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Mechanism of vanadate-induced contraction of airways smooth muscle of the guinea-pig

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1 The characteristics of vanadate-induced contraction of airways smooth muscle are described in isolated preparations of guinea-pig central and peripheral airways. Vanadate (1–1000 μM) induced sustained contractions of trachea and lung parenchymal strips within 1 min of challenge. It was more potent ($P < 0.001$) on the lung strip ($EC_{50} = 63$ μM) than on the trachea ($EC_{50} = 123$ μM). The lung strip also developed greater maximum isometric tension ($P < 0.001$) than the trachea. The efficacy on the lung strip was 2 and the trachea 0.6, relative to the response to acetylcholine (efficacy = 1).

2 Vanadate-induced contractions of the trachea were not inhibited by atropine, mepyramine, phentolamine or indomethacin, nor after mast cell depletion by compound 48/80, showing that contractions were not mediated via specific receptors or by release of endogenous mediators of tone.

3 Inorganic phosphate specifically inhibited vanadate responses in a dose-dependent and reversible manner, suggesting a common site of action.

4 Contractions could be elicited in depolarized muscle and after treatment with ouabain plus propranolol, showing that membrane depolarization and inhibition of the Na, K-ATPase system were not involved in the contractile action of vanadate.

5 Pretreatment of tracheal smooth muscle with verapamil had no influence on contractions elicited by vanadate. After removal of extracellular calcium, vanadate-induced contractions declined slowly with time, indicating that influx of extracellular calcium was not giving rise to contractions elicited by vanadate.

6 Vanadate markedly increased the rate of calcium efflux from trachealis muscle loaded with ^{45}Ca into both Ca^{2+}-free and normal Krebs solutions; this is compatible with vanadate mobilizing an intracellular store of Ca^{2+}. Such a store involving sites with Ca-ATPase activity would be consistent with the action of vanadate in isolated membrane preparations.

7 Membrane-skinned tracheal fibres contracted by micromolar Ca^{2+} were relaxed by vanadate in a reversible dose-related manner, indicating that the contractile action of vanadate was not related to its interaction with proteins at the cross-bridge level.

Introduction

Vanadate, the commonly occurring pentavalent form of the element vanadium, causes contraction of visceral and vascular smooth muscle (Jackson, 1912; Ozaki & Urakawa, 1980; Garcia, Jurkiewicz & Jurkiewicz, 1981). In man, exposure to airborne vanadate compounds during refining produces bronchial asthma and bronchitis (U.S. Department of Health, Education and Welfare Report, 1977; Musk & Tees, 1982). The mechanisms responsible for vanadate-induced contraction of smooth muscle and bronchoconstriction are unknown. Clinical studies have suggested that the pulmonary effects may involve a direct action on the airways (Musk & Tees, 1982) or be mediated via a sensitivity reaction (Zenz, Bartlett & Thiede, 1962).

In isolated tissues vanadate contracts rabbit intestinal smooth muscle (Hudgins & Bond, 1981), guinea-pig aorta (Ozaki & Urakawa, 1980), canine saphenous vein (Huot, Muldoon, Pamnani, Clough & Haddy, 1979) and rat vas deferens (Garcia *et al.*, 1981) at concentrations from 0.01–10 mM. It also inhibits several phosphate hydrolysing enzymes *in vitro*, including Na,K-ATPase (Cantley, Josephson, Warner, Yanagisawa, Lechene & Guidotti, 1977; Cantley, Cantley & Josephson, 1978a; Cantley, Resh & Guidotti, 1978b) and Ca-ATPase (O'Neal, Rhoads & Racker, 1979; Vincenzi & Ashleman, 1980; Dupont & Bennett, 1982). The presence of vanadium in trace concentrations of 0.1–1 μM in animal tissues (Underwood, 1977) has led to the

suggestion of a regulatory role for vanadium in some of these enzyme systems.

In the present paper the bronchoconstrictor action of vanadate on airways smooth muscle has been investigated in isolated preparations of central and peripheral airways of guinea-pig (Lulich, Mitchell & Sparrow, 1976). The characteristics of vanadate-induced contraction of airways smooth muscle was first determined, then elucidation of the underlying mechanism responsible for contraction was sought. This involved examining a number of possibilities, the interaction of vanadate with specific receptors on the smooth muscle cell membrane, release of endogenous mediators of tone, interaction with Ca^{2+} involved in excitation-contraction coupling, competition with phosphate at a cellular locus, and finally, an intracellular action at the level of the contractile proteins.

Methods

Organ bath experiments

Guinea-pigs of either sex and weighing between 250–400 g were stunned and exsanguinated. The trachea and lungs were excised and placed in a modified Krebs solution at 20–25°C. Its composition was (mM); NaCl 119.8, KCl 4.7, $NaHCO_3$ 14.9, $CaCl_2$ 2, dextrose 11, KH_2PO_4 1.18 and K_2H_2EGTA (ethyleneglycol-bis-(β-aminoethylene ether)-N,N'-tetraacetic acid) 0.05. The solution was buffered to pH 7.3 with NaMOPS 5 mM (morpholinopropane sulphonic acid titrated with NaOH to pH 7.3). Muscle was depolarized in a 'K^+-depolarizing Krebs' in which the NaCl in the above solution was replaced with K_2SO_4(80 mM). In Ca^{2+}-free Krebs the $CaCl_2$ was omitted. All solutions were continuously gassed with 95% O_2 and 5% CO_2 and maintained at 37°C.

