) and 291±62 mg (*n*/p=6/3) respectively. H-7, staurosporine and calphostin C each inhibited the contraction to vanadate (200 μM) (Figure 2b). Genistein (100 μM) reduced spontaneous tone by 1.08±0.94 g (*n*/p=8/3) and reduced (by approximately 30%) the contraction to vanadate (Table 1).

Effect of extracellular K^+ on vanadate-induced contraction

Human bronchus contracted on exposure to K^+ (80 mM)-rich PSS with peak tension (71.9±4.6% of the maximal response to ACh) being attained within 10 min, followed by a slow decline to a lower level (23.2±1.6% of the maximal response to ACh). After 30 min tissue incubation in K^+-rich PSS, vanadate (200 μM) induced contractions for which the peak amplitude and time course were similar to those observed in control tissues bathed by normal PSS (Table 1). Exposure of bronchial strips to a K^+-free PSS resulted in a reduction in baseline tension followed by recovery to pre-exposure levels 30 min later (Figure 3a). The subsequent addition of ouabain (10 μM) or vanadate (200 μM) evoked a contraction (Figure 3a). In the final stages of the experiments involving K^+-free, PSS, KCl (30 mM) was added to the bath fluid. In the time-matched control tissues this readmission of K^+ evoked a transient relaxation followed by a rise in tension above the pre-existing level. In test tissues that had been treated with ouabain (10 μM), the readmission of K^+ evoked contraction only. In contrast, test tissues that had been treated with vanadate responded to the readmission of K^+ with transient relaxation followed by contraction (Figure 3a).

Effect of Na^+-deficient PSS, amiloride and ouabain on vanadate-induced contraction

Exposure to a Na^+-deficient (25 mM) PSS evoked a contraction which, after a period of 4–5 min, was equivalent to 49±6% of the response to ACh (1 mM). This contraction then faded with return to baseline tension occurring after 50–60 min. Similarly, tissue treatment with amiloride (0.1 mM) evoked a transient contraction of peak value equivalent to 18±7% of the response to ACh (1 mM). Tissue tension returned to the pre-existing baseline within 25–30 min. Vanadate (200 μM)-induced contraction was significantly depressed in tissues bathed in a low Na^+ solution or treated with amiloride (Table 1 and Figure 3b). In contrast, Na^+-deficient (25 mM) PSS and amiloride (0.1 mM) each failed to modify (control response=67.3±3.6% of ACh maximal effect, response in Na^+-deficient PSS=66.5±5.4% and response after amiloride=58.7±6.3%; $P>0.05$ and *n*/p=5/3) contractile responses to ACh (50 μM). Ouabain (10 μM) evoked a sustained contraction equivalent to 43.3±5.7% (*n*/p=6/5) of the response to ACh (1 mM). This contraction was not observed in tissues subjected to 30 min preincubation in Ca^{2+}-free, EGTA (0.1 mM)-containing PSS. Vanadate (200 μM)-induced contraction was unaltered by tissue treatment with ouabain (10 μM) (Table 1).

Effects of ouabain and vanadate on the tissue content of Na^+ and K^+

Ouabain (10 μM) increased the Na^+ content but decreased the K^+ content of the human bronchial strips. Vanadate (200 μM) did not significantly modify either the Na^+ or the K^+ content of the tissue (Table 3).

Effects of vanadate on inositol phosphates

ACh (3 mM) and vanadate (3 mM) each caused time-dependent accumulation of inositol phosphates in bronchus. For each agonist, inositol phosphate accumulation increased

