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EVIDENCE FOR NON-CHOLINERGIC, NON-ADRENERGIC NERVOUS CONTROL OF MUCUS SECRETION INTO THE CAT TRACHEA

BY A. C. PEATFIELD AND P. S. RICHARDSON*

From the Department of Physiology, St George's Hospital Medical School, London, SW17 0RE

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SUMMARY

1. We have investigated the effects of electrical stimulation of the vagus nerves on the output of mucus glycoproteins (mucins), radiolabelled with ^{3}H and ^{35}S, into the trachea of anaesthetized cats.

2. In five control experiments, stimulation of the vagus nerves on four successive occasions, separated by 1 h, caused significant rises in the output of radiolabelled mucins. In these experiments repetition of stimulation did not appear to lessen the response.

3. In a parallel series of five experiments the vagus nerves were again stimulated on four occasions, but atropine was administered in increasing doses between the stimuli. Large responses, not significantly less than those seen in the corresponding control stimulations, were seen even in the presence of the highest dose of atropine. In this series of experiments, however, the effect of the last vagal stimulation (with the highest dose of atropine) was significantly less then the first (no atropine).

4. Administration of phentolamine and *l*-propranolol in addition to atropine failed to reduce the response to vagal stimulation significantly.

5. We conclude that, while cholinergic nerves can probably explain part of the increase in mucin output which occurs with vagus nerve stimulation, there is a large response mediated by a non-cholinergic, non-adrenergic neurotransmitter. Possible neurotransmitters and the relationship of these findings to those of earlier studies are discussed.

INTRODUCTION

In the course of experiments to test the effect of dust on mucus glycoprotein (mucin) secretion, we noted that atropine failed to block the mucotropic effect of vagus nerve stimulation (Peatfield & Richardson, 1983). This was a surprising result as many groups of workers have reported previously that electrical stimulation of the peripheral ends of the cervical vagus nerves promotes mucus secretion from the larger airways and that atropine would block or greatly reduce this effect (Florey, Carleton & Wells, 1932; Gallagher, Kent, Passatore, Phipps & Richardson, 1975; Ueki,

* To whom correspondence should be sent.

German & Nadel, 1980). The dose of atropine used in the dust study (Peatfield & Richardson, 1983) was the same as that used by Gallagher *et al.* (1975): 1·0 mg kg^{-1} intravenously (I.V.). In this paper we report results from experiments involving vagus nerve stimulation alone, nerve stimulation in the presence of increasing doses of atropine, and nerve stimulation in the presence of sympathetic blockade. The purpose of the study was to discover whether the cervical vagus nerves of the cat contain either adrenergic or non-cholinergic, non-adrenergic motor fibres to the secretory cells of the trachea. A brief report of some of the results has been published previously (Peatfield & Richardson, 1981).

METHODS

The method used has been described in the preceding paper (Peatfield & Richardson, 1983). In each experiment the vagus nerves were separated from the cervical sympathetics and then cut just caudal to the superior laryngeal nerves. Their peripheral ends were stimulated simultaneously up to four times during each experiment for 8 min of a 15 min collection period. Three 15 min control periods were allowed between each stimulus period. The stimulus pulses were 8 V, of 2 ms duration at a frequency of 10 Hz. The stimulating electrodes were applied to the nerves in the cranial half of the neck. During the periods of nerve stimulation the heart rate was measured via a pressure transducer (SEM 4–88) connected to the arterial catheter and recorded on a U.V. recorder (SE 6008).

The experiments were carried out in four series of five cats (Table 1). The autonomic blocking drugs were added to the reservoir of Krebs–Henseleit solution used for flushing out the tracheal segment 0·5 h before the period of nerve stimulation and were also given intravenously (I.V.). The drugs used were atropine sulphate (Antigen Ltd. and Sigma), *l*-propranolol hydrochloride (ICI) and phentolamine mesylate (Rogitine, Ciba). In the third series of experiments the stellate ganglia, through which the sympathetic nerves to the trachea pass, were excised. In some experiments of the first three groups, pilocarpine hydrochloride (Sigma) was given directly into the tracheal segment (I.S.) (0·5 $\mu g\ ml^{-1}$) after the final vagal stimulation. In the fourth series of experiments, phenylephrine hydrochloride (Boots Co. Ltd.) was administered likewise (10 $\mu g\ ml^{-1}$).