Single rings were dissected from the trachea, opened opposite the trachealis muscle and the excess cartilage trimmed away. Threads were attached to each end and the preparation mounted in a 20 ml organ bath. A load of 0.4–0.6 g was initially applied, isometric tension being measured with a Grass FTO3 or Hewlett Packard FTA-1-1 force transducer. Lung parenchymal strips (Lulich *et al.*, 1976) with approximate dimensions of 10 × 2 × 2 mm were similarly mounted and an initial load of 0.5–1.0 g tension applied to them by gentle stretching. Preparations were allowed to equilibrate for at least 30 min, the bathing solution being changed at 10 min intervals.

Concentration-response relationships were obtained by means of a cumulative dose regime with the mean concentration-response curves obtained by determining the concentration of vanadate that elicited a given percentage of the maximum response. EC_{50} values were calculated as the mean concentration producing 50% of the maximum response.

Measurement of ^{45}Ca efflux

The trachea was removed, opened lengthwise along its ventral aspect opposite the trachealis muscle and the cartilage removed, leaving a thin length of muscle about 25 × 1 × 1 mm in size. This was divided into two equal lengths to provide paired smooth muscle strips for each experiment. Each strip was lightly blotted, weighed (69.5 ± 5.9 mg, mean ± s.e.mean, $n = 8$) and suspended by a fine stainless steel hook in Krebs solution containing 10 μCi $^{45}CaCl_2$ ml^{-1} (Amersham, sp. act. 700 μCi μmol^{-1} $CaCl_2$). After equilibrating for 2 h, strips were transferred via the steel hooks to test tubes containing non-radioactive Krebs for short intervals.

Efflux experiments were conducted over 2 h. The rate of ^{45}Ca efflux, which was very rapid at first, had slowed down greatly by 40 min and followed closely the same time-course whether normal Krebs (Ca^{2+} 2 mM) or Ca^{2+}-free Krebs was used as the medium for efflux. Vanadate (1 mM) was included in tubes into which ^{45}Ca was collected 60 min after starting efflux and maintained for 10 min. The second strip from a pair was exposed to 1 mM acetylcholine (ACh) in a similar manner. Test tubes were maintained at 37°C and aerated via thin polyethylene tubes.

Radioactivity was determined by adding 2 ml aliquots of efflux solution to 15 ml of Triton based scintillant (1:2 (v/v) Triton X-100/0.9% (w/v) 2,5-diphenyloxazole in toluene). The radioactivity remaining in the tissues after 2 h of efflux was determined by dissolving them in 1 ml of Protosol (New England Nuclear) and adding 10 ml of 2,5-diphenyloxazole/toluene scintillant (0.9% (w/v) 2,5-diphenyloxazole in toluene). Radioactivity in the samples was counted in a Searle Isocap/300 Liquid Scintillation System with sufficient counts recorded to produce a standard deviation of counting of 2% or less. The counting efficiency was 75–80%.

The rate coefficient of efflux (k, with units min^{-1}) was calculated using:

$$k = \frac{[(^{45}Ca)_{t_1} - (^{45}Ca)_{t_2}]}{[(^{45}Ca)_{t_1} \times (t_2 - t_1)]}$$

where $(^{45}Ca)_{t_1}$ represents the ^{45}Ca content of the muscle (dpm) at time t_1 min.

Membrane-skinned smooth muscle

The isolated trachea was divided into sections comprising 2–3 tracheal rings and placed in a solution containing 20 mM imidazole, 5 mM K_2H_2EGTA

(pH 7.0), 50 mM KCl and 150 mM sucrose at 4°C for 15 min then transferred to the skinning solution for 4 h at 4°C. This comprised 1% Triton X-100 and 0.5 mM dithioerythritol (DTE) added to the above solution. After a 15 min rinse in the solution without Triton X-100 and DTE, preparations were stored for up to 10 days at −20°C in a relaxing solution (pH 7.0), containing 20 mM imidazole, 4 mM K_2H_2EGTA, 10 mM $MgCl_2$, 7.5 mM ATP and 0.5 mM DTE, diluted 50% v/v with glycerol. Single rings were cut and opened opposite the trachealis muscle. Strips were cut along the length of an opened ring using a scalpel blade to produce 2–3 thinner strips, each with a piece of cartilage at one end and the trachealis muscle in the centre (cross-sectional area approximately 0.03–0.06 mm^2).

A single strip was then mounted using a cellulose glue between an adjustable glass rod and a carbon rod attached to an isometric force transducer (Aksjeselskapet Mikro-Elektronikk AE 801, Norway), bathed in a 'relaxing solution' and an initial load of 5–10 mg applied. The 'relaxing solution' contained 20 mM imidazole, 4 mM K_2H_2EGTA, 10 mM $MgCl_2$ and 7.5 mM ATP adjusted to pH 6.7 with KOH and had an ionic strength of 0.08 M. Contraction was induced by increasing the free Ca^{2+} concentration ($[Ca^{2+}]$) to 21 μM by replacing EGTA with a Ca^{2+}-EGTA solution. Calmodulin (1 μM) and inorganic phosphate (6 mM) were present in these solutions to facilitate contraction and relaxation, respectively (Sparrow, Mrwa, Hofmann & Rüegg, 1981; Schneider, Sparrow & Rüegg, 1981). Solutions were contained in a 2 ml plastic bath at room temperature (20–22°C) and could be changed in less than 5 s.