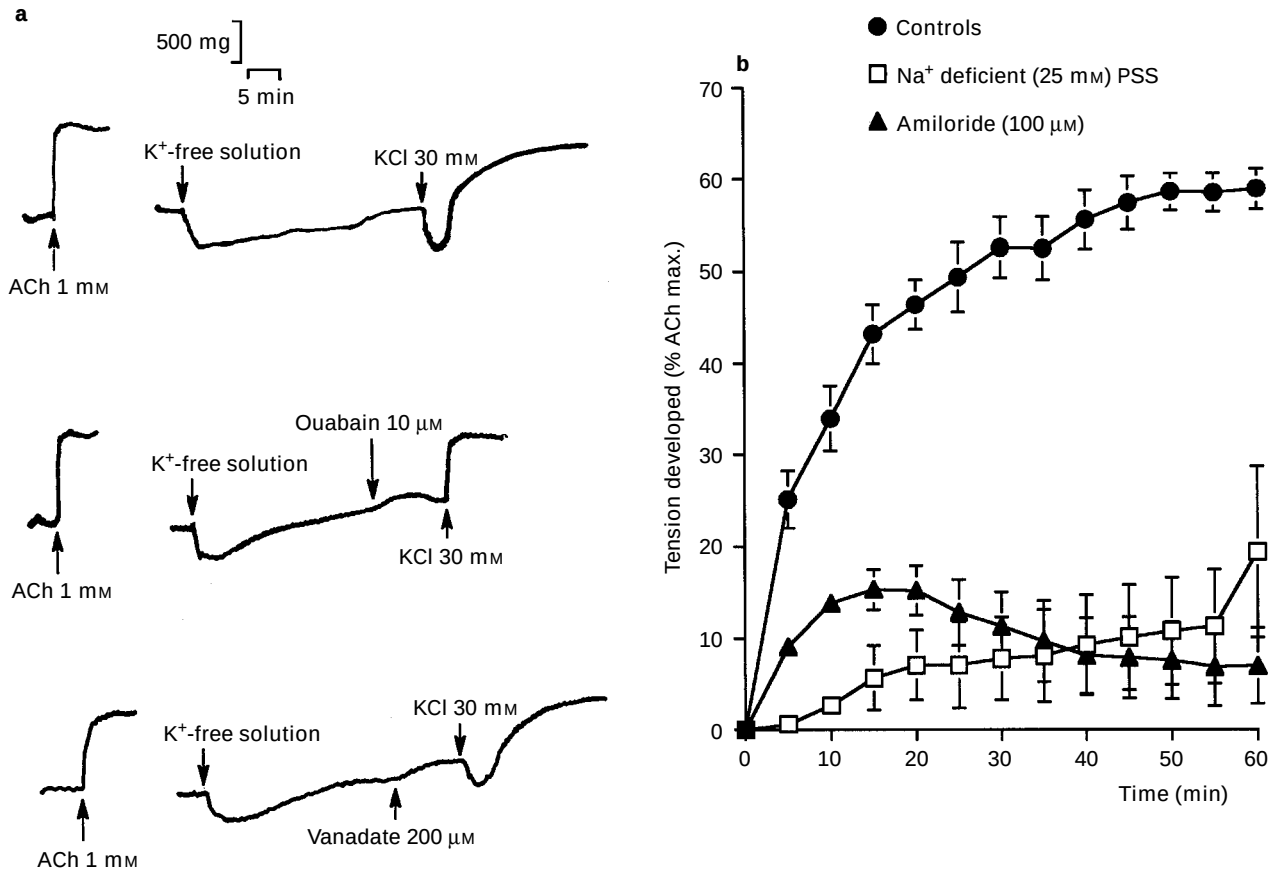

Figure 3 (a) Lack of effect of vanadate on the relaxation induced by K^+ readdition in human isolated bronchus bathed by K^+-free PSS. Responses of three preparations obtained from the same patient are illustrated. Upper traces: control tissue – note the initial contraction to ACh (1 mM), the relaxation produced by exposure to the K^+-free PSS and (30 min later) the relaxation induced by readdition of KCl. Note also that relaxation induced by KCl was followed by a rebound contraction. Centre traces: test tissue – note that pretreatment with ouabain (10 μM) abolished the relaxation induced by K^+ readdition without preventing the subsequent contraction. Lower traces: test tissue – note that, in contrast to ouabain, vanadate (200 μM) did not prevent the relaxation evoked by readdition of K^+. (b) The spasmogenic effect of vanadate (200 μM) in human isolated bronchus: its time course and the effects of Na^+-deficient (25 mM) PSS and amiloride (100 μM). Abscissa scale: time after administration of vanadate. Ordinate scale: tension developed expressed as a percentage of that evoked by ACh (1 mM). Data points are means of values from at least 6 tissues; vertical lines show s.e.mean. At all time points (except time zero) responses to vanadate obtained in the presence of the Na^+-deficient PSS or amiloride were significantly ($P<0.05$) smaller than those observed in the control tissues.