The effect of each vagal stimulation on the output of each mucin-bound radiolabel was calculated as a percentage change over the mean of the outputs in the preceding and succeeding control period. The effects of pilocarpine in the absence of atropine, and of phenylephrine, though, were normally prolonged into the following control period, so in these cases the effects were measured as percentage changes over the output during the preceding control period only. Because the distribution of the data appeared skewed we have used non-parametric methods to test for statistical significance: the sign test to test for significance of an effect above zero, and the Mann–Whitney *U* test (Siegel, 1956) to determine whether one effect was different from another. $P < 0·05$ was taken as being statistically significant.

RESULTS

1. The effect of vagus nerve stimulation without antagonists (series 1)

Vagal stimulation caused a substantial and significant increase in the release of both radiolabels in series 1 (Table 1). The size of the increase, about +158 % for ^{35}S and +41 % for ^{3}H, was similar in the first, second, third and fourth stimulations (periods I, II, III and IV) within these experiments (Table 2). In most experiments vagal stimulation caused mucus to collect at the cannula inserted into the lungs in a quantity that required removal by suction.

At the onset of vagal stimulation the heart rate fell (Fig. 1), and recovered when the stimulation ceased. The effects progressively diminished with each stimulation.

TABLE 1. Protocol of experiments

Experimental series	Period of vagal stimulation I	II	III	IV
1	Alone	Alone	Alone	Alone
2	Alone	With atropine 0·3 mg kg^{-1} I.V. +0·6 μg ml^{-1} I.S.	With atropine 1·0 mg kg^{-1} I.V.* +2·0 μg ml^{-1} I.S.	With atropine 3·0 mg kg^{-1} I.V.* 6·0 μg ml^{-1} I.S.
3	With *l*-propranolol 1·5 mg kg^{-1} I.V. 10·0 μg ml^{-1} I.S.	*l*-propranolol +atropine 3·0 mg kg^{-1} I.V. 6·00 μg ml^{-1} I.S.	*l*-propranolol, atropine+ stellate ganglia excised	—
4	Alone	With atropine, *l*-propranolol (doses as series 3)+ phentolamine, 10 μg ml^{-1} I.S.	—	—

* Represents cumulative doses.

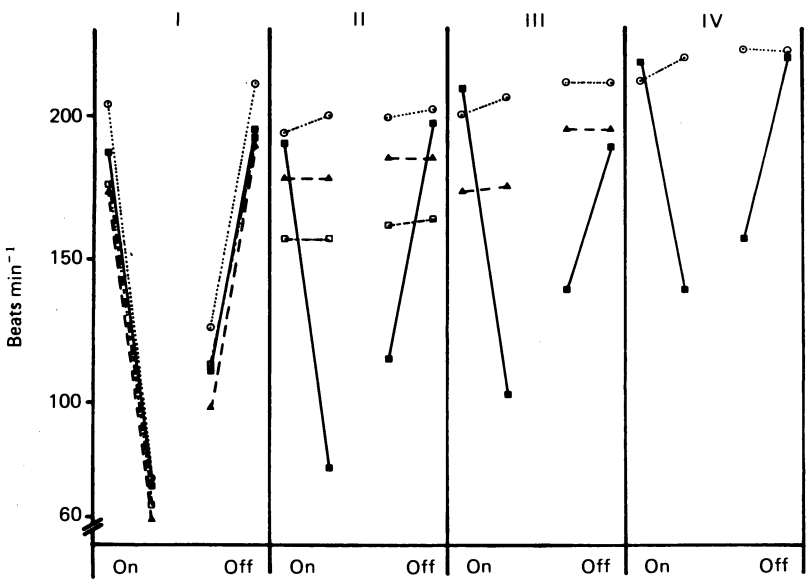

Fig. 1. Plot showing the changes in heart rate at the beginning and end of vagus nerve stimulation for each period (I–IV) in the four series of experiments: ■——■, series 1, no antagonists; ⊙·····⊙, series 2, with atropine (except in I); △----△, series 3, with atropine (except in I) and *l*-propranolol; □-·-·-·-□, series 4, with atropine, *l*-propranolol and phentolamine (for period II). See Table 1 for further details of the experimental plan.

