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Electrophysiology of neuroeffector transmission in the isolated, innervated trachea of the guinea-pig

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1 Intracellular recordings were made from cells of the guinea-pig trachealis muscle. Some cells were electrically quiescent while others exhibited spontaneous slow waves.

2 In quiescent cells, stimulation of the cervical vagus nerve evoked transient depolarization. Occasionally there was a single depolarization, but more often there were several fluctuations in potential.

3 In spontaneously active cells, vagal stimulation induced a transient increase in amplitude of the slow waves without affecting their frequency.

4 Depolarizing responses could be obtained with a single pulse applied to the vagus nerve, and responses increased in amplitude with number of pulses (up to 16 pulses), and with frequency of stimulation (up to 20 Hz). Depolarization did not give rise to spike discharge.

5 Responses to vagal stimulation were blocked by atropine.

6 In the presence of neostigmine, vagally-mediated depolarization was augmented and abortive spikes were observed in a number of cells.

7 In quiescent cells, repetitive stimulation of the sympathetic stellate ganglion evoked slight hyperpolarization.

8 In spontaneously active cells, sympathetic stimulation evoked attenuation, or temporary cessation of slow wave discharge, with or without hyperpolarization.

9 Sympathetic-induced hyperpolarization and suppression of slow waves were both blocked by propranolol, but unaffected by phentolamine.

10 Electrical changes associated with sympathetic stimulation may be of minor importance in the initiation of relaxation.

Introduction

The electrophysiological effects of stimulating the autonomic nerves supplying the trachea have so far been studied only in species in which the airways are normally devoid of tone and electrically quiescent (e.g. ox, Kirkpatrick, 1981). However, the airways of a number of species, including guinea-pig (Souhrada & Dickey, 1976) and man (Richardson, 1977), exhibit spontaneous fluctuations in tone. Single cells of the trachealis muscle of the guinea-pig also exhibit spontaneous electrical activity which can be altered by bronchoactive agents (McCaig & Souhrada, 1980; Small, 1982; Ahmed *et al.*, 1984). The aim of the present work, therefore, was to examine the electrical responses to autonomic stimulation in the spontaneously active trachea of the guinea-pig. An isolated, extrinsically-innervated preparation of guinea-pig trachea has been described (Blackman & McCaig, 1983); the chief advantage of it is that mechanical responses to stimulation of parasympathetic and sympathetic nerves may be obtained separately in the absence of muscarinic blocking agents or sympatholytic drugs. This is not possible in transmurally stimulated preparations. This extrinsically-innervated preparation has now been modified for use in electrophysiological experiments and I describe here the electrical responses to parasympathetic and sympathetic nerve stimulation recorded in single tracheal smooth muscle cells.

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Methods

Guinea-pigs (male, 250–500 g) were killed by a blow to the head. Both extra- and intrathoracic portions of the trachea were dissected, the nerve supply on the right side (recurrent laryngeal, cervical vagus, cervical sympathetic trunk and stellate ganglion) being carefully maintained, as described previously (Blackman & McCaig, 1983). In order to determine the mechanical responses of the preparation, the trachea was placed horizontally at its *in vivo* length in a tissue bath perfused with Krebs solution (4 ml min^{-1} at 21° or 37°C; composition (mM): Na^+ 127, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 121, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, HCO_3^- 25, glucose 11) and cannulated at both ends. The lumen was filled with Krebs solution, one end was closed and the other attached to a pressure transducer (Statham). Intraluminal pressure was recorded on a pen recorder (Devices). Parasympathetic and sympathetic stimulation evoked increases or decreases, respectively, in intraluminal pressure. The vagus nerve (parasympathetic supply) was stimulated with a suction electrode and the stellate ganglion (sympathetic supply) with a bipolar electrode. Stimuli, as either single pulses or trains of pulses at 1–80 Hz, were delivered from a high-current stimulator (Bell) at 40–80 V and 1 ms duration.

