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Evidence that the spasmogenic action of tetraethylammonium in guinea-pig trachealis is both direct and dependent on the cellular influx of calcium ion

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1 Tetraethylammonium (TEA, 1–8 mmol/l) evoked spasm of guinea-pig trachealis which was unaffected by atropine (1 μmol/l), mepyramine (1 μmol/l) or tetrodotoxin (3 μmol/l).

2 The spasm evoked by TEA was markedly suppressed in Ca^{2+}-free Krebs solution while that evoked by acetylcholine was much less affected.

3 Extracellular electrical recording showed that exposure to Ca^{2+}-free Krebs solution suppressed both spontaneous electrical slow wave activity of the trachea and the spasm and slow waves induced by TEA. These effects were reversible.

4 TEA (2 and 8 mmol/l) increased the lanthanum-resistant calcium fraction of trachea.

5 It is concluded that TEA acts directly on the smooth muscle of guinea-pig trachea, that the spasm and electrical slow waves evoked are Ca^{2+}-dependent and that the cellular influx of Ca^{2+} is increased.

Introduction

In the trachealis muscle of the cow and dog, tetraethylammonium (TEA) evokes spasm associated with depolarization, electrical slow waves and spike activity. These effects have been attributed to the ability of TEA to reduce the potassium conductance of the smooth muscle cells (Kirkpatrick, 1975; Kroeger & Stephens, 1975). That TEA acts directly on canine tracheal smooth muscle is suggested by the resistance of TEA-induced spasm to atropine or tetrodotoxin (Kroeger & Stephens, 1975; Kannan & Daniel, 1978). Since TEA-induced spasm is suppressed in Ca^{2+}-free Krebs solution or Krebs solution containing lanthanum, Kirkpatrick (1975) has suggested that TEA produces contraction of bovine trachealis cells by promoting the influx of Ca^{2+} into the cytoplasm.

It has been previously reported (Small, 1982; Dixon & Small, 1983) that TEA evokes both spasm of guinea-pig trachealis and electrophysiological changes similar to those seen in the trachealis of the cow and dog. In the present study we have used a variety of techniques to analyse further the action of TEA. Tissue bath experiments, electrophysiological recording and measurement of Ca^{2+} fluxes by the lanthanum technique (Van Breeman, Hwang & Siegel, 1977; Palaty, 1977; Weiss, 1981) have generated data that suggest that TEA acts directly on the smooth muscle of guinea-pig trachealis, that the spasm and electrical slow waves evoked are Ca^{2+}-dependent and that the cellular influx of Ca^{2+} is increased.

Methods

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

Tissue bath studies

Small segments of trachea were set up for the isometric recording of tension changes essentially as described by Coburn & Tomita (1973). The initial resting tension imposed on the tissue was 0.5–1.0 g

and the effects of spasmogenic drugs were studied by constructing cumulative concentration-effect curves. The contact time for each concentration of a particular agonist was such as to allow the development of almost all the tension rise attainable by that concentration of agonist. For acetylcholine this was 2 min, for histamine 5 min, for potassium chloride 12 min, and for TEA 5 min.

In the experiments where field stimulation of intramural nerves was carried out, the electrodes described by Clark (1978) were used. Frequency-response curves were constructed using biphasic pulses of supramaximal strength delivered in trains of 10 s duration every 10 min. Pulse frequency was increased in successive trains in twofold steps from 1 Hz to 64 Hz.

Antagonist drugs or Ca^{2+}-free media were allowed to equilibrate with test tissues for at least 40 min before the effects of agonists were retested. Control tissues from the same animal were not exposed to antagonists or Ca^{2+}-free media but otherwise were treated identically to test tissues.

Extracellular electrophysiological recording

Segments of trachea were set up for the extracellular recording of electrical and mechanical activity as previously described (Small, 1982). Measurements of spontaneous or TEA-induced (5 min drug contact) activity were made initially in normal Krebs solution, again after 40 min superfusion with Ca^{2+}-free Krebs solution and finally 40 min after reversion to normal Krebs solution.