TABLE 2. Comparison of the effect of four successive vagal stimulations, in the absence (series 1) and presence (series 2) of increasing doses of atropine (see text), on release of radiolabelled mucins. None of the changes in the left column were significantly different to the corresponding changes in the right column in the Mann–Whitney *U* test

Series 1					Series 2				
Test	*n*	Isotope	Mean Δ %	Range	Test	*n*	Isotope	Mean Δ %	Range	*P*
Vagal stimulation I	5	^{35}S	+165	+32 to +431	Vagal stimulation I	5	^{35}S	+254	+124 to +357	n.s.
		^{3}H	+57	+26 to +91			^{3}H	+121	+62 to +184	n.s.
Vagal stimulation II	5	^{35}S	+160	+14 to +447	Vagal stimulation II	5	^{35}S	+159	+36 to +322	n.s.
		^{3}H	+37	+2 to +64	(with atropine)		^{3}H	+61	+32 to +137	n.s.
Vagal stimulation III	5	^{35}S	+153	+81 to +252	Vagal stimulation III	5	^{35}S	+153	+53 to +250	n.s.
		^{3}H	+41	+20 to +50	(with atropine)		^{3}H	+56	+24 to +85	n.s.
Vagal stimulation IV	5	^{35}S	+153	+42 to +348	Vagal stimulation IV	5	^{35}S	+93	+47 to +176	n.s.
		^{3}H	+31	+1 to +47	(with atropine)		^{3}H	+31	+15 to +51	n.s.

n.s., not significant.

2. The effect of atropine on vagally mediated changes (series 2)

Vagal stimulation increased secretion significantly even in the presence of the highest dose of atropine. Although atropine appeared to reduce the action of vagal stimulation on the output of mucins labelled with both radio-isotopes, the differences between the effects of nerve stimulation in the presence of atropine (series 2) and the corresponding stimulation without the drug (series 1) did not reach statistical significance (Table 2). There was a surprising difference between the effect of the first nerve stimulation in the two series of experiments, though there was no difference in the protocol used since the atropine had not been added at this stage. The mean increases in output of both radiolabels diminished with the increasing doses of atropine. By the fourth stimulation (with the highest dose of atropine: 3·0 mg kg^{-1} I.V. +6·0 $\mu g\ ml^{-1}$ I.S.), the increase in output of both radiolabels was significantly less (P for $^{35}S < 0{\cdot}05$ and for $^{3}H < 0{\cdot}01$) than the outputs for the first stimulation when there was no atropine. An example of an experiment is shown in Fig. 2. In general, vagal stimulation in the presence of atropine did not cause mucus to accumulate in the most caudal cannula in a quantity that needed to be removed.

With even the lowest dose of atropine, there was no decrease in heart rate on vagal stimulation. In fact there was a small but significant increase. For the fifteen trials (series 2, 3 and 4) the heart rate increased by a mean of six (S.D. = ±7·3) beats min^{-1}. ($P < 0{\cdot}005$, sign test) (Fig. 1).

3. The effects of β-adrenoceptor blockade and sympathectomy on vagally mediated changes (series 3)

l-Propranolol had no effect on the increase in output of radiolabelled mucins which vagal stimulation elicited in period I (Table 3). When a large dose of atropine (3·0 mg kg^{-1} I.V. and 6·0 $\mu g\ ml^{-1}$ I.S.) was given subsequently (period II), the response of mucin output to vagal stimulation was less than with the equivalent vagal stimulation without autonomic blockers, but this difference was only significant for the ^{3}H-labelled mucins (Table 3). After the stellate ganglia were excised (period III) the mean responses of mucins labelled with both radio-isotopes were small and not significantly greater than zero, and they were significantly less than those found with the equivalent vagal stimulation alone (Table 3).

In the presence of *l*-propranolol (period I), the heart rate fell markedly at the onset of vagal stimulation in a manner indistinguishable from that seen in the absence of the adrenoceptor blocker, and similarly recovered when the stimulation ceased. During the next two periods of vagal stimulation in this group of experiments, one after administration of atropine and the other after excision of the stellate ganglia but still with atropine, there were no changes in heart rate (Fig. 1).

