Br. J. Pharmac. (1986), **87**, 117–127

The relaxant action of nicorandil in guinea-pig isolated trachealis

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1 Nicorandil (1–1000 μmol l^{-1}) caused concentration-dependent relaxation of guinea-pig isolated trachealis.

2 Propranolol (1 μmol l^{-1}) did not modify the relaxant action of nicorandil but antagonized isoprenaline.

3 Among K^+-channel inhibitors tested, apamin (0.1 μmol l^{-1}) and procaine (5 mmol l^{-1}) did not modify the relaxant action of nicorandil. In contrast, tetraethylammonium (TEA, 8 mmol l^{-1}) caused five fold antagonism.

4 Trachealis exposed to K^+-rich (120 mmol l^{-1}) Krebs solution developed near-maximal tension. Nicorandil relaxed the K^+-depolarized tissue though its concentration-effect curve was shifted markedly to the right.

5 In tissues in which tone was induced by histamine, methylene blue (100 μmol l^{-1}) antagonized nicorandil and sodium nitroprusside but did not modify the relaxant action of aminophylline.

6 Intracellular electrophysiological recording showed that nicorandil (1 μmol l^{-1}) could evoke some relaxation in the absence of electrical changes. Higher concentrations (10–1000 μmol l^{-1}) reduced the amplitude and frequency of spontaneous electrical slow waves. Nicorandil also caused concentration-dependent hyperpolarization and relaxation. When the hyperpolarization was sufficiently pronounced slow wave activity was abolished.

7 TEA (8 mmol l^{-1}) induced slow waves which were surmounted by a spike potential. TEA slightly reduced the maximal hyperpolarization induced by nicorandil and increased the time required for nicorandil to abolish slow wave discharge.

8 Procaine (5 mmol l^{-1}) induced slow waves of relatively low frequency. Sometimes these were surmounted by a spike potential. Procaine markedly reduced the hyperpolarization induced by nicorandil and increased the time required for abolition of slow waves.

9 In studies of the efflux of $^{86}Rb^+$ from muscle-rich strips of trachea, nicorandil (1000 μmol l^{-1}) increased the efflux rate constant, whereas isoprenaline (1 μmol l^{-1}) was without effect.

10 It is concluded that nicorandil-induced relaxation does not involve the activation of β-adrenoceptors but is partly attributable to the formation of nitric oxide from the nitrate moiety in its molecular structure. Nicorandil can evoke relaxation in the absence of membrane potential change but towards the upper end of its effective concentration range, nicorandil increases membrane K^+ conductance and thereby evokes hyperpolarization of trachealis cells. The K^+ channels opened by nicorandil are permeable to ^{86}Rb, insensitive to apamin and TEA but may be inhibited by procaine.

Introduction

Experiments *in vivo* on dogs showed that nicorandil (2-nicotinamidoethyl nitrate) and several analogues could cause tracheal dilatation. However, nicrorandil was approximately 20 times more potent than derivatives lacking a nitrate group. This suggests that the bronchodilator activity of nicorandil resides in the N-ethylnicotinamide moiety but that activity is enhanced by addition of the nitrate group (Maruyama *et al.*, 1982).

Nicorandil has also been shown to inhibit canine trachealis muscle *in vitro* (Inoue *et al.*, 1983; Nakamura *et al.*, 1984). In concentrations of 50–500 μmol l^-, nicorandil evokes hyperpolarization which is independent of $[Cl^-]_o$ in the range 18–134 mmol l^{-1}

but which is abolished when $[K^+]_o$ is raised to 20 mmol l^{-1} or more. Electrotonic potentials are reduced in amplitude when evoked during nicorandil-induced hyperpolarization. Such evidence suggests that nicorandil acts to increase K^+ conductance in canine trachealis muscle (Inoue *et al.*, 1983).

In spite of this failure to hyperpolarize canine trachealis when $[K^+]_o$ is 20 mmol l^{-1} or greater, nicorandil nevertheless reduces the K^+-induced spasm (Inoue *et al.*, 1983; Nakamura *et al.*, 1984). Furthermore, in double sucrose gap experiments on atropine-treated tissue, nicorandil was able to suppress spasm evoked by depolarizing current pulses at concentrations that did not evoke hyperpolarization. These observations suggest that the tracheal relaxant effects of nicorandil do not stem from hyperpolarization alone but also possibly from altered intracellular disposition of Ca^{2+} (Inoue *et al.*, 1983).

In the present study the relaxant action of nicorandil on airways smooth muscle has been further examined in guinea-pig isolated trachealis. Some comparisons have been made with isoprenaline because this agent, too, evokes hyperpolarization which is not essential for relaxant activity (Allen *et al.*, 1985).

Methods

Guinea-pigs (350–700 g) of either sex were killed by stunning and bleeding. Tracheae were excised, cleaned of adhering fat and connective tissue and opened by cutting longitudinally through the cartilage rings diametrically opposite the trachealis.

Tissue bath studies of mechanical activity of the trachealis

Small segments of trachea were set up for the isometric recording of tension changes as described by Foster *et al.* (1983). At the outset of each experiment tissues were subjected to imposed tension of 1 g. Approximately 20 min later aminophylline (1 mmol l^{-1}) was added in order to determine the recorder pen position at zero tone. The aminophylline was washed from the tissues and when tone subsequently became maximal, study of the relaxant drugs started.

Relaxant drugs were studied by the construction of cumulative concentration-effect curves, concentration increments being made at intervals of 4 (isoprenaline), 5 (aminophylline, sodium nitroprusside) or 6 (nicorandil) min. Following the construction of initial log concentration-effect curves for the relaxant drugs, tissues were allocated randomly in equal numbers to test or time-matched control groups. Test tissues were treated with Krebs solution containing a modifying agent (e.g., apamin, procaine, propranolol or tetraethylammonium (TEA)) or with K^+-rich Krebs solution (see below). Modifying agents or the K^+-rich medium were allowed at least 10 min preincubation with the tissue before the log concentration-effect curves for the relaxant drugs were reconstructed. Time matched control tissues were treated identically but were not exposed to the modifying agent or K^+-rich medium.