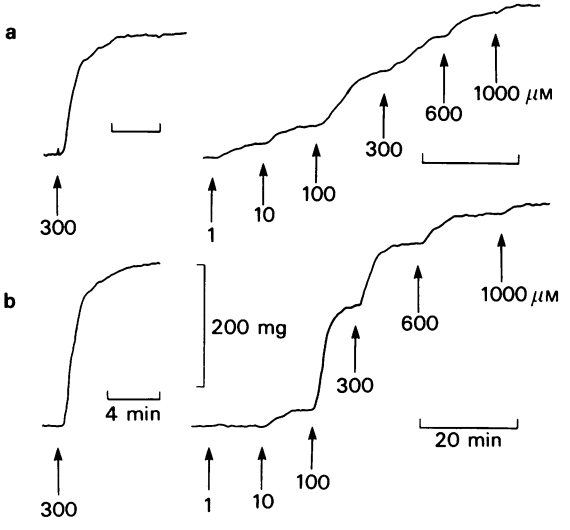

Figure 1 Isometric tension responses of (a) isolated tracheal smooth muscle and (b) lung parenchymal strips of the guinea-pig elicited by vanadate. On the left is the response to a single concentration of vanadate (300 μM); on the right is that to cumulative doses of vanadate (1–1000 μM).

Drugs

The following drugs were used: sodium metavanadate (Hopkins and Williams), acetylcholine chloride (Sigma), adrenaline tartrate (Parke-Davis), atropine sulphate (Sigma), mepyramine maleate (May and Baker), histamine acid phosphate (BDH), phentolamine (Ciba), compound 48/80 (Sigma), indomethacin (Sigma), ouabain octahydrate (Sigma), propranolol hydrochloride (Sigma), verapamil hydrochloride (Knoll Pharm.) and SITS (4 acetamido-4′-isothiocyano-2-2′-disulphonic acid stilbene, Calbiochem). Inorganic phosphate was NaH_2PO_4 adjusted to pH 7.0 with NaOH. All chemicals used were analytical grade. Indomethacin was dissolved in a 1:1 water/ethanol mixture and at the dilutions used the water/ethanol mixture had no effect on vanadate responses or intrinsic resting tone in the trachea.

Statistics

Results are expressed as the mean ± s.e.mean with the number of preparations (n) in parentheses. Statistical evaluation was performed using Student's t test with $P < 0.05$ regarded as significant.

Results

Contraction of isolated tracheal and lung strip preparations by vanadate

Isolated tracheal and lung strip preparations contracted in response to vanadate (1–1000 μM). Figure 1 shows examples of isometric tension responses of these tissues to single and cumulative concentrations of vanadate. In the trachea the onset of tension development in response to vanadate (300 μM) occurred within 10–30 s and maximum tension was achieved after 8–10 min. This tension could be maintained for up to 60 min, the longest time-span investigated, and was reversible within 1–2 min following washout. Reproducible contractions could be elicited at 20 min intervals. Lung strips responded to vanadate (300 μM) within 10–30 s of challenge with maximum tension developed after 5–8 min, slightly quicker than the trachea. As with the trachea, lung strip responses to vanadate were reversible and reproducible. Cumulative concentration-response curves of trachea and lung strip to vanadate are

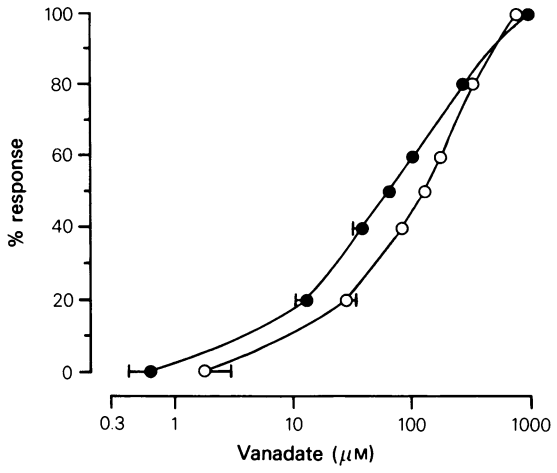

Figure 2 Concentration-response curves for vanadate on the isolated trachea (○) and lung strip (●). Responses are expressed as a percentage of the maximum isometric tension developed by each preparation to vanadate and each point is the mean of six preparations. The standard error bars are shown only when they exceed the dimension of the symbols used.

shown in Figure 2. The EC_{50} value for vanadate on the trachea ($123 \pm 11\,\mu M$, $n=6$) was greater ($P<0.001$) than on lung strip ($63 \pm 6\,\mu M$, $n=6$).

Tracheal and lung strip preparations were challenged with ACh (100 μM) which produces 85–90% of the maximum isometric tension induced by 10 mM ACh in both tissues (Lulich, 1980). This ACh (100 μM) concentration was used as a standard for comparing the effect of vanadate on the two tissues. The maximum response elicited by vanadate (1 mM) relative to that for ACh (100 μM) was $72.2 \pm 8.1\%$ ($n=6$) for the trachea and $243.2 \pm 27.5\%$ ($n=6$) for lung strip. This difference between the maximum tension response of the trachea and lung strip was significant ($P<0.001$).