Once mechanical responses had been characterized, the trachea was opened by cutting through the cartilaginous rings opposite the smooth muscle bands and pinned to the base of the chamber. Strips of mucosa overlying the smooth muscle were removed carefully. Single cells were impaled with glass microelectrodes, resistance 60–80 MΩ, filled with 0.5 M KCl. Signals were passed through a unity-gain amplifier (WPI), displayed on an oscilloscope (Tektronix) and monitored on a chart recorder (Gould). Baseline transmembrane potential and slow wave characteristics were monitored, then the vagus nerve or stellate ganglion was stimulated and the response recorded. Impalement of a single cell was maintained where possible until responses to different numbers of pulses or frequencies had been obtained, or until responses in the presence of drugs had stabilized. However, the microelectrode was often dislodged prematurely from the cell, particularly during parasympathetic stimulation at higher frequencies, presumably as a result of movement of the tissue.

The following drugs were used: atropine sulphate (McGraw Ethical Ltd); ethyleneglycol-bis-(aminoethyl ether) N,N-tetra-acetic acid (EGTA, Sigma); neostigmine methylsulphate (Sigma); phentolamine mesylate (Ciba) and (±)-propranolol HCl (ICI). Drugs were added to the reservoir of perfusing solution.

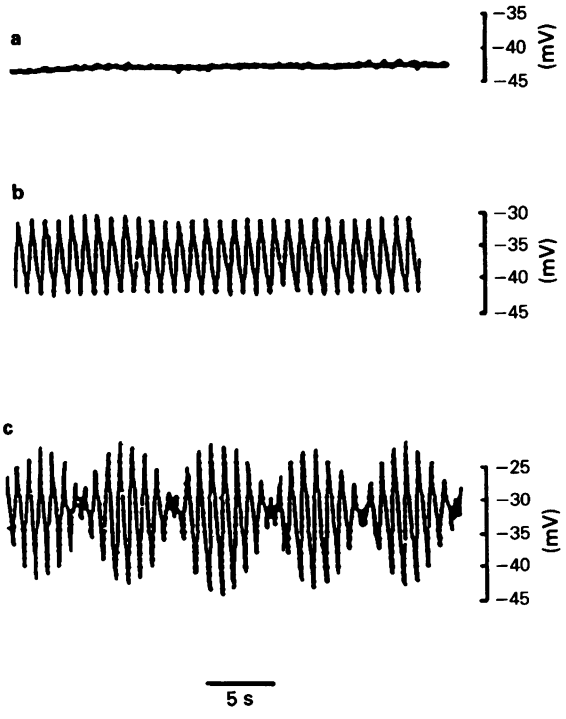

Figure 1 Intracellular recordings of electrical activity in 3 trachealis cells of the guinea-pig. (a) An electrically quiescent cell, (b) a cell exhibiting regular slow waves, (c) a cell exhibiting cyclical variations in slow wave amplitude.

Results

Resting electrical properties of tracheal cells

(a) *Intracellular recordings at 37°C* Some cells were electrically quiescent, as shown in Figure 1a, and had a stable resting membrane potential of −42 ± 0.5 mV (mean ± s.e.mean, 259 cells from 40 preparations). Other cells exhibited spontaneous fluctuations in potential, or slow waves, which were either of fairly regular amplitude (Figure 1b) or varied in a cyclical manner ('waxing and waning', Figure 1c). Spontaneously active cells were encountered in most preparations, in some throughout and in others at one or more times during the experiment. The amplitude of the slow waves varied considerably between cells, ranging from 0.5 to 22 mV (mean 6.8 ± 0.4 mV, 169 cells). Frequency varied very little between cells in any one preparation, but varied between preparations (range 0.4 to 1.4 Hz, mean 0.9 ± 0.02 Hz, 163 cells).

(b) *Effect of cooling the trachea* The effect of cooling the trachea to room temperature (approximately 21°C) was examined in 16 preparations. Cells were depolarized by about 10 mV (mean resting membrane potential -33 ± 0.4 mV, $n = 304$). Slow waves were still observed in a number of cells, although the amplitude was reduced slightly (4.2 ± 0.3 mV, $n = 71$) and the frequency reduced substantially (0.2 ± 0.5 Hz, $n = 52$).

(c) *Effect of exposure to Ca^{2+}-free solution* Trachealis cells gradually depolarized by some 15 mV when exposed to Ca^{2+}-free solution (i.e. Krebs solution from which calcium chloride had been omitted); the mean resting membrane potential was -28 ± 0.6 mV ($n = 68$). There was a gradual reduction in slow wave amplitude and slow waves disappeared completely in 20–60 min. When EGTA, 1mM, was added to the Ca^{2+}-free solution, slow waves were abolished within 10 min. On re-addition of Ca^{2+}, slow wave discharge resumed in an average of 7 min.