Tissue bath studies with methylene blue

Preliminary experiments suggested that the interaction of methylene blue and nicorandil could be obscured by methylene blue itself having relaxant activity. Accordingly these experiments were carried out in Krebs solution containing indomethacin (2.8 μmol l^{-1}) to suppress spontaneous tone. A cumulative concentration-effect curve for histamine (2, 20 and 200 μmol l^{-1} each applied for 6 min) was constructed in each tissue and from this an EC_{50} was calculated. The histamine EC_{50} was subsequently applied to each tissue and the spasm evoked was allowed to become maximal. A cumulative concentration-effect curve for a relaxant agent (aminophylline, nicorandil or sodium nitroprusside) was then constructed in the presence of the histamine EC_{50}. After washout, a cumulative concentration-effect curve for a second relaxant agent was constructed in the same way. Tissues were then allocated to test or control groups.

Test tissues were incubated with methylene blue (100 μmol^{-1}) for 10 min prior to challenge (in the presence of the histamine EC_{50}) with the same relaxant agents. Concurrent control tissues were treated similarly but were not exposed to methylene blue.

Intracellular electrophysiological recording from trachealis

Simultaneous recording of intracellular electrical activity and mechanical changes of a contiguous segment of trachea was performed by use of the technique of Dixon & Small (1983).

The effects of nicorandil on spontaneous electrical and mechanical activity were studied as follows. After impalement of a trachealis cell 3 min were allowed to elapse to check the stability of the record of electrical activity. Nicorandil (1, 10, 100 or 1000 μmol l^{-1}) was then added to the Krebs solution. The effects of nicorandil were monitored for 6 min. At the end of this period the drug was washed from the tissue and recovery of electrical and mechanical activity was monitored until the pre-nicorandil activity was regained or the microelectrode became dislodged from the cell.

Similar procedures were adopted when assessing the electrical responses to nicorandil in tissues pretreated with procaine (5 mmol l^{-1}) or TEA (8 mmol l^{-1}).

Estimation of effects of relaxant agents on $^{86}Rb^+$ efflux

Tracheae were opened as described above and pinned out, mucosal surface uppermost, on a paraffin wax block. That section of tracheal wall containing the trachealis muscle (and minimal cartilage) was dissected from the organ by making two cuts running longitudinally at the tips of the cartilage arches. The strip of tissue thus prepared was divided into three equal segments (laryngeal, central and carinal). Tissue segments were subsequently allocated to control or test experimental groups in such a way that laryngeal, central and carinal tissue was present in equal proportion in each group.

After preincubation at 37°C in 5 ml of Krebs solution bubbled with 95% O_2:5% CO_2, all tissues were loaded with $^{86}Rb^+$ by 150 min incubation with 37 MBq l^{-1} and 6.6 μmol l^{-1} $^{86}RbCl$ in Krebs solution. Each tissue was then transferred to the first of a series of 17 washing samples of 5 ml of Krebs solution at 37.5°C. Tissues remained in each washing sample for 4 min. In the case of test tissues the 7th and 8th washing samples contained either isoprenaline (1 μmol l^{-1}) or nicorandil (1 mmol l^{-1}). These concentrations of isoprenaline and nicorandil were chosen for study because they produced not only maximal relaxation but also maximal hyperpolarization of trachealis cells (Allen *et al.*, 1985; present study, Table 1). In this situation opening of the ion channels responsible for the hyperpolarization should be maximal and therefore detection of radiotracer efflux through them should be optimized.

At the end of the efflux period the tissues were blotted, weighed and solubilised using 0.5 ml Soluene 350 (Packard) in the warm, overnight; 0.5 ml of HCl (0.5 mol l^{-1}) was subsequently added to each tissue digest. One ml aliquots of the loading medium and the washing samples were taken for radioassay along with the tissue digests. Nine ml of Optiphase Safe (LKB) was added and radioassay was by liquid scintillation counting at efficiencies greater than 90%. Back accumulation of the tissue: loading medium ratio (apparent volume of distribution of ^{86}Rb) at 68 min with the losses by efflux allowed calculation of the efflux rate constant for each 4 min of the efflux period for each tissue.