Table 3 Effects of vanadate and ouabain on the Na^+ and K^+ contents of human isolated bronchus

	Na^+	K^+
Control	5.9±1.5	28.7±2.7
Ouabain (10 μM)	11.3±1.6*	18.4±1.9*
Vanadate (200 μM)	6.3±1.8	23.0±2.5

Data are mean (±s.e.mean) ion contents (nmol mg^{-1} wet weight of tissue) from four tissue segments derived from three subjects. *$P<0.05$ compared with the control value.

linearly over a period of 60 min and no saturation of accumulation was oberved (data not shown). When inositol phosphate accumulation was measured 30 min after agonist challenge, ACh (1 μM – 3 mM) and vanadate (100 μM – 3 mM) were each found to increase inositol phosphate accumulation in a concentration-dependent manner (Figure 4a). In contrast to ACh, the maximal effect of vanadate was not reached, even at a concentration of 3 mM. When equal concentrations were compared, the inositol phosphate accumulation produced by vanadate was significantly smaller than that evoked by ACh (Figure 4a). The accumulation of inositol phosphates induced either by vanadate or by ACh was unaffected by tissue exposure to Ca^{2+}-free EGTA 0.1 mM PSS with or without ryanodine (10 μM) (Figure 4b).

Effects of vanadate on $[Ca^{2+}]_i$

Histamine (100 μM) evoked contraction of human bronchus equivalent to that evoked by vanadate (200 μM). This concentration of histamine increased the $[Ca^{2+}]_i$ of cultured tracheal smooth muscle cells from a basal value of 97.0±14.3 nM to a peak value of 366.7±36.6 nM (n/p = 7/4). The peak of the histamine-induced increase in $[Ca^{2+}]_i$ occurred approximately 4 s after drug addition. Thereafter $[Ca^{2+}]_i$ declined towards the basal value. Compared with histamine, vanadate (200 μM) produced a smaller ($P<0.05$) rise in $[Ca^{2+}]_i$, from a basal value of 99.9±18.2 nM to a peak value of 168.6±20.5 nM (n/p = 5/4). $[Ca^{2+}]_i$ reached its peak value in approximately 6 s. $[Ca^{2+}]_i$ returned to the basal level within the next 10 s.

Electrophysiological studies

The resting membrane potential of the human bronchial muscle was −45.7±1.3 mV (range −36 mV to −52 mV; n/p = 15/8). In 5 out of 15 cells examined, spontaneous slow, oscillatory potential changes (slow waves) were recorded. These slow waves occurred at a frequency of 29.2±3.3 min^{-1} and were of variable amplitude (1–5 mV in one cell, 10–15 mV in the remainder). The other cells exhibited irregular potential changes of small amplitude (1–5 mV) and, in one case, these irregular potential changes were interrupted by bursts of more regular slow waves. The resting membrane