Trachea contracted on exposure to K^+-depolarizing solution with peak tension being attained within 30 s, followed by relaxation to levels close to the initial resting tension. After 20 min in K^+-depolarizing solution, vanadate (300 μM) contractions were elicited. These showed a similar time course to control responses in normal Krebs solution but developed less tension ($46.7 \pm 4.4\%$ of control, $n=3$). The response to vanadate (300 μM) recovered to control values ($94.7 \pm 16.3\%$, $n=3$) after tracheae were returned to normal Krebs.

Role of receptors and endogenous mediators of tone in vanadate-induced contractions of the trachea

The trachea, its smooth muscle being almost exclusively airway, was chosen to investigate the involvement of membrane receptors in the action of vanadate. Vanadate did not appear to act via direct or indirect stimulaton of α-adrenoceptors, muscarinic or histamine H_1-receptors that may be located on the tracheal smooth muscle cell membrane. In 2 tracheal preparations, atropine (1 μM) given 3 min before challenge diminished the response to ACh (100 μM) to 7.7% and 14.0% of that before atropine treatment. Using this regime of atropine treatment the effect of vanadate (300 μM) was unchanged ($97.2 \pm 6.2\%$ of control, $n=4$). Mepyramine (1 μM, 3 min before challenge) inhibited the response of 2 tracheal preparations to histamine (100 μM) by 91.9% and 100% but had no effect on the response to vanadate (300 μM) in 4 preparations. Contractions to vanadate (300 μM) after phentolamine (1 μM, 5 min before vanadate) were $106.8 \pm 10.2\%$ ($n=6$) of those before phentolamine treatment.

The release of mediators by mast cells was not involved in the action of vanadate. Initial exposure to

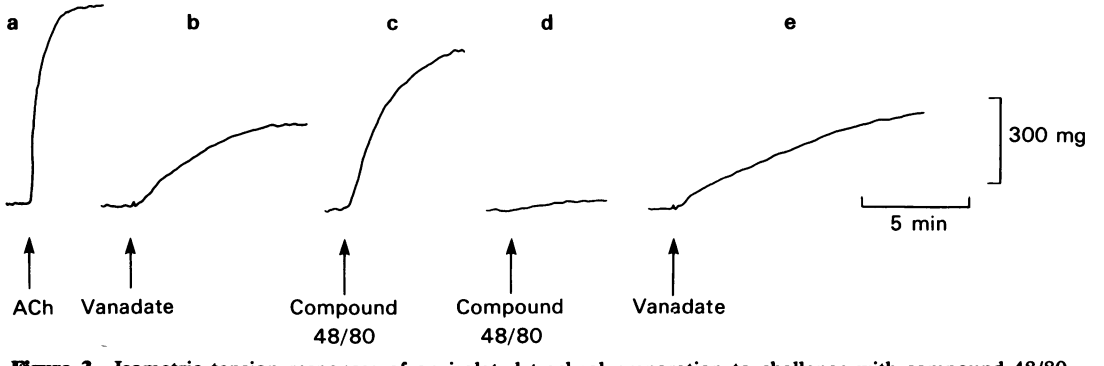

Figure 3 Isometric tension responses of an isolated tracheal preparation to challenge with compound 48/80 followed by challenge with vanadate. Contractions in response to (a) acetylcholine (ACh, 100 μM), (b) vanadate (300 μM), (c) compound 48/80 (100 $\mu g\,ml^{-1}$), (d) subsequent challenge with compound 48/80, (e) challenge with vanadate (300 μM).

compound 48/80 (100 μg/ml) produced a strong contraction (Figure 3) of the trachea (84.8 ± 11.2% of the response to 100 μM ACh, $n = 4$), but the response to a subsequent challenge was markedly reduced ($P < 0.001$) to 5.3 ± 2.6% ($n = 4$). The response to vanadate (300 μM) was no different (109.7 ± 15.8% of control, $n = 4$) after two exposures to compound 48/80.

To see whether vanadate contractions were mediated via prostaglandin production, tracheal preparations were incubated with indomethacin (30 μM) for 25 min before vanadate challenge. Indomethacin (30 μM) produced about a 50% decrease in resting tension within 2–4 min of administration, this new tension remaining stable thereafter. The response to vanadate (300 μM) after 25 min was 135.3 ± 30.4% of control ($n = 6$), which was not significantly different from that before indomethacin, indicating that prostaglandins did not play a part in the action of vanadate.

Effect of ouabain, phosphate and an anion exchange inhibitor on vanadate contractions

Ouabain and vanadate are both potent inhibitors of Na,K-ATPase activity in isolated membrane preparations and inhibition of this enzyme in vascular smooth muscle may lead to contraction (Broekaert & Godfraind, 1973; Karaki, Ozaki & Urakawa, 1978). The response of tracheal smooth muscle to ouabain was first examined and vanadate subsequently added. Propranolol (1 μM, 5 min before ouabain) was included to block the inhibitory effect of catecholamines released by ouabain (Karaki & Urakawa, 1977). Ouabain (100 μM) contracted the trachea initially and peak tension (21.9 ± 2.5% of that to 100 μM ACh, $n = 3$) was reached within 5 min. This was followed by relaxation. Tension stabilized after 25 min at levels below that prior to ouabain exposure. Challenge at this time with vanadate (300 μM) produced a contraction of the trachea of similar magnitude to the initial vanadate response (112.0 ± 7.4% of control, $n = 3$).