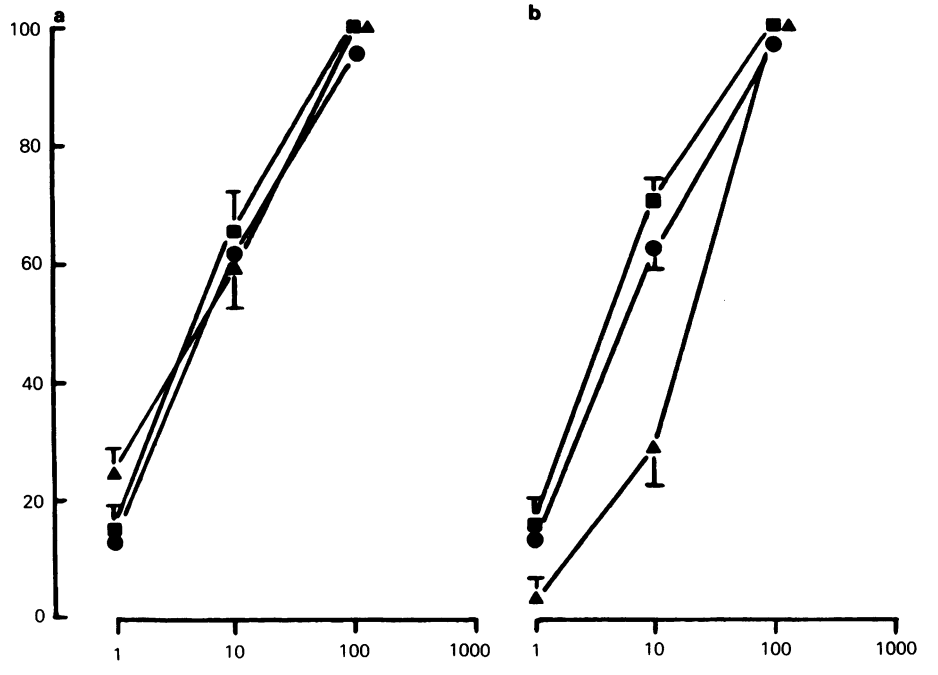

Figure 1 The effects of (a) procaine (5 mmol l^{-1}) and (b) tetraethylammonium (TEA, (8 mmol l^{-1}) on the relaxant action of nicorandil in guinea-pig isolated trachealis. The abscissae indicate the concentration (μmol l^{-1}) of nicorandil on a log scale. The ordinates represent relaxation as a % of the initial response to nicorandil 100 μmol l^{-1}: (●) = pooled initial log concentration-effect curve; (■) = log concentration-effect curve obtained after 10 min incubation with vehicle (control tissues); (▲) = log concentration-effect curve obtained after 10 min incubation of test tissues with (a) procaine (5 mmol l^{-1}) or (b) TEA (8 mmol l^{-1}). Data indicate the means of values from at least 6 tissues; s.e.mean shown by vertical bars.

Drugs and solutions/statistical analysis of results

Drug concentrations are expressed in terms of the molar concentration of the active species. The following substances were used: aminophylline (BDH), apamin (Sigma), histamine acid phosphate (Sigma), indomethacin (Sigma), (−)-isoprenaline hydrochloride (Sigma), methylene blue (Sigma), nicorandil (Chugai, Japan), procaine hydrochloride (Sigma), (±)-propranolol hydrochloride (ICI), sodium nitroprusside (BDH), tetraethylammonium bromide (Sigma).

Stock solutions of indomethacin and isoprenaline were prepared in absolute ethanol and 0.1 mol l^{-1} HCl respectively. Other stock solutions were prepared in twice-distilled water. Stock solutions of apamin were kept frozen at −20°C until ready for use. Dilutions of isoprenaline were prepared in distilled water containing 0.57 mmol l^{-1} ascorbic acid as an antioxidant.

The Krebs solution used in the majority of experiments had the following composition (mmol l^{-1}): Na^+ 143.5, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 125, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.1.

The K^+-rich Krebs solution was of identical osmolality to Krebs solution and had the following composition (mmol l^{-1}): Na^+ 26, K^+ 120, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 125, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.1.

The significance of differences between pairs of means was assessed by means of either a one-tailed or a two-tailed unpaired *t* test while larger groups were assessed using analysis of variance and the Studentised range test (Goldstein, 1964). A difference between means was assumed to be significant when $P < 0.05$.

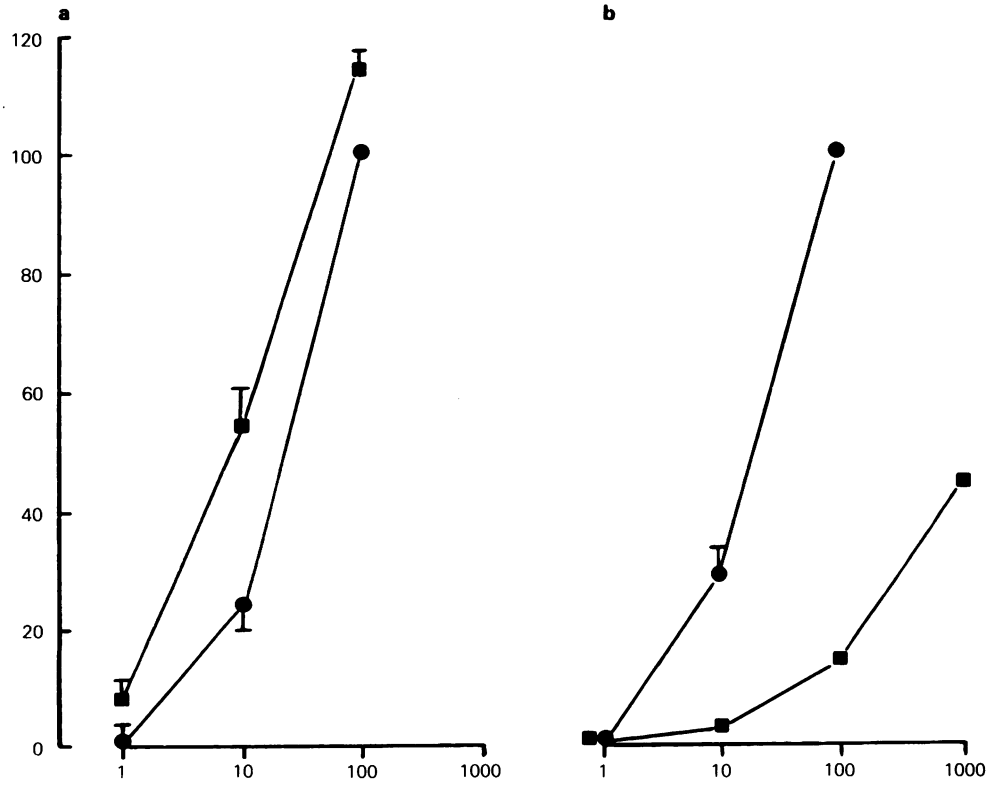

Figure 2 The effects of K^+-rich Krebs solution on the relaxant action of nicorandil in guinea-pig isolated trachealis. The abscissae indicate the concentration (μmol l^{-1}) of nicorandil on a log scale. The ordinates represent relaxation as a % of the initial response to nicorandil 100 μmol l^{-1}: (●) = initial log concentration-effect curves for control (a) and test (b) tissues; (■) = subsequent log concentration-effect curve constructed after 40 min further incubation in Krebs solution (a) or after 40 min incubation with K^+-rich (120 mmol l^{-1}) Krebs solution (b). Data indicate the means of values from 6 tissues; s.e.mean shown by vertical bars.