Measurement of the lanthanum-resistant calcium fraction

The lanthanum technique has been widely used to estimate the cellular influx of Ca^{2+} induced by drugs. The basis of the technique is that tissue is exposed to ^{45}Ca together with the drug being studied. Subsequent tissue exposure to $LaCl_3$ at 0°C displaces extracellularly-bound ^{45}Ca and greatly reduces ^{45}Ca loss from the cell interior. The ^{45}Ca remaining in the tissue, the lanthanum-resistant calcium fraction, thus provides an estimate of drug-induced calcium influx (for a review of the lanthanum technique see Daniel, Crankshaw & Kwan, 1979).

Measurements of the lanthanum-resistant calcium fraction were carried out in MOPS physiological salt solution (MOPS–PSS) as prepared by Jetley & Weston (1980). Tracheae were opened as described above and then pinned out, mucosal surface uppermost, on a block of paraffin wax under MOPS–PSS.

That segment of tracheal wall lying between the natural tips of the cartilage arcs was excised by means of two cuts in the longitudinal axis of the organ. Preparation of a tissue strip in this way ensured a maximal content of smooth muscle relative to other material.

The muscle-containing tissue strip was cut into 3 pieces, one piece acting as control, and the other two as test pieces. Control tissues were mounted on a stainless steel hook and allowed to equilibrate in MOPS-PSS for 75 min at 37.5°C. The two groups of test tissues were treated similarly. Each group of tissues was then exposed to $^{45}Ca^{2+}$, 500 nCi/ml, for 5 min to allow the $^{45}Ca^{2+}$ to equilibrate with the extracellular space of the tissue. Then, to each tissue group was added either vehicle or sufficient TEA solution to achieve a TEA concentration of either 2 mmol/l or 8 mmol/l. Five minutes later tissues were removed from these media and placed in ice-cold oxygenated MOPS-PSS containing $LaCl_3$ 10 mmol/l; 20 min later tissues were transferred to an identical solution for a further 40 min. Tissues were then blotted, weighed and solubilized in Soluene 350 (Packard). The $^{45}Ca^{2+}$ content of tissues and media was determined by scintillation counting. The tissue $^{45}Ca^{2+}$ content was converted to a tissue:medium ratio (nCi of $^{45}Ca^{2+}$ per g of tissue/nCi of $^{45}Ca^{2+}$ per ml of incubation medium).

In a separate series of experiments a tissue strip was used which was prepared from a section of tracheal wall diametrically opposite the trachealis muscle.

Drugs and solutions/statistical analysis of results

Drug concentrations are throughout expressed in terms of the molar concentration of the base. The following drugs were used: acetylcholine chloride (BDH), atropine sulphate (Sigma), histamine acid phosphate (BDH), mepyramine maleate (M & B), potassium chloride (Hopkins & Williams), sodium nitrate (Sigma), tetraethylammonium bromide (Sigma) and tetrodotoxin (Sigma). A stock solution of acetylcholine was prepared in absolute ethanol. Stock solutions of other drugs were prepared in double distilled water.

$^{45}Ca^{2+}$ was supplied as an aqueous solution of $CaCl_2$ by the Radiochemical Centre, Amersham. The specific activity of the material was 2 mCi/170 μg Ca^{2+}.

Most of the tissue bath and electrophysiological studies were carried out in Krebs solution as described by Small (1982). Ca^{2+}-free Krebs solution was prepared by omitting $CaCl_2$ from the formulation.

The significance of differences between means was assessed using a two-tailed, unpaired *t* test.

Table 1 Failure of atropine or mepyramine (each 1 μmol/l) to antagonize tetraethylammonium (TEA) in guinea-pig trachealis

Antagonist	*Agonist*	*LDR* ± s.e. mean		
		With Antagonist	*Time-matched control*	*Net shift due to antagonist*
Atropine	Acetylcholine	2.24 ± 0.19	−0.29 ± 0.12	2.53 ± 0.22
	TEA	0.08 ± 0.05	0.01 ± 0.05	0.07 ± 0.07
Mepyramine	Histamine	>1.28 ± 0.16	−0.07 ± 0.12	>1.35 ± 0.20
	TEA	0.06 ± 0.08	−0.08 ± 0.08	0.14 ± 0.14

Data represent $\log_{10}$ dose-ratio for the indicated agonist ± s.e.mean ($n = 7$); a negative sign signifies a leftward shift. > indicates that in tissues where profound antagonism was seen, the log dose-ratio could only be observed to exceed a limiting value set by our ability to increment histamine concentration: the s.e.mean is necessarily then imprecise.