(d) *Effect of autonomic drugs on resting properties* Atropine (6×10^{-7} M), propranolol (3.5×10^{-6} M), phentolamine (4×10^{-6} M) and neostigmine (5×10^{-8} M) were each without effect on the resting membrane potential or spontaneous activity of trachealis cells.

Responses to stimulation of the vagus nerve

(a) *Responses recorded at 37°C* Approximately 75% of cells responded to vagal stimulation by depolarizing. Since the extrinsic nerve supply on the right side only was maintained in the preparation, this value was perhaps higher than anticipated. It has been shown in mechanical studies, however, that at least some of the muscle is innervated by nerves arising on both sides, which could account for this (Blackman & McCaig, 1983). Alternatively some of the responses, particularly those of low amplitude, could be due to intercellular spread of excitation rather than direct neuronal input to the cell.

In some quiescent cells stimulation of the vagus nerve with single pulses elicited a transient depolarization. This response occurred after a delay of the order of 300 ms, and reached a maximum in a further 300 ms. The duration of the response at half-maximal amplitude was 477 ± 21 ms ($n = 8$). The amplitude of the depolarization varied from 0.3 to 11 mV and examples of the two extremes of response are shown in Figure 2 a and b. The mean response was 2 ± 0.4 mV ($n = 43$). In the record shown in Figure 2c, the response, typical of that in many cells, consisted of a series of oscillations in potential. In some quiescent preparations, vagal stimulation using a single pulse was followed by a slow wave discharge which persisted

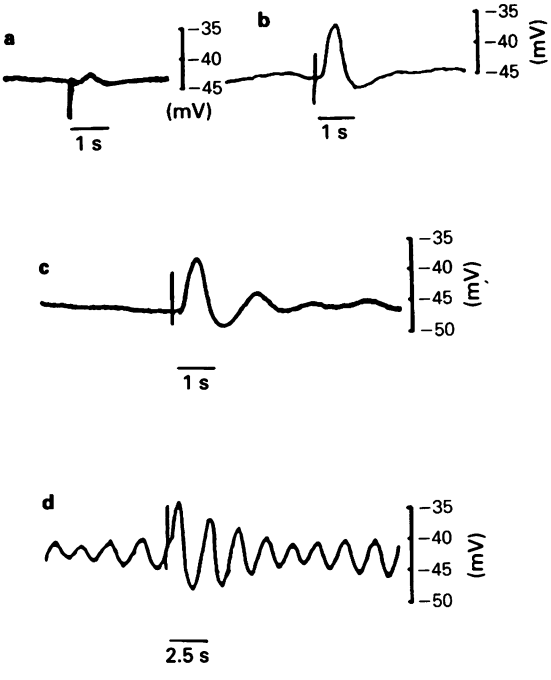

Figure 2 Intracellular recordings of responses from 5 different guinea-pig trachealis cells, evoked by a single stimulus applied to the vagus nerve (stimulus artefact appears as vertical line on record). (a) A cell exhibiting a small depolarizing response, (b) a cell exhibiting a large depolarizing response, (c) a cell showing several fluctuations in potential, (d) a spontaneously active cell in which a single stimulus evoked a transient increase in slow wave amplitude.

indefinitely. In cells which were discharging slow waves spontaneously, a single pulse caused a transient enhancement of slow wave amplitude, as shown in Figure 2d. Frequency of discharge was unaltered. In cells with spontaneous slow waves of large amplitude, vagal stimulation, with a single pulse or a train of pulses, appeared to be without affect.