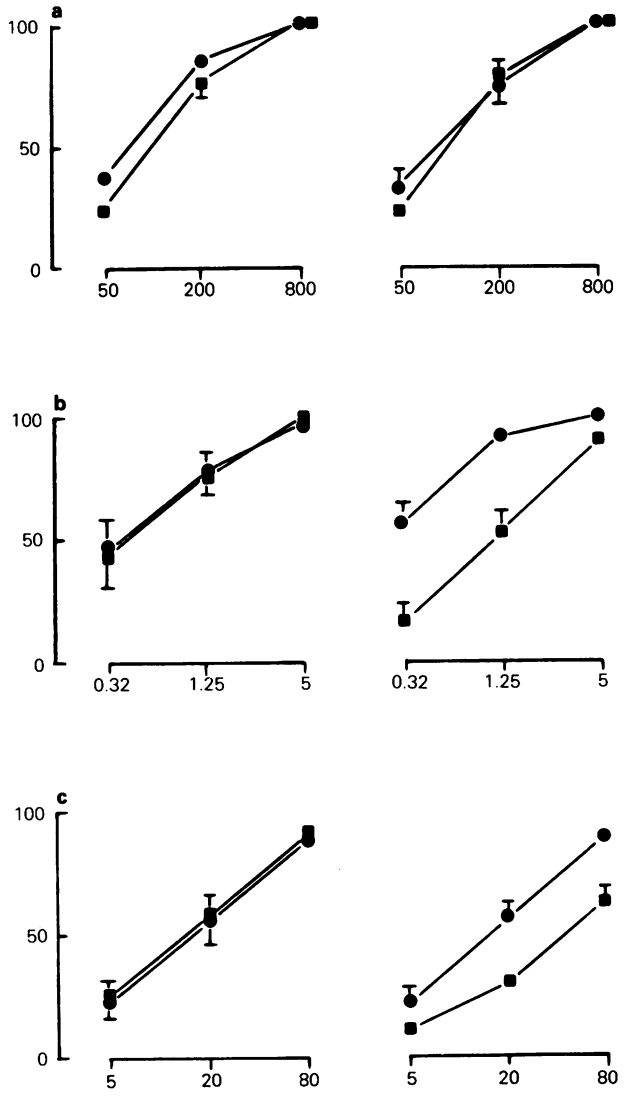

Figure 3 The effects of methylene blue on the relaxant actions of aminophylline, sodium nitroprusside and nicorandil in guinea-pig isolated trachealis. The abscissae indicate the concentration (μmol l^{-1}) of (a) aminophylline (b) sodium nitroprusside or (c) nicorandil on a log scale. The ordinates represent relaxation as a % of the maximal relaxation induced by aminophylline: (●) = initial log concentration-effect curve for control (left-hand panels) and test (right hand panels) tissues; (■) = subsequent log concentration-effect curve constructed after 10 min incubation with vehicle (control tissues) or with 100 μmol l^{-1} methylene blue. Data indicate the means of values from 6 tissues; s.e.mean shown by vertical bars.

Results

Tissue bath studies of mechanical activity

Effects of propranolol on actions of isoprenaline and nicorandil Isoprenaline (0.001–0.1 μmol l^{-1}) and nicorandil (1–100 μmol l^{-1}) each caused concentration-dependent suppression of the spontaneous tone of guinea-pig isolated trachealis. The use of time-matched control tissues showed that the log concentration-effect curves of both isoprenaline and nicorandil moved slightly to the left after tissue incubation

with vehicle. In the test tissues, propranolol (1 μmol l^{-1}) caused no change in tone but evoked marked antagonism of isoprenaline. Propranolol did not modify the action of nicorandil for the log concentration-effect curve moved slightly to the left as observed in the control tissues.

Effects of K^+-channel inhibitors Apamin (0.1 μmol l^{-1}) caused little or no change in tracheal tone, nor did it significantly modify the relaxant action of nicorandil on the trachealis.

TEA (8 mmol l^{-1}) and procaine (5 mmol l^{-1}) both evoked spasm of the trachealis which was tonic initially but which subsequently often assumed a phasic pattern. Procaine caused little, if any, change in the shape or position of the log concentration-effect curve for nicorandil (Figure 1a). In contrast TEA antagonized nicorandil, moving its log concentration-effect curve approximately 0.7 log units to the right relative to the time-matched controls (Figure 1b).

Effects of K^+-rich Krebs solution Exposure of test preparations of trachealis to Krebs solution containing 120 mmol l^{-1} K^+ evoked spasm which was tonic and well-maintained. Nicorandil (1–1000 μmol l^{-1}) was able to relax the K^+-depolarized trachealis but the nicorandil log concentration-effect curve was moved markedly to the right (Figure 2). Responses to 1000 μmol l^{-1} nicorandil in test tissues were much smaller than responses to 100 μmol l^{-1} nicorandil in the control tissues. This suggests that the maximal response to nicorandil may have been reduced in the