Results

Tissue bath studies

Acetylcholine (1.0–1000 μmol/l) and histamine (0.1–100 μmol/l) each evoked tonic (smoothly developing) spasm of the trachealis. The spasm evoked by histamine developed with a slower time course than that produced by acetylcholine but the slopes of the central portions of the log concentration–effect curves of the two agonists were similar.

The spasm evoked by TEA (1–8 mmol/l) often developed in a phasic manner, particularly at the higher concentrations tested. The slope of the log concentration–effect curve for TEA was more than twice that of acetylcholine or histamine.

While atropine (1 μmol/l) caused profound antagonism of the action of acetylcholine, it did not

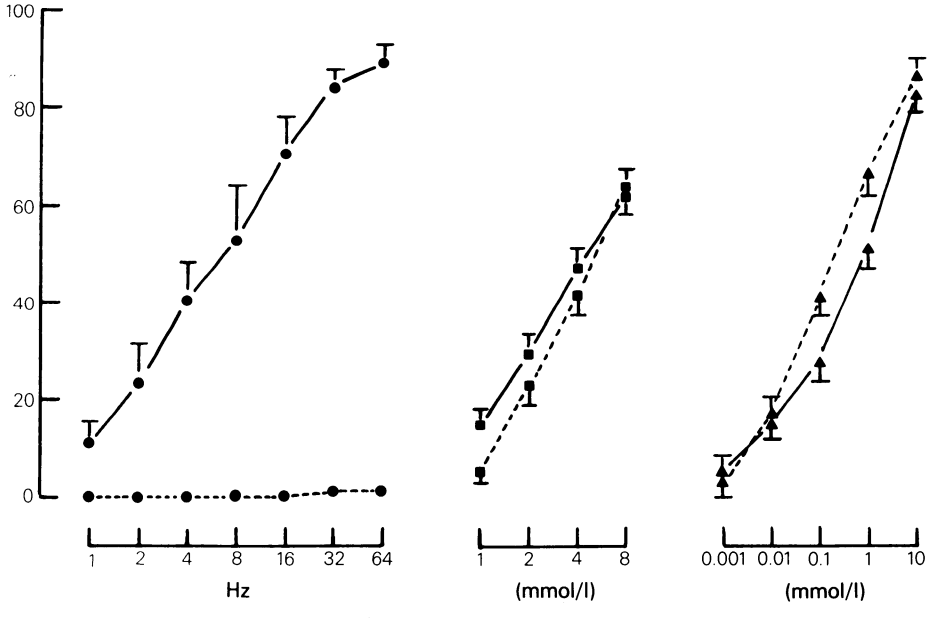

Figure 1 The effects of tetrodotoxin (3 μmol/l) on the relaxant response of guinea-pig trachea to field stimulation and the spasmogenic responses of the tissue to tetraethylammonium (TEA) and acetylcholine. The abscissa scale represents the pulse frequency (Hz) of field stimulation (●) or the concentration of TEA (■) or acetylcholine (▲) (mmol/l) on a log scale. The ordinate scale represents the response as a % of the maximal relaxation evoked by sodium nitrite (field stimulation) or as a % of the initial maximal contraction evoked by acetylcholine (TEA and acetylcholine). Data represent the means of values from 9 tissues; s.e. shown by vertical lines. Solid lines indicate responses obtained in normal Krebs solution and broken lines those obtained in the presence of tetrodotoxin.

modify the action of TEA tested on the same tissue. Similarly, mepyramine (1 μmol/l) profoundly antagonized the action of histamine but did not affect TEA tested on the same tissue (Table 1).

The response of the trachea to field stimulation was generally biphasic. The initial phase of the response comprised a spasm of relatively rapid rise and fall. The secondary phase of the response was a relaxation which developed and waned less rapidly than the spasmogenic response. The spasmogenic and relaxant phases of the response to field stimulation were both frequency-dependent. Spasmogenic responses to field stimulation were less reproducible than relaxant responses, particularly at low frequencies of stimulation.