In many cells there was no response to a single pulse, but responses became evident when 2 or more pulses were applied to the vagus. The amplitude of the depolarizing response increased both with number of pulses and frequency of stimulation (up to 20 Hz), presumably as a result of increasing concentration of neurotransmitter at the postsynaptic membrane. Figure 3a shows results typical of a quiescent cell in which the initial depolarization increased in amplitude as the number of stimuli was increased from 1 to 8 pulses at 10 Hz. With increasing number of pulses there was also a tendency for the induced oscillations

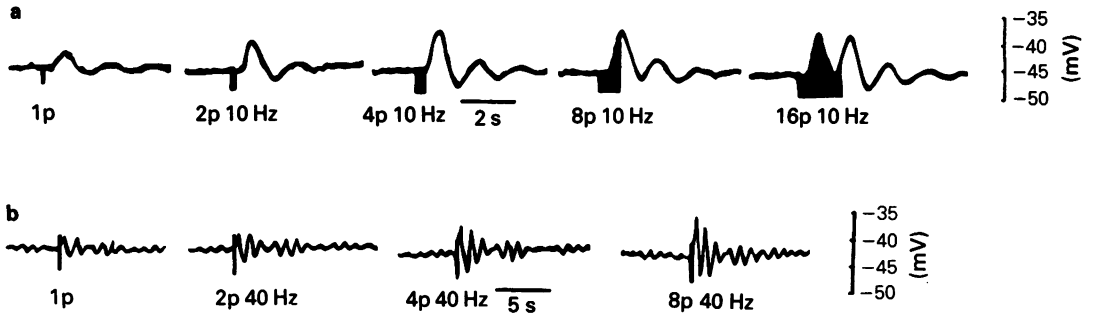

Figure 3 Responses to single and repetitive stimulation of the vagus nerve (stimulus artefacts appear as vertical lines on record), recorded intracellularly in 2 smooth muscle cells of the guinea-pig trachea. (a) A quiescent cell, (b) a cell discharging low amplitude slow waves at rest. Number of pulses (p) and frequency of stimulation as indicated.

to become both larger and more numerous (Figure 3a). From Figure 3b it can be seen that enhancement of slow wave activity in spontaneously active cells increased also with number of pulses. There was no further increase in response when the number of pulses exceeded 16, or occasionally 32, regardless of frequency of stimulation. The largest depolarization recorded in 60 cells varied between 1.5 and 15 mV with a mean of 5.9 ± 0.4 mV. On no occasion did the depolarization give rise to an action potential.

(b) *Effect of cooling the trachea* When recordings were made at room temperature, vagal stimulation still elicited depolarization. In 14 cells studied, the amplitude of the depolarizing response was within the relatively wide range (0.3–11 mV) seen at 37°C. However, the time course of the response was considerably prolonged, depolarization beginning at an average of 680 ms (cf. 300 ms at 37°C) after the application of a single pulse and peaking after a further 750 ms (cf. 300 ms at 37°C).

(c) *Effect of atropine on vagal responses* The effect of atropine (6×10^{-7} M) on vagal responses was examined in 3 separate tracheal preparations. Responses to vagal stimulation in a single quiescent cell before, and 12 min after, the application of atropine, are shown in Figure 4a. The depolarization was blocked completely by atropine. When the response to vagal stimulation consisted of two or more fluctuations in potential, all components of the response were blocked by atropine, as shown in Figure 4b. Electrical responses to vagal stimulation, therefore, seem to be mediated by muscarinic cholinoceptors on the tracheal smooth muscle.

(d) *Effect of neostigmine on vagal responses* The effect of neostigmine on vagally-mediated responses was studied in 5 preparations. Neostigmine (5×10^{-8} M)

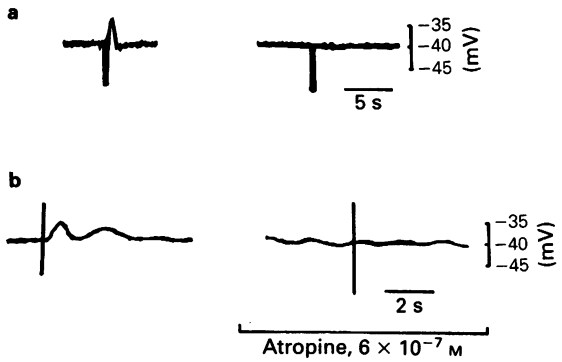

Figure 4 The effect of atropine, 6×10^{-7} M, on responses evoked by vagal stimulation and recorded intracellularly in 2 trachealis cells of the guinea-pig. (a) Responses to 8 pulses at 40 Hz (constant amplitude and duration; stimulus artefacts appear as vertical lines) before and 12 min after the addition of atropine, (b) responses to identical single pulses, before and 12 min after the addition of atropine.