An interaction between phosphate and vanadate on tension development in the trachea was examined because phosphate and vanadate are analogues of transition state elements (Lindquist, Lynn & Lienhard, 1973) and compete with one another in a number of cellular systems, such as the red blood cell anion exchange system (Cantley *et al.*, 1978b). Tracheal preparations were exposed to increasing concentrations of inorganic phosphate 2 min before administration of vanadate (500 μM). Phosphate up to 40 mM caused a dose-dependent inhibition of the vanadate response (Figure 4). Although maximum tension was decreased, the time to attain this tension was the same as in control responses. After removal

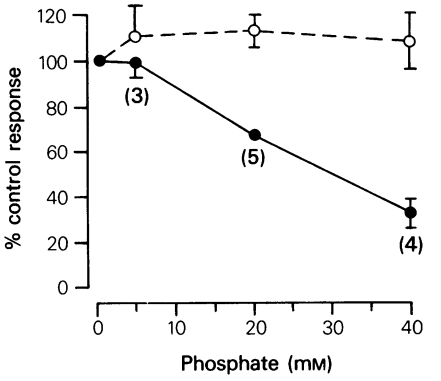

Figure 4 The inhibitory effect of inorganic phosphate on the maximum tension of vanadate-induced contractions of tracheal smooth muscle (solid line). Control responses to vanadate (500 μM) were obtained and preparations then incubated with either 5, 20 or 40 mM phosphate for 2 min prior to vanadate (500 μM) challenge. Complete recovery of tension to vanadate challenge occurred 30 min after phosphate removal. ACh (100 μM) contractions were not influenced by phosphate (broken line). Each point represents the mean ± s.e.mean and the number of preparations are shown.

of phosphate (40 mM), full recovery of responses to vanadate occurred (97.1 ± 11.8% of control, $n = 4$). No action of phosphate itself was seen except at 40 mM phosphate when preparations relaxed by about 20% of their resting tension, this decrease in tension stabilizing within 2 min and remaining unaltered for at least 10–15 min. Phosphate up to 40 mM had no effect on responses to 100 μM ACh (see Figure 4).

In red blood cells the initial uptake of vanadate via the anion exchange system is specifically inhibited by 4 acetamido-4'-isothiocyano-2,2'-disulphonic acid stilbene (SITS) (Cantley *et al.*, 1978b). SITS (2.5 mM) caused an initial marked relaxation of the trachea with tension gradually recovering towards the original resting tension after approximately 20 min. Contractions to vanadate (500 μM) at this time were the same as controls ($n = 3$).

Interaction of calcium and vanadate in tracheal smooth muscle

In an attempt to see whether vanadate was affecting Ca^{2+} influx involved in excitation-contraction coupling, the effect of verapamil, an antagonist of calcium entry through the membrane, was investigated on responses to vanadate and ACh. Vanadate-induced contractions over the concentration range 10–1000 μM were unaffected by verapamil (100 μM) given 5 min before challenge.

In contrast, contractions elicited with ACh

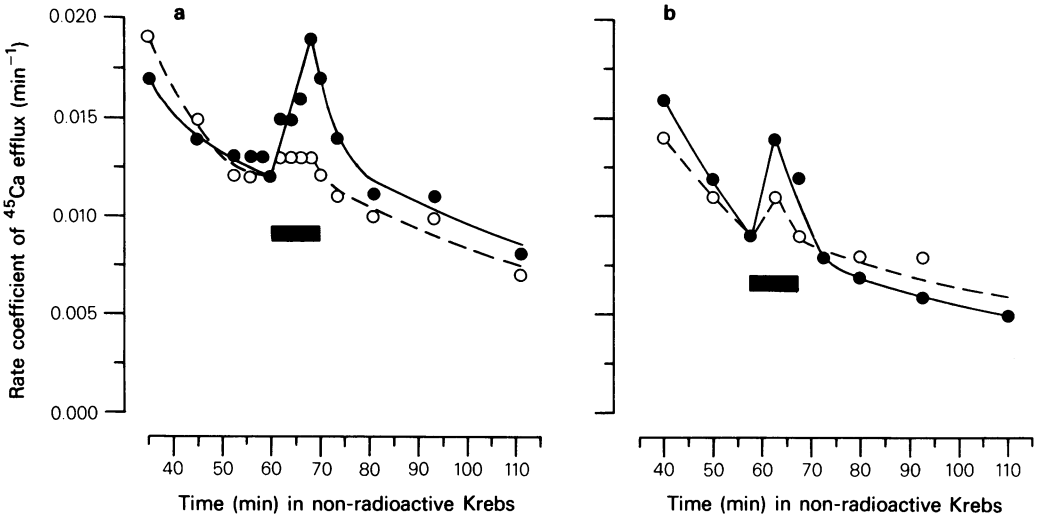

Figure 5 The effect of vanadate and acetylcholine (ACh) on the rate of ^{45}Ca efflux from paired strips of trachealis muscle. Muscle strips were incubated in ^{45}Ca containing Krebs (2 mM Ca^{2+}) for 2 h then transferred to non-radioactive solutions containing either (a) Ca^{2+}-free Krebs or (b) normal Krebs (2 mM Ca^{2+}) and challenged with 1 mM vanadate (●) or 1 mM ACh (○) after approximately 60 min (solid bar indicates period of challenge).