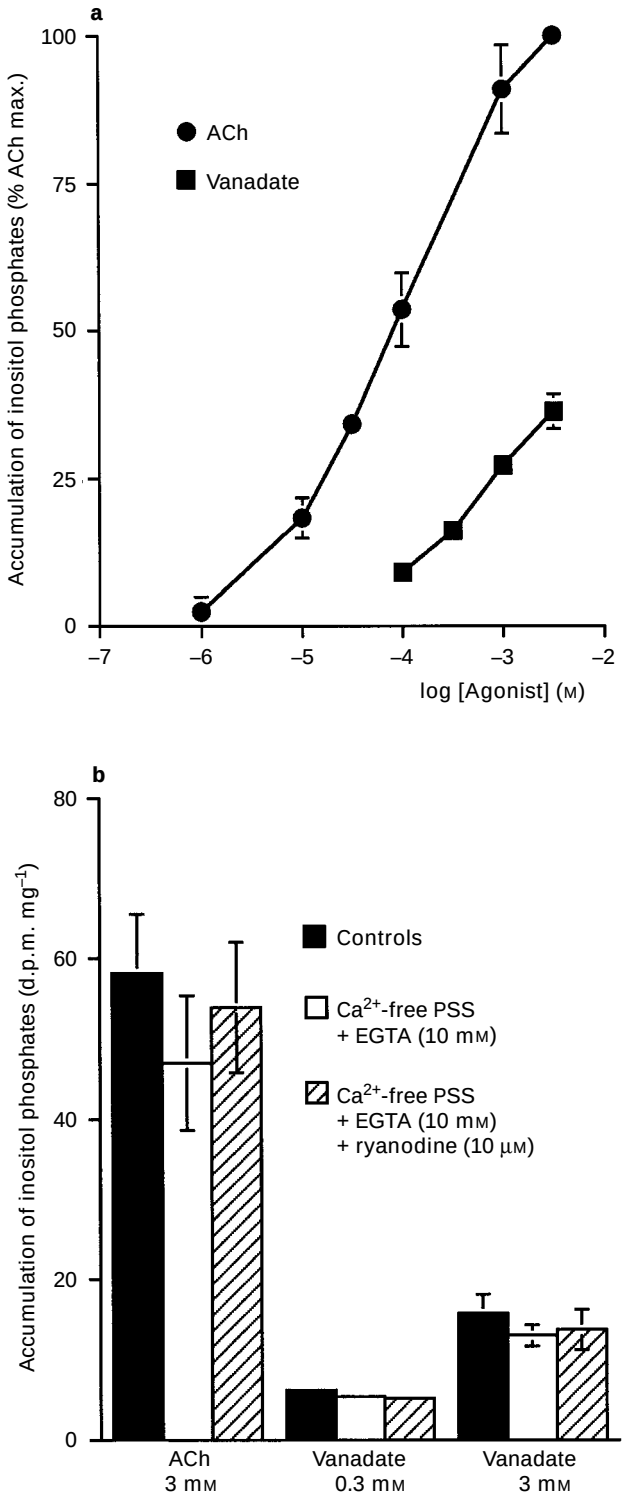

Figure 4 Accumulation of inositol phosphates in human isolated bronchus. (a) Log concentration-response relationships for vanadate and acetylcholine. (b) Inositol phosphate accumulation induced by acetylcholine or vanadate in PSS containing Ca^{2+} (2.5 mM), in Ca^{2+}-free EGTA (0.1 mM)-containing PSS, or in Ca^{2+}-free, EGTA (0.1 mM)-containing PSS + ryanodine (10 μM). All measurements were made 30 min after tissue exposure to vanadate or ACh. Data are means of 4–8 experiments; vertical lines show s.e.mean. When equal concentrations were compared, responses to vanadate were significantly ($P<0.05$) smaller than the corresponding values for ACh (a). Responses to ACh or vanadate were not significantly modified in a Ca^{2+}-free medium or a Ca^{2+}-free medium containing ryanodine (b).

potential in cells subsequently exposed to vanadate was -42.8 ± 2.1 mV ($n/p=5/4$). The addition of vanadate (200 μM) to the superfusate reduced the amplitude of the oscillatory slow waves and evoked depolarization (25.4 ± 1.9 mV; $n/p=5/4$). These electrical changes were accompanied by an increase (640 ± 154 mg) in tissue tension (Figure 5).

Discussion

Direct basis of vanadate-induced contraction in human bronchus

The spasmogenic action of vanadate in human bronchus does not result from stimulation of prostaglandin synthesis since indomethacin (2.8 μM, a concentration sufficient to block prostaglandin synthesis by human bronchial tissue; Brink *et al*., 1980) failed to antagonize vanadate (Table 1). Zileuton (10 μM) is known markedly to inhibit the activity of 5-lipoxygenase in human tissue (McMillan *et al*., 1992). The failure of zileuton to antagonize vanadate-induced spasm of human bronchus (Table 1) therefore suggests that vanadate does not act to stimulate leukotriene production. The resistance of vanadate-induced spasm to both atropine (1 μM) and phentolamine (1 μM) (Table 1) suggests that such spasm is not mediated by the release of parasympathetic or sympathetic neurotransmitters. Tissue treatment with either compound 48/80 or with mepyramine (1 μM) did not modify the action of vanadate (Table 1). These two findings suggest that the spasmogenic action of vanadate is not mediated by the release of histamine from mast cells. Furthermore epithelial removal did not modify the action of vanadate (Table 1). Therefore, the spasmogenic action of vanadate in human bronchus is unlikely to involve the release of mediators from structures within the bronchial wall but, instead, reflects a direct effect of vanadate on the smooth muscle cells.