augmented vagal responses, the effect developed over about 20 min and persisted for as long as the recording was continued (up to 2 h), even when the tissue was returned to neostigmine-free solution after 20 min. The amplitude of depolarization induced by a given stimulus was enhanced and maximal depolarization was often elicited by a single pulse. In a number of cells, small spikes were observed at the height of the vagally-induced depolarization, as can be seen in Figure 5a. The number and amplitude of fluctuations in potential evoked by vagal stimulation at increasing frequencies or increased number of pulses were also enhanced in the presence of neostigmine. In control cells, there were typically a few fluctuations which rapidly decreased in amplitude, but in the

presence of neostigmine, there were more fluctuations and their amplitude decreased much more slowly, as shown in Figure 5b. In spontaneously active cells both the increase in slow wave amplitude and the duration of the increase were augmented by neostigmine.

Responses to stimulation of sympathetic nerves

(a) *Responses at 37°C* It is of interest that in many preparations none of the cells impaled responded to sympathetic stimulation, despite the fact that sympathetic-induced decreases in intraluminal pressure had been observed in these tissues before commencing electrical recording. Very few cells responded electrically to sympathetic stimulation at frequencies below 10 Hz, although mechanical inhibitory responses to sympathetic stimulation were already up to 40% of maximum at 5 Hz (for 5 s) in the same preparations.

In those cells responding to sympathetic stimulation a single pulse applied to the sympathetic stellate ganglion did not evoke an electrical response. Responses were obtained, however, with prolonged repetitive stimulation, at, for example, 10 Hz for 5 s. In quiescent cells sympathetic stimulation evoked membrane hyperpolarization, as indicated in Figure 6a. Hyperpolarization began during a 5 s train of stimuli and lasted about 10 s. Responses increased in amplitude with frequency of stimulation up to 40 Hz, where the hyperpolarization ranged from 1–5.5 mV (mean + 3.1 ± 0.3 mV, $n = 16$, see Figure 6a). Increasing the period of stimulation from 5 to 30 s only occasionally evoked a greater hyperpolarization (up to 9 mV).

When cells were spontaneously active, sympathetic stimulation caused a decrease in amplitude or temporary cessation of slow wave discharge, examples of

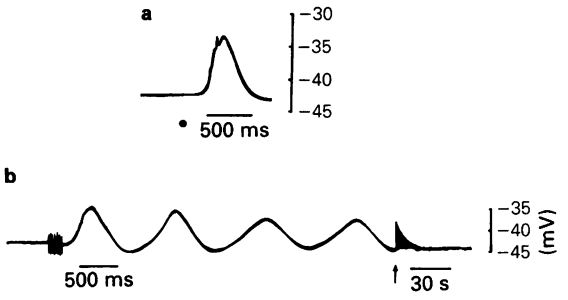

Figure 5 Responses to stimulation of the vagus nerve recorded in 2 guinea-pig trachealis cells in the presence of neostigmine (5×10^{-8} M). (a) A single pulse (indicated by dot) induced a large depolarization with spike component superimposed (recorded 90 min after addition of neostigmine), (b) 8 pulses at 40 Hz (stimulus artefacts appear as vertical lines) induced a series of fluctuations in potential which lasted about 20 s, in a quiescent cell (recorded 60 min after the addition of neostigmine). The portion of the record to the right of the arrow was recorded at a slower speed and shows the gradual attenuation and final cessation of the slow waves elicited by vagal stimulation.