(0.1–10 μM) were inhibited ($P < 0.02$) by verapamil treatment, in agreement with previous observations on dog trachea (Farley & Miles, 1978).

The need for extracellular Ca^{2+} was next investigated. Contractions to vanadate (300 μM) elicited 10 min after the removal of extracellular Ca^{2+} were the same as control responses obtained in normal Krebs containing 2 mM Ca^{2+}. After 30 min in Ca^{2+}-free Krebs the response to vanadate (300 μM) declined to 10.2 ± 4.3% of control ($n = 3$) and by 50 min was 8.1 ± 5.6% ($n = 3$). This demonstrated that Ca^{2+} was necessary for the maintenance of vanadate-induced contractions of airways smooth muscle, so an attempt was made to see whether vanadate affected the flux of Ca^{2+} in the smooth muscle cell.

The influence of vanadate (1 mM) and ACh (1 mM) on the rate of ^{45}Ca efflux was tested on paired strips of tracheal smooth muscle 60 min after measurement of efflux began (see Methods). Figure 5 shows two typical efflux experiments from a total of four where the medium for efflux was Ca^{2+}-free Krebs (Figure 5a) or normal Krebs containing 2 mM Ca^{2+} (Figure 5b). Vanadate (1 mM) increased the rate of ^{45}Ca efflux on average by 60% (range 40–85%, $n = 4$) above that prior to challenge. There was no significant difference between the increase in the rate of efflux from muscle strips bathed in normal or Ca^{2+}-free Krebs. An increase in efflux rate above that before challenge was maintained for not less than 8 of the 10 min exposure to vanadate. ACh had much less effect on the rate of ^{45}Ca efflux than vanadate. In 4 strips the average increase in ^{45}Ca efflux rate was 9% (range 0–22%) over that before ACh challenge. In all cases this increase was less than that elicited by vanadate (1 mM) in the corresponding paired muscle strip.

Effect of vanadate on membrane-skinned tracheal fibres

One possible interpretation of the ^{45}Ca efflux experiments was that vanadate was acting intracellularly to mobilize bound or stored Ca^{2+}. To see whether an intracellular action of vanadate was compatible with the observations made hitherto, its effect on bundles of tracheal smooth muscle in which membranes had been removed with the detergent Triton X-100 was investigated. In these membrane-skinned tracheal fibres, reproducible contractions could be elicited by Ca^{2+} (21 μM), the developed tension being maintained while Ca^{2+} was present. Removal of Ca^{2+} (i.e. $Ca^{2+} < 10$ nM) completely relaxed the fibre bundles. Figure 6a shows the effect of increasing concentrations of vanadate on a fully developed Ca^{2+}-activated contraction. Progressive increases in the concentration of vanadate inhibited tension development with an EC_{50} averaging 95 μM ($n = 3$). Removal of vanadate (cumulative 500 μM) lead to the re-development of tension to levels approaching those obtained before vanadate administration (average recovery to 85.0 ± 6.3%, $n = 3$). The relationship between inhibition of tension and vanadate concentration is shown in Figure 6b. Incubation of skinned tracheal fibres

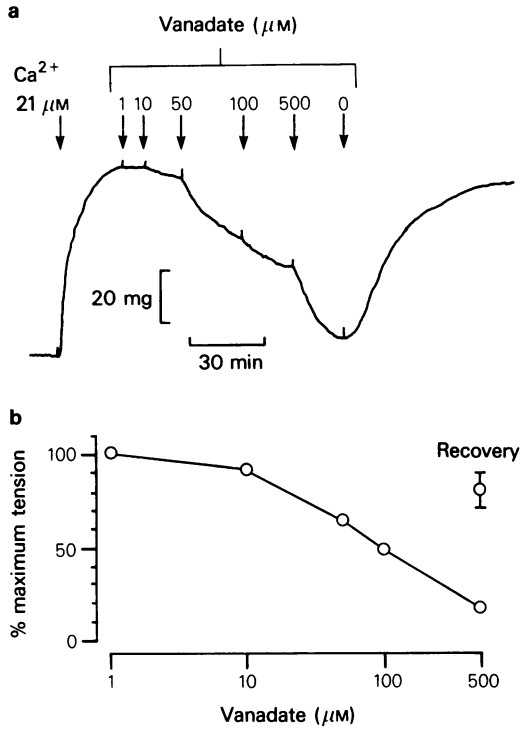

Figure 6 (a) A typical tension tracing of a membrane-skinned tracheal fibre bundle in MgATP salt solution showing the relaxant effect of cumulative concentrations of vanadate on a fully developed Ca^{2+}-activated contraction (21 μM Ca^{2+}). After removal of vanadate the tension redeveloped. (b) Relationship between the concentration of vanadate and the relaxation of tension developed to a maximal concentration of Ca^{2+} (21 μM). Inhibition of tension was dose-related, 95 μM vanadate producing 50% inhibition, and reversible on vanadate removal. Each point is the mean of the isometric tension produced by 3 tracheal fibre bundles.

with vanadate (300 μM) 6 min before the addition of Ca^{2+} (21 μM) also resulted in the partial inhibition of tension development in response to Ca^{2+}, tension development recovering upon the removal of vanadate.