Effects of vanadate on $[Ca^{2+}]_i$ and source of activator Ca^{2+} utilized by vanadate

It proved easier to dissect smooth muscle from the trachea than from bronchus. It was for this reason that we elected to study the effects of vanadate on $[Ca^{2+}]_i$ in trachealis cells. That the trachealis cells would act as a good model of bronchus cells was suggested by the results of pilot experiments in which

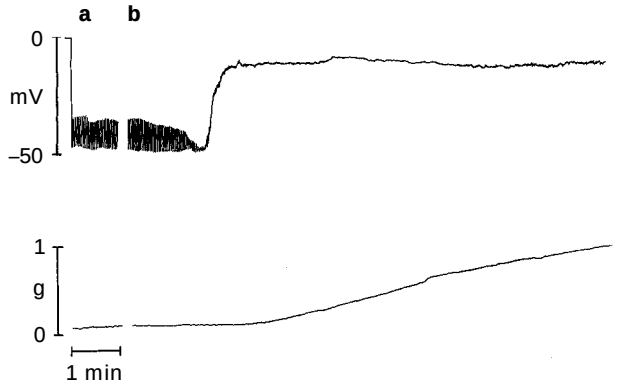

Figure 5 The effects of vanadate on the intracellular electrical and mechanical activity of the human isolated bronchus. The upper trace indicates membrane potential changes while the lower trace indicates tension changes recorded from a contiguous segment of bronchus. The electrical recordings were taken from the same cell. (a) Microelectrode impalement of the cell and control recording showing spontaneous slow waves. (b) Activity recorded following addition of vanadate (200 μM) to the superfusate. Note that vanadate initially suppressed the electrical slow waves and then evoked marked depolarization of the impaled cell. These electrical effects were accompanied by tension development.

vanadate was observed to cause contraction of human trachealis with a potency and time course similar to those observed in bronchus.

The rise in $[Ca^{2+}]_i$ evoked by vanadate in human trachealis cells was very transient. A similar response was described by Sandirasegarane & Gopalakrishnan (1995) for vanadate acting on cultured aortic smooth muscle cells. In human bronchus it is difficult to ascribe an activator role to this brief increase in $[Ca^{2+}]_i$ because it did not evoke an early phasic, component in the mechanical response. The mechanical responses to vanadate developed slowly, was monophasic and, for the vast majority of its time course, was associated with a value of $[Ca^{2+}]_i$ that was not significantly different from the basal value. We therefore presume that the activator Ca^{2+} utilized by vanadate either comprises discrete, very localized increases in $[Ca^{2+}]$ within the cell or comprises a more widespread, but very small, increase in $[Ca^{2+}]_i$. In either case such events could have been below the limits of detection of our method of measuring $[Ca^{2+}]$.

Verapamil and nifedipine can inhibit Ca^{2+} influx through L-type channels in airway smooth muscle cells (Drazen *et al*., 1983; Kohrogi *et al*., 1985; Black *et al*., 1986). The failure of these two agents to reduce vanadate-induced contraction (Table 1) indicates that the spasmogenic action of vanadate does not involve promotion of Ca^{2+} influx through such channels. It might be argued that vanadate acts, instead, to promote Ca^{2+} influx through channels resistant to these agents. However, the contraction of human isolated bronchus induced by vanadate was unaffected by tissue exposure to a Ca^{2+}-free PSS containing EGTA (0.1 or 1 mM). Such concentrations of EGTA are envisaged to chelate extracellular Ca^{2+} and Ca^{2+} bound at superficial sites on the plasmalemma, but to leave the intracellular stores of Ca^{2+} largely intact. Hence, the failure of the EGTA-containing, Ca^{2+}-free PSS to suppress vanadate-induced contraction suggests that such contraction does not depend on an extracellularly-located source of activator Ca^{2+} or on the release of Ca^{2+} from a superficial pool that is accessible to EGTA. This finding contrasts with the results of studies on airways smooth muscle from laboratory animals where vanadate-induced contraction was markedly reduced in a Ca^{2+}-free medium (Nayler & Sparrow, 1983; Ueda *et al*., 1985; Cortijo *et al*., 1993b; Lee *et al*., 1994). It may be that these contrasting results reflect different sources of activator Ca^{2+} for vanadate in human and animal airways smooth muscle. Alternatively, they may reflect differences in the susceptibility of the intracellular Ca^{2+} stores to depletion by the Ca^{2+}-free, EGTA-containing medium.