4. The effects of α-adrenoceptor blockade on vagally mediated changes in mucin release (series 4)

To test whether the residual effect of vagal stimulation in the presence of atropine and *l*-propranolol on mucin release might be due to activation of α-adrenoceptors, phentolamine was administered intrasegmentally in addition to atropine and l-propranolol (series 4). The increase in output of radiolabelled mucins in the presence

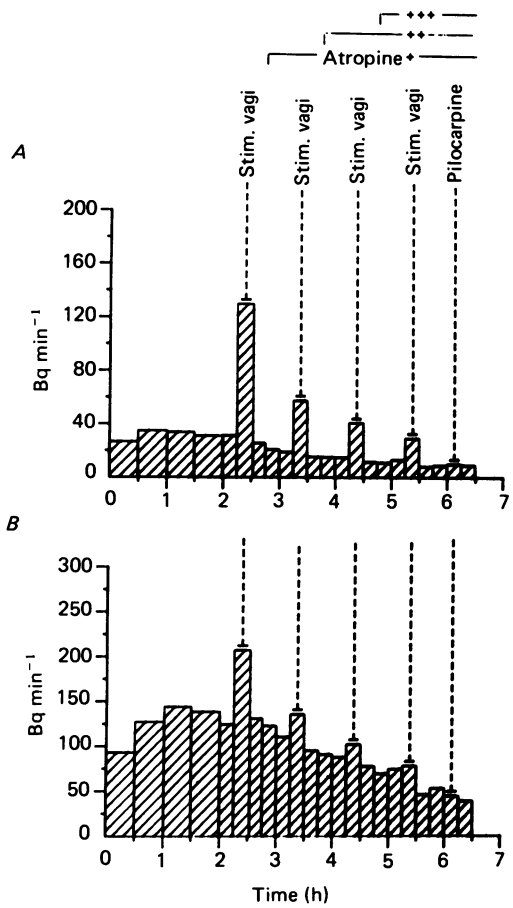

Fig. 2. Graph of the rate of output of mucin-bound radioactivity from the cat trachea (*A*, ^{35}S; *B*, ^{3}H) against time. Electrical stimulation of the vagus nerves stimulated mucin release both in the absence and in the presence of increasing doses of atropine. The normally potent effect of pilocarpine (0·5 μg ml^{-1} I.S.) was not observed when atropine was present.

Atropine + = 0·3 mg kg^{-1} I.V. and 0·6 μg ml^{-1} I.S.
Atropine + + = 1·0 mg kg^{-1} I.V. and 2·0 μg ml^{-1} I.S.
Atropine + + + = 3·0 mg kg^{-1} I.V. and 6·0 μg ml^{-1} I.S.

of the three antagonists (period II) was not significantly less than that seen in the equivalent period of vagal stimulation in the absence of antagonists (series 1, period II) (Table 4). Similarly the effect of vagal stimulation in the presence of phentolamine, *l*-propanolol and atropine (period II) was no less than that in the presence of only the latter two drugs (series 3, period II) (Table 4). The increase in mucin release during vagal stimulation in the presence of the three autonomic antagonists was significantly greater than zero ($P < 0.05$ for both isotopes). The phentolamine was given at a dose sufficient to block the effect of phenylephrine administered intrasegmentally towards the end of these experiments. Under these conditions, phenylephrine elicited only a +1 % (range: −5 to +7) increase in release of ^{35}S-labelled mucins and a −8 % (range: −32 to +5) change in release of ^{3}H-labelled mucins ($n = 5$). These results are not

TABLE 3. Comparison of the effect of three successive vagal stimulations, in the absence (series 1) and presence (series 3) of various forms of autonomic blockade, on release of radiolabelled mucins. The *P* values refer to the changes in the left column compared with changes in the right column by the Mann–Whitney *U* test

Series 1					Series 3				
Test	*n*	Isotope	Mean Δ%	Range	Test	*n*	Isotope	Mean Δ%	Range	*P*
Vagal stimulation I	5	^{35}S	+165	+32 to +431	Vagal stimulation I	5	^{35}S	+249	+20 to +643	n.s.
		^{3}H	+57	+26 to +91	(with *l*-propranolol)		^{3}H	+53	+17 to +107	n.s.
Vagal stimulation II	5	^{35}S	+160	+14 to +447	Vagal stimulation II	5	^{35}S	+61	−6 to +135	n.s.
		^{3}H	+37	+2 to +64	(with *l*-propranolol and atropine)		^{3}H	+14	−1 to +34	< 0·05
Vagal stimulation III	5	^{35}S	+153	+82 to +252	Vagal stimulation III	4	^{35}S	+38	−12 to +73	< 0·01
		^{3}H	+41	+20 to +50	(with *l*-propranolol, and stellates excised)		^{3}H	+2	−13 to +10	< 0·01

n.s., not significant.