Discussion

Vanadate contracted both trachea and lung parenchymal strips, tension being sustained throughout exposure showing that vanadate can exert prolonged effects on the tone of airways smooth muscle. Using the respective EC_{50} values as a measure of potency, vanadate was about twice as potent on lung strips as on the trachea. Lung strips also developed greater relative maximum tension. In comparison with ACh, which is assumed to have an efficacy of 1, the efficacy of vanadate on trachea and lung strips is approximately 0.6 and 2, respectively. Although the lung strip is assumed to represent peripheral airway smooth muscle, its contractile response may consist of a component arising from its vascular tissue content. A minor involvement of this smooth muscle cannot be excluded, nor can tension development by the contractile interstitial myofibroblasts (Kapanci, Assionacoupoulas, Irle, Zwaahlen & Gibbiany, 1974). The greater apparent efficacy of vanadate on the lung strip may therefore reflect the vascular component in the lung strip which ACh would be predicted to relax and vanadate contract. On the other hand it may indicate a true difference in the characteristics of smooth muscle from central and peripheral airways. *In vivo*, this would mean that, if vanadate was equally distributed throughout the airways, it would be expected to affect lung compliance to a greater extent than airways resistance. The greater potency of vanadate on lung strips could also be explained by their vascular component if the potency of vanadate on pulmonary vascular tissue was greater than on airways smooth muscle. However, we have no evidence for this. The EC_{50} for vanadate on guinea-pig aorta is approximately 800 μM (Ozaki & Urakawa, 1980) compared with 63 μM for the lung strip. The potency for the trachea (EC_{50} = 123 μM) was about twice that reported for rat vas deferens (Garcia *et al.*, 1981).

Threshold responses to vanadate were observed at micromolar concentrations in both the trachea and lung strip, with contraction occurring within 1 min. These *in vitro* actions would suggest that vanadate can contract airways smooth muscle *in vivo* at low concentrations and with rapid onset following exposure. *In vivo* such effects would depend on the extent to which exogenous vanadate dissolved in the layer of fluid lining the airways. The distribution of particulate vanadate in the lungs is unknown but it seems feasible that micromolar concentrations may arise in localized regions of the airways.

Stimulation of muscarinic, histamine H_1-receptors and possibly α-adrenoceptors located on the airways smooth muscle cell membrane leads to the development of tension. The lack of effect of receptor blockade on vanadate-induced contractions of the trachea *in vitro* showed that it did not act either directly or indirectly via these receptors. Pretreatment of the tissues with compound 48/80 showed that the action of vanadate was not mediated via mast cell degranulation. The relaxation of tracheal smooth muscle after indomethacin, an inhibitor of prostaglandin biosynthesis, implied that the resting tone in this tissue was related to prostaglandin production, in agreement with Farmer, Farrar & Wilson (1974).

Inhibition of prostaglandin synthesis by indomethacin had no effect on vanadate responses indicating that they were not due to prostaglandin production.

Ouabain contracts some vascular smooth muscle (Broekaert & Godfraind, 1973; Karaki *et al.*, 1978) but did not produce a sustained contraction of the trachea, even after treatment with propranolol which was included to block the possible inhibitory influence of released catecholamines (Karaki & Urakawa, 1977). This suggests that inhibition of the Na,K-ATPase system by ouabain does not have a significant influence on the development of tone in tracheal smooth muscle, consistent with the weak nature of the Na-K pump in tracheal smooth muscle (see Kirkpatrick, 1981). The ability of vanadate to contract the trachea normally after ouabain plus propranolol treatment indicates that it is not acting via the Na,K-ATPase system. Vanadate contractions of the trachea in K^+-depolarizing solution also argue against the involvement of the Na,K-ATPase system and show that vanadate can contract tracheal smooth muscle independently of membrane depolarization.

Vanadate and phosphate compete in a number of systems, such as the red blood cell anion exchange pathway (Cantley *et al.*, 1978b) and the Ca-ATPase from skeletal sarcoplasmic reticulum (Dupont & Bennett, 1982). Phosphate inhibited contractions elicited by vanadate in a reversible manner but did not affect responses to ACh, suggesting a common site of action of these analogues on or within the smooth muscle cell. The initial uptake of vanadate in the red blood cell can be inhibited by SITS, a specific inhibitor of the anion exchange system (Cantley *et al.*, 1978b). The chemical similarity of vanadate and phosphate might suggest that vanadate enters the cytoplasm of other tissues by this system (Macara, Kustin & Cantley, 1980). Inhibition of vanadate uptake into tracheal smooth muscle cells by phosphate could then account for its specific inhibitory effect on vanadate-induced contractions if the site of action of vanadate was intracellular. SITS had no effect on vanadate-induced contractions in the trachea but it is not known whether it is effective on anion transport pathways in smooth muscle.