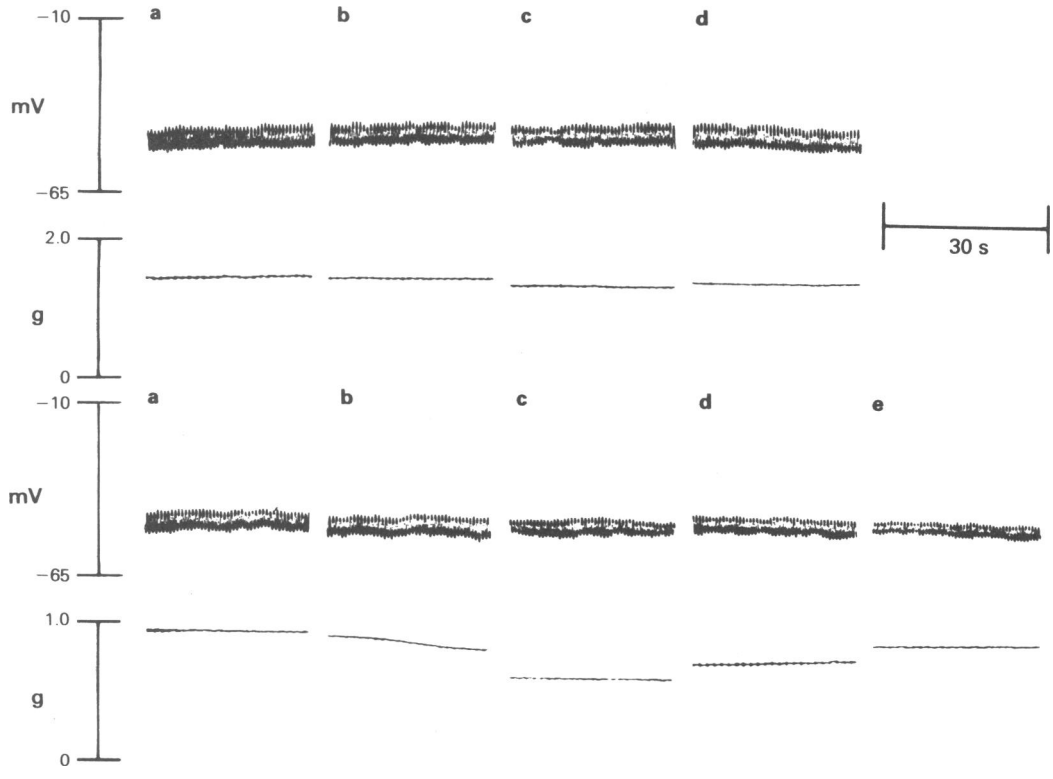

Figure 4 Effects of nicorandil (1 and 10 μmol l^{-1}) on the electrical and mechanical activity of guinea-pig isolated trachealis. The upper and lower rows of records indicate results obtained from two different cells. In each row the upper trace represents membrane potential and the lower trace the mechanical activity of a contiguous segment of trachea. Upper row: activity was recorded before (a) and 1 min (b) and 6 min (c) after addition of nicorandil (1 μmol l^{-1}). Panel (d) shows activity recorded 3 min after washout. Lower row: Activity was recorded before (a) and 1 min (b) and 6 min (c) after addition of nicorandil (10 μmol l^{-1}). Panels (d) and (e) show activity recorded 3 min and 6 min after washout respectively. Note the ability of nicorandil (1 and 10 μmol l^{-1}) to evoke relaxation with little change in electrical activity.

K^+-rich medium. However, solubility problems prevented our further increasing the concentration of nicorandil to test this hypothesis.

Effects of methylene blue on relaxant actions of aminophylline, sodium nitroprusside and nicorandil In tissues treated with indomethacin and histamine, aminophylline (50–800 μmol l^{-1}), sodium nitroprusside (0.32–5 μmol l^{-1}) and nicorandil (5–80 μmol l^{-1}) each evoked concentration-dependent relaxation. The use of control tissues showed that the log concentration-effect curves of these relaxant agents changed very little in shape or position when reconstructed after tissue incubation with Krebs solution containing vehicle.

In test tissues methylene blue (100 μmol l^{-1}) was found not to modify the relaxant action of aminophylline but to cause approximately four fold antagonism of both sodium nitroprusside and nicorandil (Figure 3).

Intracellular electrophysiological recording Nicorandil (1–1000 μmol l^{-1}) produced concentration-dependent changes in both the electrical and mechanical behaviour of guinea-pig isolated trachealis. However, relaxant effects of nicorandil could be detected at concentrations which failed to evoke electrical changes. Minor relaxation was evoked by nicorandil 1 μmol l^{-1} though this concentration of nicorandil did not affect spontaneous electrical slow waves or resting membrane potential (Figure 4). Nicorandil 10 μmol l^{-1} caused more marked relaxation and, in some cells,