TABLE 4. Comparison of the effects of vagal stimulation with all three autonomic antagonists (series 4) with vagal stimulation alone (series 1) or with just atropine and *l*-propranolol (series 3)

Test	*n*	Isotope	Mean Δ%	Range	Test	*n*	Isotope	Mean Δ%	Range	*P*
Vagal stimulation II (series 1)	5	^{35}S	+160	+14 to +447	Vagal stimulation II (with atropine, *l*-propranolol and phentolamine) (series 4)	5	^{35}S	+59	+4 to +129	n.s.
		^{3}H	+37	+2 to +64			^{3}H	+19	+3 to +43	n.s.
Vagal stimulation II (with atropine and *l*-propranolol (series 3)	5	^{35}S	+61	−6 to +135	Vagal stimulation II (with atropine, *l*-propranolol and phentolamine) (series 4)	5	^{35}S	+59	+4 to +129	n.s.
		^{3}H	+14	−1 to +34			^{3}H	+19	+3 to +43	n.s.

n.s., not significant.

significantly different from zero but are significantly less than the changes elicited by the same dose of phenylephrine, administered in the absence of antagonists, reported in a previous publication ($\Delta^{35}S = +454\%$, range: +51 to +922; $\Delta^{3}H = +169\%$, range: +27 to +507%, $n = 12$, $P < 0.001$ for both isotopes; Peatfield & Richardson, 1982).

5. The effect of pilocarpine on mucin release

Pilocarpine was only given to two cats in the absence of atropine. On both occasions it increased the release of both radiolabels substantially ($\Delta^{35}S = +643\%$ and $+416\%$; $\Delta H = +212\%$ and $+196\%$). It was also given on seven occasions to atropinized cats when the mean changes were: $\Delta^{35}S$: +5% (range: −10 to +33), $\Delta^{3}H$: 0% (range: −16 to +25). The difference in response was statistically significant ($P < 0.05$).

DISCUSSION

The large increases in radiolabelled mucin output elicited by stimulation of the cervical vagus nerves described here confirm the findings of earlier studies. The increase in output of ^{35}S-labelled mucins was approximately three times that of ^{3}H-labelled mucins. Autoradiographic studies have demonstrated that sulphate is mainly incorporated into submucosal glands and tritiated glucose is taken up predominantly by epithelial cells (Gallagher, Hall, Jeffery, Phipps & Richardson, 1978). Nerve fibres, believed on ultrastructural grounds to be cholinergic, have been observed adjacent to the mucous and serous cells of the cat tracheal submucosal glands (Silva & Ross, 1974; Murlas, Nadel & Basbaum, 1980), while epithelial secretory cells such as goblet cells are not thought to be innervated (Florey *et al.* 1932). The radiolabelling pattern (rich in ^{35}S, poor in ^{3}H) of secretions collected in response to vagal stimulation can be explained if they originate mainly from the submucosal glands.

There were no significant differences between the effects of vagal stimulation on mucin release in the presence of atropine and the effects of the corresponding nerve stimulations without atropine. This finding is contrary to those of Florey *et al.* (1932), Gallagher *et al.* (1975) and Ueki *et al.* (1980), all of whom reported an abolition or marked reduction of effect by atropine. Even the highest dose of atropine used in the present experiments failed to block the effects of vagal stimulation or to lessen it significantly when compared with the effect of vagal stimulation at the same stage of control experiments. There was, however, some evidence of an atropine-sensitive component: in control experiments later vagal stimulations gave as great an effect on mucin output as earlier ones, but in the presence of the highest dose of atropine the effect of vagal stimulation was significantly less than that seen in the same cats before atropine treatment. To what can the difference between these results and those of earlier workers be attributed?