Tetrodotoxin (3 μmol/l) virtually abolished both the spasmogenic and (Figure 1) the relaxant responses to field stimulation. In contrast, the spasmogenic effects of TEA and acetylcholine were unaffected by tetrodotoxin. The small changes in the positions of the log concentration–effect curves for TEA and acetylcholine apparent in Figure 1 were also observed in control tissues and therefore were not attributable to an action of tetrodotoxin.

In studies where the effects of Ca^{2+}-free Krebs solution were examined, control experiments run at the same time showed that the shape and position of the log concentration–effect curve for TEA changed little, if at all, when TEA was retested following 40 min periods of incubation in normal Krebs solution (Figure 2).

Exposure of test tissues to Ca^{2+}-free Krebs solution evoked a loss of tone. Tone loss was complete by the time of the 80 min agonist challenge. Exposure of test tissues to the Ca^{2+}-free medium for 40 min caused a marked depression of the log concentration–effect curve for TEA. Little further depression of the curve occurred if a further 40 min treatment with Ca^{2+}-free Krebs solution was employed (Figure 3). The log concentration–effect curve for acetylcholine also underwent some depression and a shift to the right (Figure 3). However, these effects were less marked than for TEA and in part may have been attributable to the decrease in tissue sensitivity evident in the concurrent controls (Figure 2).

A series of tissue bath experiments was performed to check whether the medium used in the radioisotope studies (MOPS-PSS) had any effect on the sensitivity of the trachea to agonist drugs. The sensitivity of the trachea to potassium chloride, TEA and acetylcholine was little affected by MOPS-PSS. In these experiments tracheal contractility was assessed in terms of the maximal spasm evoked by acetylcholine. In MOPS-PSS the mean maximal response to acetylcholine ± s.e. was 1.55 ± 0.07 g and in nor-

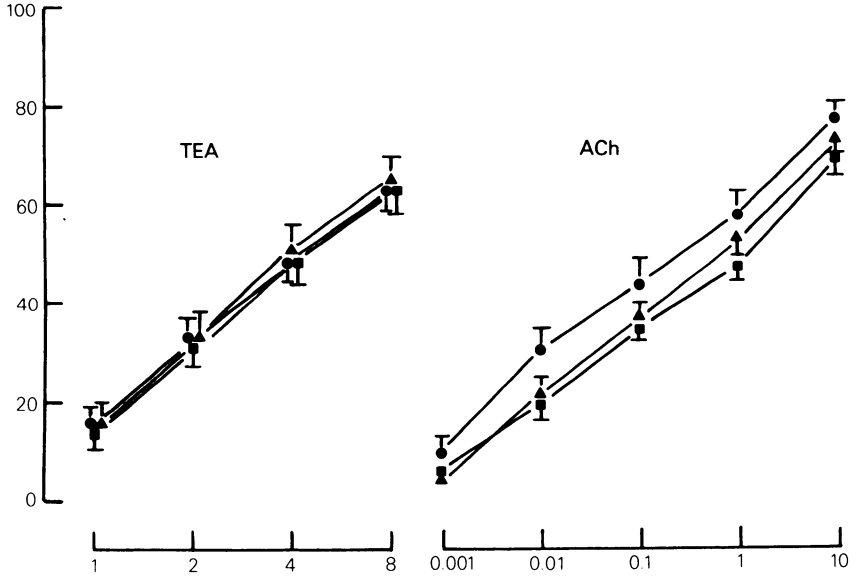

Figure 2 The effects of incubation in normal Krebs solution on the spasmogenic responses of guinea-pig trachealis to tetraethylammonium (TEA) and acetylcholine (ACh). The abscissa scale represents the concentration of drug (mmol/) on a log scale. The ordinate scale represents the response as a % of the initial maximal response to acetylcholine. (●) Initial log concentration–effect curve; (■) concentration–effect curve obtained after 40 min incubation in normal Krebs solution; (▲) concentration–effect curve obtained after a further 40 min incubation in normal Krebs solution. Data represent the means of values from 12 tissues; s.e. shown by vertical lines.