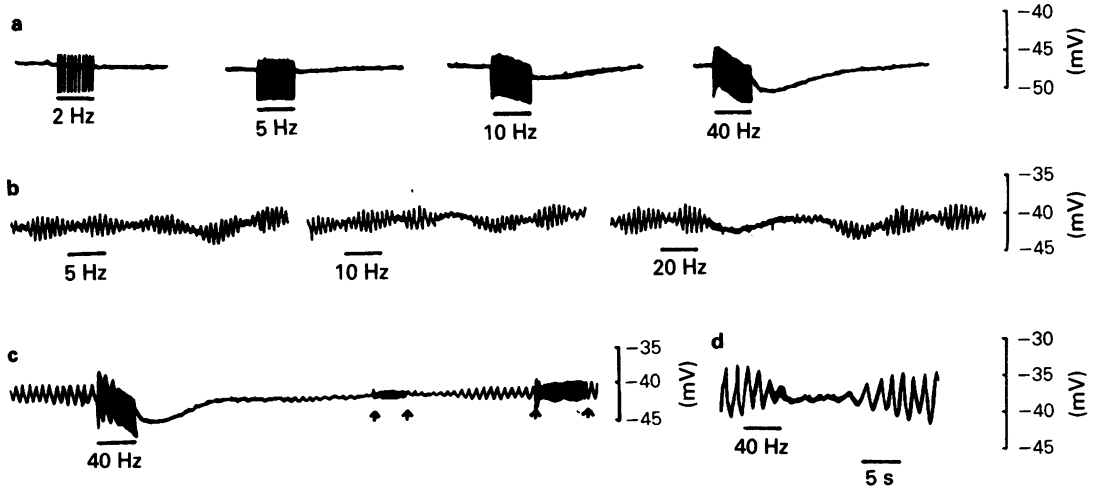

Figure 6 Responses to sympathetic nerve stimulation (for 5 s at frequencies indicated, represented by horizontal lines) recorded intracellularly in 4 trachealis cells of the guinea-pig. (a) Hyperpolarizing response, discernible at 5 Hz and increasing with frequency to a maximum at 40 Hz. (b) Attenuation of slow wave discharge, also increasing with frequency of stimulation. (c) Hyperpolarization and concomitant attenuation of slow waves lasting > 1 min. Note reduction in speed of recording as indicated by 2 sets of arrows, where horizontal calibration represents 25 s. (d) Attenuation of slow waves with no hyperpolarization. Note that in (a) and (c) stimulus artefacts appear as vertical lines on the records, but in (b) and (d) cells were further from the stimulating electrode and no artefact is visible.

which can be seen in Figure 6b–d. In some cells attenuation of slow waves was accompanied by hyperpolarization (Figure 6c) but in others the inhibition of slow waves occurred without hyperpolarization (Figure 6d). The degree of slow wave depression increased with frequency of stimulation (up to 40 Hz) and the duration of the effect also increased with frequency, with mean duration at 5, 10, 20 and 40 Hz of 5.3, 7.6, 9.8 and 10.1 s, respectively. In a few cells, the suppression of slow waves lasted for more than a minute after sympathetic stimulation for 5 s, as shown in Figure 6c.

(b) *Effect of cooling the trachea* Both hyperpolarization and supression of slow waves (with or without hyperpolarization) were observed in a number of cells following sympathetic stimulation at room temperature.

(c) *Effect of propranolol and phentolamine on sympathetic responses* The effect of propranolol on sympathetic responses was studied in 4 experiments. In Figure 7a, it can be seen that sympathetically-induced hyperpolarization was blocked completely in the presence of propranolol (3.5×10^{-6} M). Sympathetic attenuation of slow waves was likewise blocked completely by propranolol (Figure 7b). It is likely, therefore, that both the hyperpolarization and suppression of slow waves are mediated by β-adrenoceptors on the smooth muscle cells.

In 4 experiments it was found that phentolamine (4×10^{-6} M) had no effect on either the hyperpolarization or the attenuation of slow waves associated with sympathetic stimulation.

Discussion

Stimulation of the vagus nerve evoked a transient depolarization in cells of the trachealis muscle of the guinea-pig, which was similar in magnitude to that recorded in airways of other species (ox, Cameron & Kirkpatrick, 1977; dog, Ito & Tajima, 1981; cat, Ito & Takeda, 1982; ferret, Coburn, 1984), and had a relatively slow time-course which is commonly observed in smooth muscle. Depolarization increased in amplitude with number of pulses and frequency of stimulation, but did not give rise to action potential discharge, a finding also consistent with results obtained in other species. In some smooth muscles, including that of the airways, depolarization may be limited by the efflux of K^+ ions from the cell through voltage-dependent K^+-channels (Bolton & Large, 1986). In a recent patch-clamp study of canine trachealis cells such K^+-channels were found to predominate in the cell membrane (McCann & Welsh,