That vanadate utilizes an intracellular source of activator Ca^{2+} in human bronchial smooth muscle is also suggested by the present experiments with amiloride or a PSS deficient in Na^+. Blaustein (1989) proposed that a Na^+/Ca^{2+} antiporter exists in airways smooth muscle. This Na^+/Ca^{2+} antiporter is located in regions of the smooth muscle cell membrane, that are in close apposition to the endoplasmic reticulum, and plays an important role in maintaining the Ca^{2+} content of the intracellular store (Moore *et al*., 1993). Amiloride inhibits the Na^+/Ca^{2+} antiporter (Bova *et al*., 1988). Such an action could lead to a reduction in the cellular extrusion of Ca^{2+} and could explain the present finding that amiloride caused contraction of human bronchus. Exposure of smooth muscle to a Na^+-deficient PSS can evoke a reverse-mode action of the Na^+/Ca^{2+} antiporter (Ohata *et al*., 1996) and may explain our finding that such a medium also induced contraction. In the long term, tissue exposure to a Na^+-deficient PSS would result in a reduction in $[Na^+]_i$. In this situation the Na^+/Ca^{2+} antiporter would be compromised and the intracellular Ca^{2+} store would become depleted (Brading *et al*., 1980). Tissue treatment with amiloride would have the same end effect. The present findings that tissue exposure to amiloride and a PSS deficient in Na^+ could each reduce the contractile action of vanadate therefore also indicate that vanadate uses an intracellular source of Ca^{2+} as an activator.

Ryanodine interferes with Ca^{2+} mobilization from the endoplasmic reticulum in a variety of tissues including airway smooth muscle (Ito *et al*., 1986; Gerthoffer *et al*., 1988; Hay, 1990; Chideckel & Anireddy, 1991). Ryanodine receptors are located on the cytosolic end of Ca^{2+} channels that permeate the membrane of the endoplasmic reticulum. Their activation can promote channel opening in a reduced conductance state, but can also promote irreversible channel closure (Sitsapesan *et al*., 1995). It may be that the opening of Ca^{2+} channels in the endoplasmic reticulum explains our finding that ryanodine evoked transient spasm of human bronchus. The ability of ryanodine to reduce vanadate-induced contraction of bronchus bathed by a Ca^{2+}-free, EGTA-containing PSS (Figure 2a and Table 1) suggests that vanadate utilizes activator Ca^{2+} that is released from an intracellular store. Since ryanodine did not evoke contraction equivalent to that produced by cyclopiazonic acid (see below), it may be that the principal effect of ryanodine in antagonizing vanadate is not so much to deplete intracellular Ca^{2+} stores but more to prevent Ca^{2+} release from the stores into the cytosol.

Mechanisms of vanadate-induced release of Ca^{2+} from intracellular stores

Vanadate has been shown to inhibit Ca-ATPase in the endoplasmic reticulum of cardiac muscle, skeletal muscle and smooth muscle with a K_i value in the range 12–50 μM (Wang *et al*., 1979; Wibo *et al*., 1981). This K_i value for vanadate lies within the effective concentration range of vanadate in contracting human bronchus (Figure 1). As suggested by Nayler and Sparrow (1983), inhibition of Ca-ATPase would promote Ca^{2+} release from the endoplasmic reticulum. In the present study, cyclopiazonic acid was able to mimic the contractile effects of vanadate, suggesting that inhibition of Ca-ATPase can release sufficient Ca^{2+} to trigger contraction. However, it is unlikely that inhibition of Ca-ATPase in the endoplasmic reticulum is, alone, sufficient to explain the contractile effects of vanadate. For the vast majority of its time course, the contractile effect of vanadate was generated at values of $[Ca^{2+}]_i$ that were not significantly different from the basal value. This suggests the concomitant operation of mechanisms that increase the sensitivity of the contractile machinery to Ca^{2+} (see below).

In addition to inhibiting Ca-ATPase in the endoplasmic reticulum, vanadate may also promote the release of stored Ca^{2+} by stimulating the production of inositol phosphate second messengers. In canine trachealis muscle (Lee *et al*., 1994) and in human bronchus (present study; Figure 4a) ACh and vanadate each evoked a concentration-dependent increase in the accumulation of inositol phosphates. It is likely that the effect of vanadate on inositol phosphate accumulation stems from its ability to activate phospholipase C (PLC). Three major isoforms (PLCβ, PLCγ and PLCδ) of this enzyme have been described in mammalian cells. PLCβ is activated by heterotrimeric G-proteins while PLCγ is activated following phosphorylation by protein tyrosine kinase. The mechanisms by which PLCδ is activated remain unclear (Rhee & Choi, 1992; Clapham, 1995).