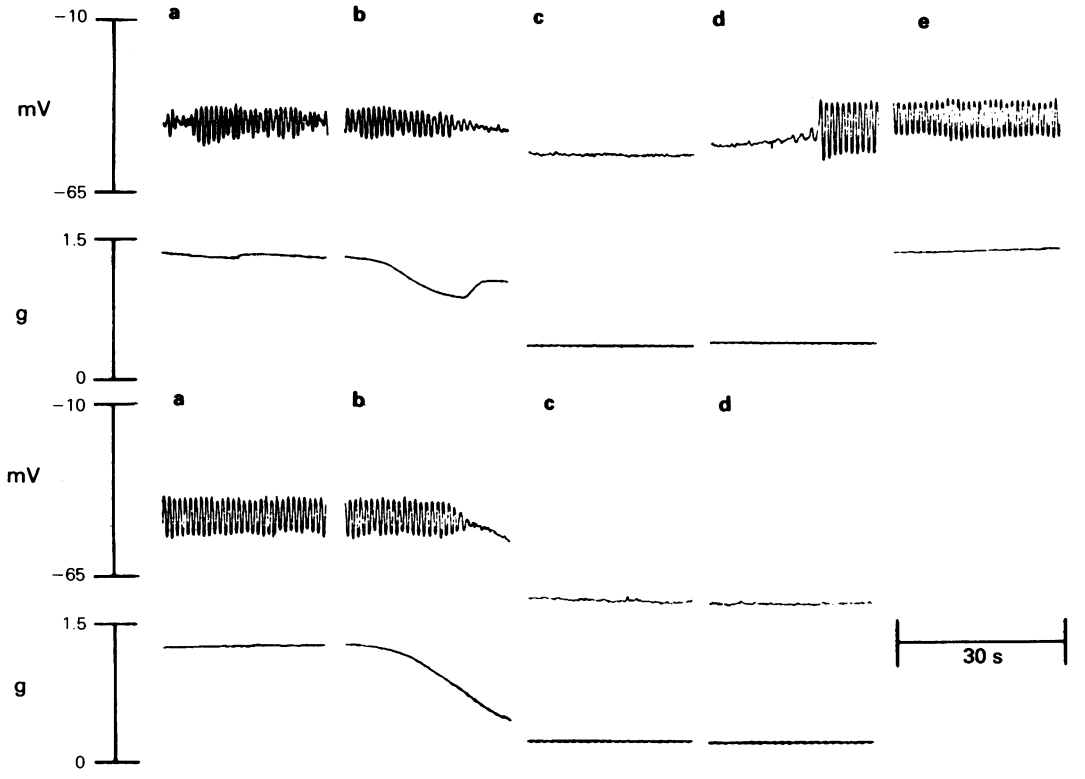

Figure 5 Effects of nicorandil (100 and 1000 μmol l^{-1}) on the electrical and mechanical activity of guinea-pig isolated trachealis. The upper and lower rows of records indicate results obtained from two different cells. In each row the upper trace represents membrane potential and the lower trace the mechanical activity of a continguous segment of trachea. Upper row: activity was recorded before (a) and 1 min (b) and 6 min (c) after addition of nicorandil (100 μmol l^{-1}). Panels (d) and (e) show activity recorded 2 min and 13 min after washout respectively. Lower row: activity was recorded before (a) and 30 s (b) and 2 min (c) and 6 min (d) after addition of nicorandil (1000 μmol l^{-1}). Note the hyperpolarization which accompanies the relaxant effects of nicorandil (100 and 1000 μmol l^{-1}).

Table 1 Effects of nicorandil on the spontaneous electrical and mechanical activity of guinea-pig isolated trachealis

	Properties of cells prior to exposure to nicorandil		*Measurements made 6 min after exposure to nicorandil*			
Nicorandil concentration (μmol l^{-1})	*Maximal amplitude of slow waves* (mV)	*Slow wave frequency* (Hz)	*Maximal amplitude of slow waves* (mV)	*Slow wave frequency* (Hz)	*Change in resting membrane potential* (mV)	*Change in mechanical tone* (mg)
1	6.8 ± 0.5	1.5 ± 0.1	5.8 ± 0.8	1.5 ± 0.1	+ 0.3 ± 1.1	−112 ± 10*
10	10.4 ± 2.6	1.45 ± 0.1	6.8 ± 2.6	0.88 ± 0.3	− 1.7 ± 1.8	−233 ± 63*
100	9.1 ± 1.4	1.07 ± 0.1	0	0	+16.5 ± 2.7*	−615 ± 100*
1000	5.1 ± 1.6	1.35 ± 0.1	0	0	+25.3 ± 2.5*	−717 ± 135*

Data indicate mean ± s.e.mean of observations from at least six cells. A positive change in membrane potential indicates hyperpolarization. A negative change in mechanical tone indicates relaxation. *indicates a significant ($P < 0.05$, two-tailed t test) change in membrane potential or mechanical tone.

abolished slow wave activity without changing the resting membrane potential. Higher concentrations (100 and 1000 μmol l^{-1}) of nicorandil always abolished slow wave discharge within 2 min, hyperpolarized the trachealis cells and caused profound relaxation (Table 1 and Figure 5).

Trachealis muscle treated with TEA (8 mmol l^{-1}) often discharged slow waves which were surmounted by a spike potential. Nicorandil was able to relax TEA-treated tissues and (in concentrations of 100–1000 μmol l^{-1}) also abolished slow wave activity and hyperpolarized the trachealis. The maximal hyperpolarization induced in TEA-treated cells was slightly but significantly smaller than that observed in untreated (control) cells (Table 2). However, TEA prolonged the time required for nicorandil to abolish slow wave discharge (Table 2 and Figure 6).

Slow waves surmounted by spike potentials were sometimes a feature exhibited by trachealis cells treated with procaine (5 mmol l^{-1}). Nicorandil (100 and 1000 μmol l^{-1}) relaxed procaine-treated tissues and the relaxation was accompanied by slow wave abolition. However, the time required for slow wave abolition was greater than that observed in untreated (control) cells (Table 2). In the presence of procaine the ability of nicorandil to hyperpolarize trachealis cells was markedly reduced (Table 2 and Figure 6).

Table 2 The effects of tetraethylammonium (TEA) and procaine on some electrical responses of guinea-pig isolated trachealis to nicorandil

	Change in resting membrane potential (mV) 6 min after exposure to nicorandil		
Nicorandil concentration (μmol l^{-1})	*Cells bathed by normal Krebs solution*	*Cells treated with TEA* (8 mmol l^{-1})	*Cells treated with procaine* (5 mmol l^{-1})
10	− 1.7 ± 1.8	− 0.3 ± 2.7	–
100	16.5 ± 2.7	18.2 ± 3.7	2.9 ± 1.5*
1000	25.3 ± 2.5	18.0 ± 1.4*	2.7 ± 2.6*
	Time required for slow wave abolition (s)		
Nicorandil concentration (μmol l^{-1})	*Cells bathed by normal Krebs solution*	*Cells treated with TEA* (8 mmol l^{-1})	*Cells treated with procaine* (5 mmol l^{-1})
100	68.3 ± 7.8	167 ± 25.2*	> 156*
1000	75.6 ± 15.7	151 ± 25.4*	101.7 ± 6.4

Data indicate mean ± s.e.mean of at least 5 experimental results. A negative change in resting membrane potential signifies depolarization. *indicates a significant ($P < 0.05$) difference from cells bathed by normal Krebs solution (two-tailed unpaired t test).