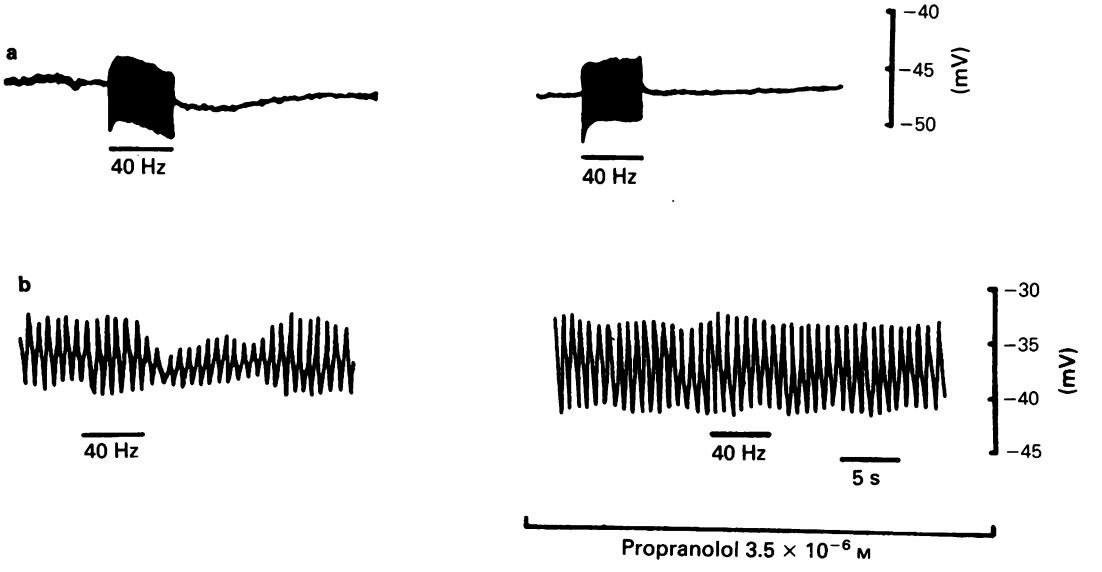

Figure 7 The effect of propranolol, 3.5×10^{-6} M, on sympathetic responses recorded intracellularly in 2 guinea-pig trachealis cells. (a) Hyperpolarizing response in a quiescent cell and (b) attenuation of slow waves in a spontaneously active cell, before, and 10 min after the addition of propranolol. Both types of response were blocked by propranolol. Sympathetic stimulation at 40 Hz for 5 s, indicated by horizontal lines.

1986). When K^+-channels are blocked, for example by tetraethylammonium, action potentials can be elicited in the trachealis muscle (McCaig & Souhrada, 1980; Kirkpatrick, 1981), indicating that the cell membrane has the capacity to undergo regenerative activity, should depolarization reach the necessary threshold. In the present experiments, spiking could be elicited by vagal stimulation during cholinesterase inhibition with neostigmine. This suggests that acetylcholine, if present in a sufficiently high concentration for long enough, can evoke threshold depolarization, even when K^+ efflux is uninhibited. It should be noted, however, that action potentials are not a prerequisite for contraction in airway smooth muscle, and depolarization of only a few millivolts is associated with a significant increase in tension in bovine or feline trachea (Suzuki *et al.*, 1976; Kirkpatrick, 1981; Ito & Itoh, 1984).

The situation in guinea-pig trachea is complicated by the widespread occurrence of spontaneous slow wave discharge. Slow waves have some relationship to the development of tone but the precise nature of the link is not known (Dixon & Small, 1983). Slow waves were not blocked by atropine, indicating that they do not arise from the spontaneous release of acetylcholine (ACh) from cholinergic nerve terminals, but are possibly myogenic. The slow waves are however Ca^{2+}-dependent since they are abolished in Ca^{2+}-free solution (present work) or in the presence of the Ca^{2+}-channel blocker, nifedipine (Ahmed *et al.*, 1985). They could arise from cyclical bursts of opening of Ca^{2+}-channels in the cell membrane. In cells discharging slow waves, vagal stimulation was associated with an increase in amplitude, an effect which would be consistent with the promotion of Ca^{2+}-channel opening by ACh. The frequency of discharge was unaffected, however, indicating that the period of the slow wave cycle is determined by a mechanism which is not susceptible to alteration by ACh. It is likely, therefore, that amplitude and frequency of slow waves are determined by largely independent mechanisms, as has been suggested in some intestinal smooth muscle (Tomita, 1981).