Vanadate has been shown to activate G-proteins in various tissues by forming a stable complex with guanosine 5′-diphosphate (GDP) at the nucleotide binding site (Krawietz *et al*., 1982; Paris & Pouyssegur, 1987). It may therefore be that vanadate can activate PLCβ by such a mechanism. Similarly, vanadate has been shown to inhibit phosphotyrosine phosphatase (Swarup *et al*., 1982). This would mimic the effect of activating protein tyrosine kinase and would, in turn, activate PLCγ. Whether vanadate can also activate PLCδ remains to be established.

Since the PLC isoform content of human airway smooth muscle cells has not been documented (Chilvers *et al*., 1994), it is difficult to assess the relative roles of PLCβ, PLCγ and PLCδ in mediating the presently-observed contractile effects of vanadate. However, we have generated some evidence to

suggest that the activation of PLCγ may play only a minor role. In taenia coli and vascular smooth muscle (Di Salvo *et al*., 1993; Filipeaunu *et al*., 1995) genistein and other tyrosine kinase inhibitors markedly attenuated vanadate-induced contraction. In contrast, genistein (100 μM, a concentration about five times higher than that required for half-maximal inhibition of protein tyrosine kinase phosphorylation; Akiyama & Ogawara, 1991) produced only a 30% inhibition of vanadate-induced contraction of human bronchus. This suggests that protein tyrosine kinase phosphorylation of PLCγ (and other intracellular proteins) is only of limited importance in mediating the spasmogenic effects of vanadate in this tissue.

For agonists that stimulate the hydrolysis of phosphoinositides by PLC, inositol trisphosphate (IP_3) is the dominant second-messenger molecule provoking the release of Ca^{2+} from intracellular stores (Berridge, 1993; Clapham, 1995). Several authors (Hashimoto *et al*., 1985; Katsuyama *et al*., 1990; Challiss *et al*., 1992) have shown that, in airways smooth muscle, spasmogenic drugs evoke a transient increase in the tissue content of IP_3. It may be that the vanadate-induced increase in inositol phosphates observed in the present study incorporates a similar IP_3 signal. If so, this could account for the transient rise in $[Ca^{2+}]_i$ induced by vanadate. However, as discussed above, this Ca^{2+} signal seems unimportant for expression of the mechanical response to vanadate.

Role of protein kinase C in vanadate-induced contraction of human bronchus

Since vanadate can activate certain of the isoforms of phospholipase C, it is therefore likely to stimulate the production of diacylglycerol, an activator of protein kinase C (PKC). The activation of PKC is involved in the tonic contraction of smooth muscle that can be maintained at, or very near, basal levels of $[Ca^{2+}]_i$ (Rasmussen *et al*., 1987). Yang and Black (1995) have explored the role of PKC in mediating contractile responses of human bronchus to histamine (10 μM). These authors showed that GF 109203X, a highly selective inhibitor of PKC, did not affect the initial peak tension induced by histamine but reduced the secondary, sustained phase of the response. Yang and Black (1995) concluded that PKC is important in mediating only the latter part of the response to histamine.

In the present study the contractile response to vanadate appeared to be monophasic, the peak tension being reached between 25–45 min (Figure 2) and thereafter being well-sustained. The PKC inhibitors H-7, staurosporine and calphostin reduced vanadate-induced contraction over its entire time-course (Figure 2b). Like GF 109203X, calphostin is a highly selective inhibitor of PKC (Kobayashi *et al*., 1989). The present findings therefore suggest that mechanisms underlying the contractile responses of human bronchus to vanadate differ from those of responses to histamine. In the case of histamine, PKC seems to be important in mediating only the secondary, sustained phase of the response. However, in the case of vanadate, PKC plays an important role throughout the time course of the response.