Vanadate inhibits the Ca-ATPase from skeletal and cardiac sarcoplasmic reticulum as well as from red blood cell plasma membranes (O'Neal *et al.*, 1979; Vincenzi & Ashleman, 1980; Dupont & Bennett, 1982) and phosphate competitively antagonizes this effect of vanadate (Dupont & Bennett, 1982). Inhibition of Ca^{2+} transport in the sarcoplasmic reticulum, mitochondria and plasma membrane of tracheal smooth muscle cells would lead to an increase in the concentration of Ca^{2+} in the cytoplasm ($[Ca^{2+}]_{cyt}$), resulting in tension development. The contribution by the sarcoplasmic reticulum would be expected to be greater than mitochondrial and plasma membranes because it is a more dynamic system (Vallieres, Scarpa & Somlyo, 1975). The competition between phosphate and vanadate on Ca-ATPase activity could then explain the inhibition of contraction to vanadate after exposure to phosphate. Thus, from the evidence available the most likely sites in tracheal smooth muscle for interaction of vanadate and phosphate are the anion transport and Ca-ATPase systems. Both systems may be involved in the action of phosphate although the relative importance of each is unclear. It is unlikely that phosphate inhibits the contractile response to vanadate through precipitation of Ca^{2+}, a property used in locating intracellular deposits of Ca^{2+} in smooth muscle, because the contractile response to 100 μM ACh, which relies to a large extent on intracellular stores of Ca^{2+} (Farley & Miles, 1978; see Kirkpatrick, 1981), was not affected after 40 mM phosphate.

Vanadate required Ca^{2+} in the bathing solution if contractions were to be maintained. In Ca^{2+}-free Krebs, contractions diminished slowly over 50 min indicating that the source of Ca^{2+} was not extracellular, but more likely from a bound intracellular store. In support of this was the ability of vanadate to elicit contractions initially after the removal of extracellular Ca^{2+} and the lack of effect of verapamil, which is reported to block the influx of extracellular Ca^{2+} through potential-dependent channels (Mironneau, 1973), on vanadate-induced contractions.

Stimulation of ^{45}Ca efflux by vanadate provided further evidence that vanadate mobilizes Ca^{2+} from an intracellular store. An increased rate of ^{45}Ca efflux, of a similar magnitude to that found for tracheal smooth muscle, has been reported from skeletal muscle during vanadate challenge (Clausen, Andersen, Stürup-Johansen & Petkova, 1981). The uptake of ^{45}Ca in visceral smooth muscle during the loading procedure is initially very rapid and by 2 h is virtually complete (Bauer, Goodford & Hüter, 1965; Potter, Sparrow & Simmonds, 1970). Efflux of ^{45}Ca from the muscle was rapid for the first 15 min in non-radioactive Krebs, probably corresponding to the exchange of ^{45}Ca from the extracellular space and superficial binding sites. The later slow phase of ^{45}Ca efflux is likely to represent efflux from slowly exchanging sites, generally assumed to be intracellular. The increased rate of ^{45}Ca efflux by vanadate and ACh after 60 min may reflect the mobilization of ^{45}Ca from these slowly exchanging intracellular stores. Stimulation of ^{45}Ca efflux by vanadate in the Ca^{2+}-free Krebs supports this view because the EGTA (0.05 mM) in this solution should chelate extracellular Ca^{2+} and Ca^{2+} bound to superficial binding sites on membranes.

Increased ^{45}Ca efflux has been noted from aortic smooth muscle after treatment with dinitrophenol, a metabolic inhibitor which inhibits active Ca^{2+} trans-

port (van Breemen, Wuytack & Casteels, 1975). Loss of Ca^{2+} from mitochondria and sarcoplasmic reticulum was postulated to raise the $[Ca^{2+}]_{cyt}$, leading to a nett increase in ^{45}Ca efflux. Inhibition of Ca-ATPase activity by vanadate would serve the same function as metabolic inhibition and be expected to lead to an increase in ^{45}Ca efflux. It is difficult to assign intracellular locations to the slowly exchanging ^{45}Ca stores seen in these experiments, although the mitochondrial pool particularly, and the sarcoplasmic reticulum to a lesser extent, appear as likely sites. ACh stimulation is unlikely to affect the mitochondrial storage sites and the small increase in ^{45}Ca efflux may reflect ^{45}Ca release from the sarcoplasmic reticulum.

Vanadate appears to enter the cytoplasm of most tissues (Macara *et al.*, 1980) and the evidence presented here suggests an intracellular site of action. However, when vanadate was allowed to enter skinned fibres freely, these comprising a structurally intact contractile apparatus, it relaxed fibres previously contracted by maximal Ca^{2+} in a dose-related manner and when pre-incubated it inhibited tension development to Ca^{2+}. This shows that interaction with the contractile proteins is not the mechanism through which vanadate elicits contraction in the trachea. The relaxant effect of vanadate in skinned vascular and cardiac muscle preparations has been reported previously (Peterson, 1980; Solaro, Holroyde, Herzig & Peterson, 1980) and attributed to a decreased binding of phosphorylated myosin to actin by vanadate (Peterson, 1980).

In conclusion, our evidence suggests that the bronchoconstrictor effects of vanadate exposure *in vivo* can be attributed, at least in part, to a direct action on airways smooth muscle. Vanadate appears to enter the cytoplasm of airways smooth muscle cells, probably via anion exchange pathways, to inhibit the Ca-ATPase system, thereby increasing the $[Ca^{2+}]_{cyt}$ and causing contraction. A further test of this hypothesis would be to demonstrate that vanadate inhibits membrane Ca-ATPases from airways smooth muscle. The possibility cannot be excluded that if vanadate does penetrate intracellularly then contractions resulting from vanadate challenge could involve a balance between excitatory (eg. Ca^{2+} mobilization) and inhibitory (at the level of the contractile apparatus) influences on the pathways leading to contraction. The distribution of vanadate within the airways smooth muscle cell, determined by the extent to which vanadate is bound to various intracellular proteins, would be a strong determinant in these actions.

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