It is possible that depolarization induced by any means triggers cyclical changes in the properties of the cell membrane. Alternatively, there may be cyclical changes in the membrane at rest, even in cells not discharging slow waves, which only become apparent during depolarization. Vagal stimulation, for example, often gave rise to a series of potential oscillations, and these were augmented during cholinesterase inhibition with neostigmine. Transmural stimulation in bovine airways can also elicit fluctuations in potential (Kirkpatrick, 1981). In addition, similar effects have been observed during depolarization induced by bronchoconstrictors (Kirkpatrick, 1981). The question of how spontaneous and neurally-evoked electrical changes interact in the regulation of tone remains to be answered.

The depolarizing response did not increase in amplitude when the number of pulses in a train exceeded around 16 pulses. Mechanical responses, however, measured separately in the same preparations, increased with number of pulses up to the maximum tested (100). Increases in contraction occur, therefore, without concomitant increases in the amplitude of the neurally-evoked depolarization. It is possible that the inward current, carried perhaps by Ca^{2+}, continues to increase but is matched by K^+ efflux. Alternatively, ACh may act through potential-independent mechanisms to promote contraction, especially at higher concentrations (see Bolton & Large, 1986). In keeping with this, it has been demonstrated that the effects of carbachol at low concentrations on guinea-pig trachea can be antagonized by verapamil which is thought to block voltage-operated Ca^{2+}-channels, whereas the effects of high concentrations are resistant to verapamil, suggesting that receptor-operated channel opening predominates at high concentrations (Baba *et al.*, 1985). In canine and feline trachea the contraction associated with endogenous or exogenous ACh is greater than that induced by equivalent direct depolarization, indicating that potential-independent actions of ACh are involved in the contractile response (Farley & Miles, 1977; Ito & Itoh, 1984). The exact contribution of potential-dependent and independent mechanisms to the development and maintenance of smooth muscle tone has not been established (Bolton & Large, 1986), but the role of potential-independent mechanisms may have been underestimated.

Electrical responses to sympathetic stimulation took the form of slight hyperpolarization, inhibition of slow waves or both and repetitive stimulation was necessary to elicit these responses. Hyperpolarization evoked by sympathetic stimulation has been observed previously in cat trachea (Ito & Takeda, 1982). Small α-adrenoceptor-mediated contractions can be elicited in guinea-pig trachea by repetitive stimulation during β-adrenoceptor blockade, but it is not yet clear whether such responses are associated with electrical changes.

Although the electrical responses to sympathetic stimulation were mediated by β-adrenoceptors, as shown by blockade with propranolol, several pieces of information bring into question the role of electrical changes in sympathetic inhibitory responses. Firstly, electrical responses were not seen in a number of preparations which had exhibited normal sympathetic relaxations. Secondly, electrical responses were seen only at frequencies of stimulation in excess of those required to elicit substantial relaxations. Thirdly, electrical responses were unaffected by phentolamine, which augments sympathetic relaxation in this

preparation (McCaig, 1986). It is possible that phentolamine acts by blocking presynaptic α-adrenoceptors, thus removing feedback inhibition of noradrenaline release and increasing the amount of the transmitter reaching the postsynaptic membrane, hence potentiation of electrical responses would be anticipated. It seems likely, therefore, that potential-independent actions, such as alterations in Ca^{2+} availability to the contractile apparatus, play an important role in sympathetic inhibition. It has been shown that isoprenaline hyperpolarizes guinea-pig trachealis (Allen *et al.*, 1985), but that the hyperpolarization is not critical for relaxation. It has still to be established whether electrical effects of sympathetic stimulation play a part in the attenuation of vagally-mediated electrical and/or mechanical responses.

It would be of interest to examine the electrical responses to autonomic nerve stimulation in the trachea of chronically-sensitized guinea-pigs, which have been used as a model for bronchial asthma. The tracheal smooth muscle cells of such animals are depolarized, as compared to normal controls (McCaig & Souhrada, 1980; Souhrada & Souhrada, 1981). This might be explained in terms of a decrease in number, or inactivation of K^+-channels in the membrane. The consequences of this would be a reduced capacity to counteract neural- or mediator-induced depolarization, which could lead to action potential discharge and produce the airway hyperreactivity which characterizes asthma (Nadel, 1983). Similarly, if K^+-channel opening is involved in relaxation, inhibitory mechanisms might be compromised.

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