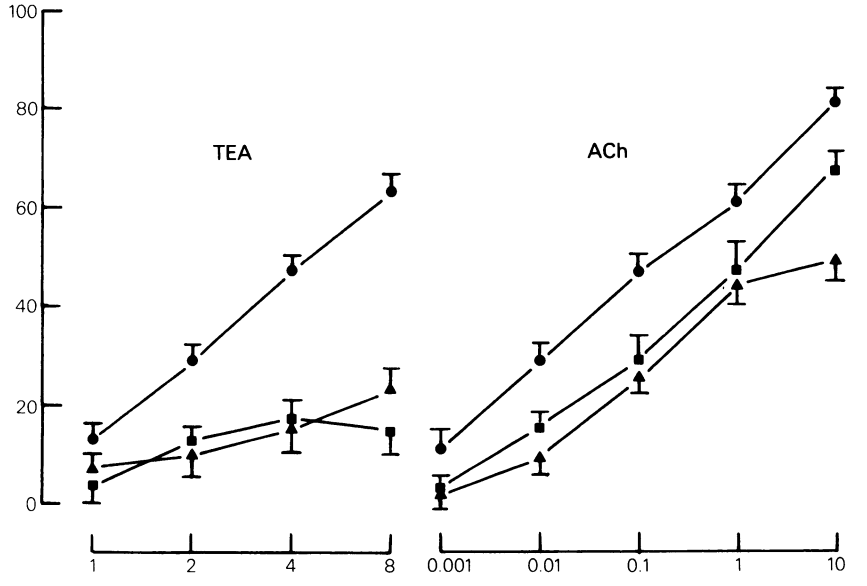

Figure 3 The effects of Ca^{2+}-free Krebs solution on the spasmogenic responses of guinea-pig trachealis to tetraethylammonium (TEA) and acetylcholine (ACh). The abscissa scale represents the concentration of drug (mmol/l) on a log scale. The ordinate scale represents the response as a % of the initial maximal response to acetylcholine. (●) Initial concentration–effect curve obtained in normal Krebs solution; (■) concentration–effect curve constructed after 40 min exposure to Ca^{2+}-free Krebs solution; (▲) concentration–effect curve constructed after a further 40 min exposure to Ca^{2+}-free Krebs solution. Data represent the means of values from 12 tissues; s.e. shown by vertical lines.

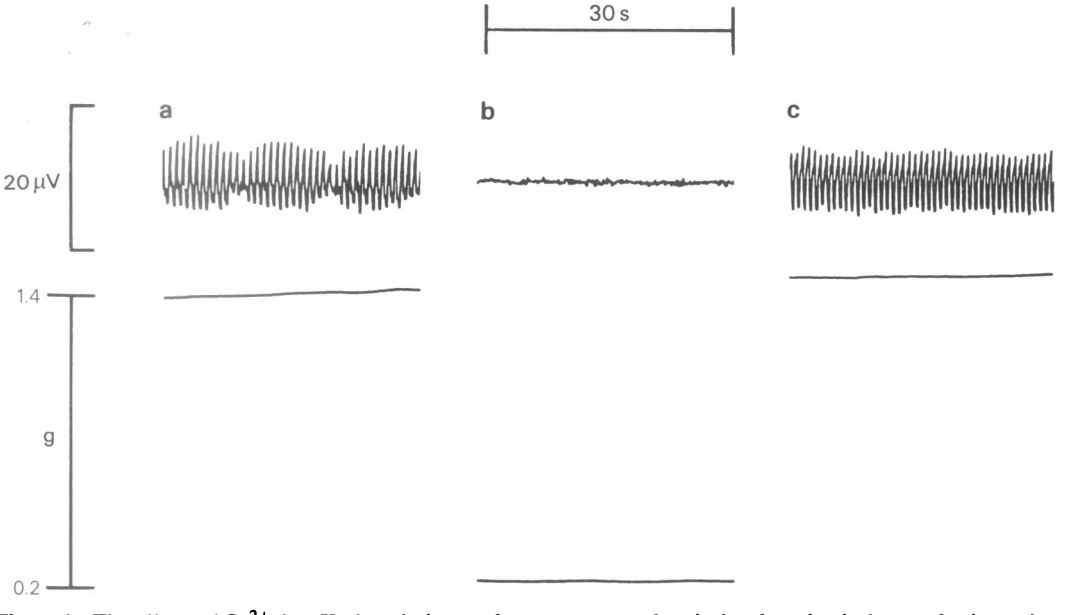

Figure 4 The effects of Ca^{2+}-free Krebs solution on the spontaneous electrical and mechanical tone of guinea-pig trachealis. All records are from the same preparation. In each case the upper trace represents extracellularly-recorded electrical activity and the lower trace the tension developed. (a) Initial recordings in normal Krebs solution; (b) recordings made after 40 min superfusion with Ca^{2+}-free Krebs solution; (c) recordings made 10 min after returning the tissue to normal Krebs solution.

mal Krebs solution the corresponding value was 1.35 ± 0.09 g. There was no significant difference between these means.