Role of plasmalemmal transport mechanisms in the contractile action of vanadate

The plasmalemma of human airway smooth muscle cells contains Na^+/K^+-ATPase and ouabain, an inhibitor of this enzyme, contracts human isolated trachea and bronchus (Chideckel *et al*., 1987; Knox *et al*., 1990; present study). Vanadate is known to be a potent inhibitor of Na^+/K^+-ATPase in a number of tissues (Nechay, 1984) and it is possible that inhibition of this enzyme accounts for the vanadate-induced contraction of human bronchus. However, several of the present findings do not support this idea. For example, contraction produced by ouabain was abolished in Ca^{2+}-free, EGTA-containing PSS while contraction to vanadate remained unaltered (Table 1). Tissue pretreatment with ouabain in a concentration sufficient to produce marked inhibition of Na^+/K^+-ATPase did not inhibit vanadate-induced contraction (Table 1). Na^+/K^+-ATPase can be inhibited by reducing $[K^+]_o$. This manoeuvre resulted in tissue relaxation followed by secondary recovery to the pre-existing level of tension (Figure 3). This relaxation-contraction sequence may result from the initial effects of the increase in the concentration gradient for K^+ across the cell membrane followed by the secondary effects of Na^+/K^+-ATPase inhibition. The contractile response to vanadate remained unaltered in tissue incubated in K^+-free solution, a result consistent with the resistance of vanadate-induced contraction to ouabain. Readdition of K^+ after incubation of human trachea or bronchus in K^+-free PSS induces a ouabain-sensitive relaxation (Chideckel *et al*., 1987; Figure 3) that is attributable to the re-activation of Na^+/K^+-ATPase (Chideckel *et al*., 1987). In the present study K^+-induced relaxation was fully inhibited by ouabain (10 μM) whilst vanadate (20 μM) had no such effect (Figure 3). Finally, the present atomic absorbtion spectrophotometry studies (Table 3) showed that while ouabain (10 μM) evoked the changes in tissue Na^+ content and K^+ content that might be expected following inhibition of Na^+/K^+-ATPase, such changes were not observed in response to vanadate. Hence, although vanadate may be a potent inhibitor of Na^+/K^+-ATPase (Nechay, 1984) in certain tissues, this does not seem to explain its ability to contract human isolated bronchus.

The importance of inhibition of Ca-ATPase in the endoplasmic reticulum to the action of vanadate has been discussed above. However, in muscle tissue of various kinds, vanadate is more potent in inhibiting the Ca-ATPase of the plasmalemma than that of the endoplasmic reticulum (Wang *et al*., 1979; Caroni & Carafoli, 1981; Wibo *et al*., 1981). It is thus possible that, by inhibiting the extrusion of Ca^{2+} across the plasmalemma mediated by Ca-ATPase, vanadate augments the effects of the Ca^{2+} that it releases from the intracellular store.

Role of depolarization in the contractile action of vanadate in human bronchial muscle cells

In the present study the resting membrane potential and the pattern of spontaneous electrical activity of human bronchial smooth muscle cells was similar to that described by Ito *et al*. (1989). As discussed above, it seems likely that vanadate acts to promote the release of Ca^{2+} from intracellular stores. The depolarization of human isolated bronchus induced by vanadate (200 μM; Figure 5) could therefore result from the opening of the Ca^{2+}-dependent Cl-channels that have been suggested to exist in the plasmalemma of airways smooth muscle cells (Janssen & Sims, 1992). However, vanadate-induced depolarization does not appear to be crucial for its spasmogenic action since tissue treatment with a K^+-rich (80 mM) PSS did not suppress the mechanical response of human bronchus to vanadate.

Conclusions

It is concluded that vanadate acts directly on human bronchial smooth muscle, promoting the release of Ca^{2+} from an intracellular store. This store is maintained by the plasmalemmal Na^+/Ca^{2+} antiporter and by Ca-ATPase of the endoplasmic reticulum. The process of vanadate-induced Ca^{2+} release involves both the production of inositol phosphate second messengers and inhibition of Ca-ATPase. The activation of PKC plays an important role in mediating vanadate-induced contraction which, for most of its time-course, is associated with values of $[Ca^{2+}]_i$ that are close to basal.

The concentration of vanadium normally found in the human lung is in the range 0.25–2.8 μM (Nechay, 1984) though in city-dwellers this may rise to 78 μM (Tipton & Shafer, 1964). It is possible that vanadium concentrations in the lungs of workers exposed to vanadium-containing dusts may exceed this value though, as far as we are aware, such data has yet to be published. While lung vanadate concentrations in the range 1–

100 μM might be expected directly to cause some contraction of airways smooth muscle (Figure 1) it is possible that vanadate, in a lower concentration, may induce airway hyper-reactivity by sensitizing the muscle to other bronchoconstrictor stimuli. This hypothesis awaits the test of further experimentation.

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