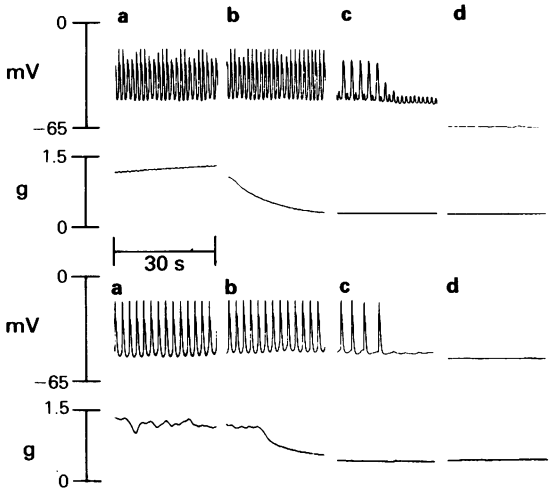

Figure 6 Effects of nicorandil (1000 μmol l^{-1}) on the electrical and mechanical activity of guinea-pig isolated trachealis treated with tetraethylammonium (TEA, 8 mmol l^{-1}) or procaine (5 mmol l^{-1}). The upper and lower rows of records indicate results obtained from two different cells. In each row of records the upper trace represents membrane potential and the lower trace the mechanical activity of a contiguous segment of trachea. Upper cell: from tissue treated with TEA (8 mmol l^{-1}). Activity was recorded before (a) and 1 min (b) 2 min (c) and 6 min (d) after addition of nicorandil (1000 μmol l^{-1}). Lower cell: from tissue treated with procaine (5 mmol l^{-1}). Activity was recorded before (a) and 1 min (b) 2 min (c) and 6 min (d) after addition of nicorandil (1000 μmol l^{-1}). Note marked hyperpolarization induced by nicorandil in TEA-treated tissue and minor hyperpolarization observed in procaine-treated tissue.

^{86}Rb efflux studies

The pooled control plot of ^{86}Rb efflux rate constant against time showed that, after an initial very high value, the efflux rate constant quickly (< 10 min) settled to a low and consistent value. More detailed examination of the variation of efflux rate constant with time was applied to the values obtained after the initial 20 min had elapsed. A decline of mean efflux rate constant with time tended to be obscured by the larger variation of values between tissue segments at each time. This was minimised by a standardisation procedure.

For each tissue segment the average of the last four efflux rate constant values was subtracted from all values. The decline in mean standardised efflux rate constant with elapsed time was then revealed as significant, emphasising the necessity for possible drug effects to be examined against time-matched controls.

Figure 7 shows the enhancement of ^{86}Rb efflux which occurs in the presence of nicorandil (1 mmol l^{-1}). This spike was present in the unstandardised curve of every tissue yet, in spite of this consistency, only stood out with high significance ($P < 0.01$) by the

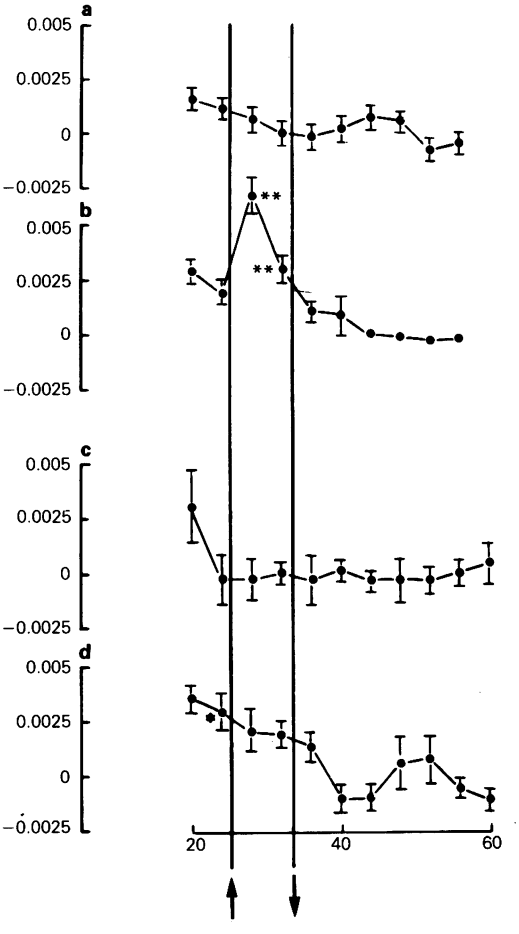

Figure 7 The effects of nicorandil (1000 μmol l^{-1}) and isoprenaline (1 μmol l^{-1}) on the efflux of ^{86}Rb from muscle-rich strips of guinea-pig isolated trachea. The abscissa scale indicates time (min) and the ordinates represent the relative efflux rate constant (min^{-1}). Drug (test tissues) or vehicle (time-matched control tissues) was present during the period between the arrows. (a) Time-matched controls and (b) test tissues in experiments with nicorandil; (c) time-matched controls and (d) test tissues in experiments with isoprenaline. Data points are the means of at least 8 experimental values and vertical bars indicate s.e.mean. * and ** indicate significant ($P < 0.05$ and $P < 0.01$ respectively) differences from the corresponding point in time-matched control tissues.

Studentised range test after the application of the above standardisation procedure.

No comparable spike occurred in the presence of isoprenaline (1 μmol l^{-1}). The one point in the test curve revealed as 'significantly' ($P < 0.05$) different from the corresponding time point in the time-matched control curve preceded the drug treatment. The 'difference' is attributed to the operation of random variation.

Discussion

Involvement of β-adrenoceptors or the nitrate moiety in the relaxant action of nicorandil

Since propranolol markedly antagonized isoprenaline on guinea-pig isolated trachealis but did not modify the relaxant action of nicorandil we conclude that the action of nicorandil does not involve the activation of β-adrenoceptors.