One explanation was that the atropine had deteriorated. This is unlikely because atropine consistently blocked the vagally induced slowing of the heart rate and the pilocarpine-induced rise in mucin secretion.

The second explanation was that during these experiments we may have been stimulating some sympathetic fibres running in the vagus nerves (Ranson, Foley &

Alpert, 1933; Muryobayashi, Mori, Fujiwara & Shimamoto, 1968). The increase in heart rate at the onset of vagal stimulation in atropinized cats is evidence for this. Our results show that administration of the β-adrenoceptor blocker, *l*-propranolol, which largely blocks the promotion of mucin release elicited by stellate ganglia stimulation (Peatfield & Richardson, 1982), had no effect on the mucotropic action of vagal stimulation. When the stellate ganglia were excised the response to nerve stimulation was significantly less than the response of the equivalent stimulation without atropine for both radio-isotopes. It is possible that preganglionic sympathetic fibres originate from the upper segments of the thoracic sympathetic outflow, pass up the vagi, loop, and then descend the nerve again to relay in the stellate ganglia. Some bronchomotor fibres run a similar course (Daly & Mount, 1951). This hypothesis may go some way to explain the ineffectiveness of atropine as a blocker, but fails to explain the discrepancy between these results and those of Gallagher *et al.* (1975).

The third explanation was that there are some parasympathetic efferent nerve endings which release non-cholinergic, non-adrenergic transmitters. In the experiments in which all three autonomic blockers were used, vagal stimulation still elicited a significant increase in radiolabelled mucin output. There is evidence for a non-cholinergic, non-adrenergic transmitter controlling mucus secretion from ferret trachea. Field stimulation elicits a marked increase in radiolabelled mucin release from tracheal explants, a component of which is not blocked by the combined action of muscarinic, α- and β-adrenoceptor antagonists (Borson, Charlin, Gold & Nadel, 1982). There is now mounting evidence for the coexistence of peptides with amines or acetylcholine in neurones (see Hökfelt, Johansson, Ljungdahl, Lundberg & Schultzberg, 1980; Schultzberg, Hökfelt & Lundberg, 1982). Substance P and vasoactive intestinal polypeptide (VIP) are present in the human vagus nerve (Lundberg, Hökfelt, Kewenter, Petterson, Ahlman, Edin, Dahlström, Nilsson, Terenius, Uvnäs-Wallenstein & Said, 1979), and both have been observed in tracheal tissue, the latter specifically around submucosal glands (Uddman, Alumets, Densert, Håkanson & Sundler, 1978; Nilsson, Dahlberg, Brodin, Sundler & Strandberg, 1977). Substance P increases the release of macromolecules from canine tracheal explants (Baker, Hillegass, Holden & Smith, 1977) and VIP has been shown to increase radiolabelled mucin output from ferret trachea (Peatfield, Barnes, Bratcher, Nadel & Davis, 1983). Thus either or both of these peptides are strong contenders for possible co-release with acetylcholine in vagally induced tracheal mucus secretion. Again, this explanation does not account for the differences in findings between this study and that of Gallagher *et al.* (1975).

Specific pathogen-free cats were used for this study while Gallagher *et al.* (1975) used animals some of which had respiratory infections at the time of the experiment and others had probably recovered from infections. Animals that have recently had respiratory tract infections display mucous gland hypertrophy (Jones, Baskerville & Reid, 1975), and human bronchi with hypertrophied glands have different sensitivities to acetylcholine stimulation and atropine block (Sturgess & Reid, 1972). It is likely that hypertrophied glands undergo a change in the nature or number of end-organ receptors, and that this alters their response to neurotransmitters.

The most important practical conclusion that can be drawn from these results is that atropine and adrenoceptor antagonists together can no longer be relied upon to

block the autonomic efferent pathways of reflex promotion of cat tracheal mucin secretion as has been done in the past (see e.g. Richardson, Phipps, Balfré & Hall, 1978). The failure of atropine and *l*-propranolol to block the reflex increase in secretion caused by dust inhalation (Peatfield & Richardson, 1983), does not exclude the vagus nerves as the efferent pathway of the reflex. We conclude that, in the cats used in this study, the tracheal secretory cells were innervated by some vagal efferent fibres which produced a non-cholinergic, non-adrenergic transmitter.

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