Sodium nitroprusside, sodium nitrite and organic nitrates can liberate nitric oxide within smooth muscle cells and there is evidence (Ignarro *et al.*, 1981) that relaxation induced by these agents is associated with the conversion of nitric oxide to S-nitrosothiols and the subsequent activation of guanylate cyclase. Such evidence includes the observation that methylene blue can antagonize sodium nitroprusside, sodium nitrite and organic nitrates both as regards their relaxant activity and their ability to stimulate guanylate cyclase (Gruetter *et al.*, 1979; 1981). Methylene blue is believed to antagonize these agents by oxidising a haemoprotein associated with the guanylate cyclase enzyme (Gruetter *et al.*, 1979; Martin *et al.*, 1985).

In the present study methylene blue (100 μmol l^{-1}) failed to modify the relaxant action of aminophylline but antagonized both sodium nitroprusside and nicorandil. This suggests that methylene blue exhibits selectivity of action and that sodium nitroprusside and nicorandil relax guinea-pig isolated trachealis (at least in part) by the common mechanism of guanylate cyclase activation. These results lend support to the earlier finding (Maruyama *et al.*, 1982) that the nitrate moiety of the nicorandil molecule contributes to its relaxant effects in trachealis muscle.

Membrane potential changes evoked by nicorandil and underlying mechanisms

The marked hyperpolarization of guinea-pig trachealis evoked by nicorandil (100–1000 μmol l^{-1}) could result from an increase in membrane K^+ conductance, a decrease in membrane Cl^- conductance or the stimulation of electrogenic ion exchange. Electrophysiological experiments in canine trachealis (Inoue *et al.*, 1983) suggested that nicorandil might act to increase membrane K^+ conductance and the present studies of ^{86}Rb efflux were performed to test the validity of that suggestion.

^{86}Rb has been used as a tracer for K^+ efflux in a wide variety of excitable cells and its use for that purpose in canine trachealis has been justified by Imaizumi & Watanabe (1981). The present observation that nicorandil (1 mmol l^{-1}) stimulates ^{86}Rb efflux from the tissue strongly indicates that nicorandil-induced hyperpolarization results from an increase in K^+-conductance of the trachealis cell membrane. In this respect the present findings lend support to the electrophysiological evidence obtained in canine tissue (Inoue *et al.*, 1983).

The change in the ^{86}Rb efflux rate constant evoked by nicorandil was quite small despite the use of nicorandil in a concentration known to evoke near-maximal hyperpolarization of the trachealis muscle. This may have been a reflection of the small mass of smooth muscle relative to other tissue in our 'muscle rich' strips of trachea.

Isoprenaline (1 μmol l^{-1}) evokes hyperpolarization of guinea-pig trachealis (Allen *et al.*, 1985) of similar amplitude to that evoked by nicorandil (1 mmol l^{-1}). There is electrophysiological evidence, too, that isoprenaline-induced hyperpolarization of trachealis muscle is the result of an increase in membrane K^+ conductance (Ito & Tajima, 1982; Cameron *et al.*, 1983). In view of this it is curious that isoprenaline failed to increase ^{86}Rb efflux from guinea-pig trachealis. Airways smooth muscle may contain several kinds of K^+ channel (Inoue *et al.*, 1983) and the K^+ channels of excitable cells may not all be permeable to ^{86}Rb (Petersen & Maruyama, 1984). This raises the possibility that isoprenaline opens trachealis K^+-channels which are impermeable to ^{86}Rb.

Nature of K^+-channels opened by nicorandil

Apamin-sensitive K^+-channels do not seem to be involved in the maintenance of spontaneous tone of guinea-pig isolated trachea or in the mechanical and electrical responses of the tissue to isoprenaline (Allen *et al.*, 1985). The present failure of apamin to modify the relaxant action of nicorandil suggests that apamin-sensitive K^+-channels do not play a crucial role in mediating nicorandil-induced relaxation in this tissue.

The K^+-channels opened by nicorandil do not seem to be sensitive to TEA, for TEA only weakly antagonized the hyperpolarizing action of nicorandil in guinea-pig trachealis. Similar resistance of nicorandil-induced hyperpolarization to TEA has been reported in canine trachealis (Inoue *et al.*, 1983).

In contrast, tracheal hyperpolarization induced by nicorandil is markedly inhibited by procaine (Inoue *et al.*, 1983; present study). Together with the present radioisotope studies such findings suggest that the

K^+-channels opened by nicorandil are permeable to ^{86}Rb, inhibited by procaine but resistant to apamin or TEA.

Dependency of nicorandil-induced relaxation on hyperpolarization

Several pieces of evidence suggest that the relaxation of guinea-pig trachealis induced by nicorandil is not entirely dependent on cellular hyperpolarization. Firstly, low concentrations (1–10 μmol l^{-1}) of nicorandil evoked relaxation which was not accompanied by detectable electrical changes – hyperpolarization (and stimulation of ^{86}Rb efflux) was only observed with concentrations of nicorandil causing maximal or near-maximal relaxation. Secondly, procaine markedly reduced the hyperpolarization induced by nicorandil but did not inhibit relaxant activity. Thirdly, nicorandil retained some relaxant activity when tested in a K^+-rich (120 mmol l^{-1}) medium. In such a medium the opening of K^+ channels cannot evoke hyperpolarization of trachealis cells because the K^+ equilibrium potential approaches the resting membrane potential. Our observations therefore lend support to the suggestion of Inoue *et al.* (1983) that the inhibitory effects of nicorandil on trachealis muscle do not result simply from hyperpolarization of trachealis cell membranes but also, perhaps, from alteration of the intracellular disposition of Ca^{2+}. The latter postulated mechanism may entirely explain the relaxant actions of nicorandil at the lower end of its effective concentration range.

The financial support of the Asthma Research Council, the Mason Medical Research Foundation, May and Baker Ltd., the North Western Regional Health Authority, and the SmithKline (1982) Foundation is gratefully acknowledged. We thank Miss D. Gray for expert technical assistance. We thank Mrs V. Sillavan and Mrs J. Stafford for typing the manuscript.

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(Received August 26, 1985.
Revised October 11, 1985.
Accepted October 21, 1985.)