Extracellular electrophysiological recording

The effects of Ca^{2+}-free Krebs solution were examined in six tissues exhibiting spontaneous slow waves and mechanical tone. The Ca^{2+}-free medium abolished slow wave activity in all six tissues following a contact time of 19.1 ± 1.7 min (mean $\pm$ s.e.).

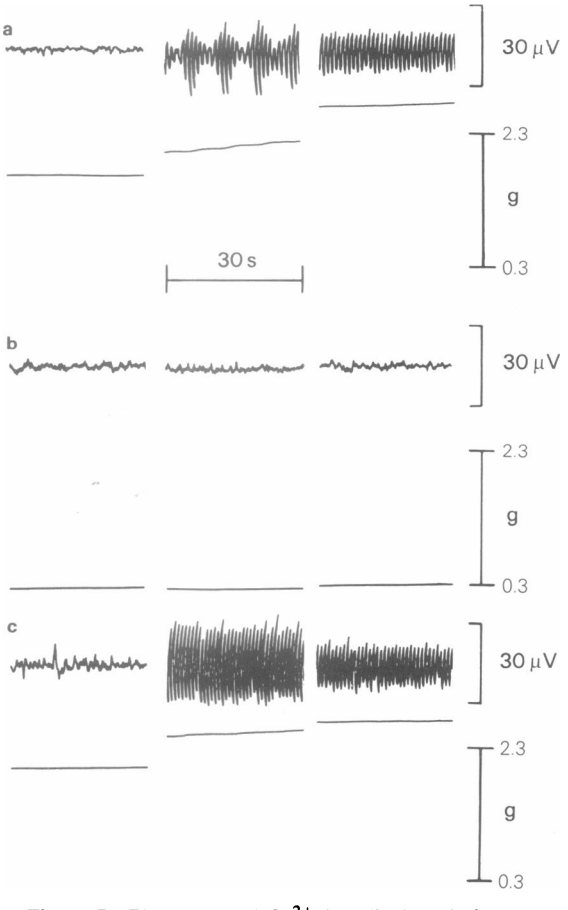

Figure 5 The effects of Ca^{2+}-free Krebs solution on the electrical and mechanical responses of guinea-pig trachealis to tetraethylammonium (TEA) 2 mmol/l. All records are from the same preparation. In each case the upper trace represents extracellularly-recorded electrical activity and the lower trace the tension developed. (a) Initial recordings in normal Krebs solution; (b) recordings made after 40 min superfusion with Ca^{2+}-free Krebs solution; (c) recordings made 40 min after returning the tissue to normal Krebs solution. In each case records are shown of activity prior to TEA and 1.5 and 5 min after exposure to TEA.

Mechanical tone started to decline before the abolition of slow waves and continued thereafter. Following 40 min exposure to Ca^{2+}-free Krebs solution, tone loss was 997 ± 87 mg (mean $\pm$ s.e.). Restoration of normal Krebs solution evoked full recovery of tone and slow wave activity in some tissues (Figure 4) but only partial recovery in others.

Tissues challenged with TEA 2 mmol/l responded by developing tension and generating slow waves. Often these slow waves were of greatest amplitude shortly after the onset of the action of TEA. Thereafter their amplitude declined somewhat (Figure 5).

Following washout of the TEA and the start of superfusion with Ca^{2+}-free Krebs solution, a marked loss of tissue tone occurred. Slow wave activity eventually ceased. When the TEA challenge was repeated following 40 min superfusion with the Ca^{2+}-free medium, the drug often failed to trigger any electrical activity or tension development.

Following washout of the TEA and a return to superfusion with normal Krebs solution, tissue tone increased and spontaneous slow wave activity restarted. Following 40 min superfusion with normal Krebs solution, the electrical and mechanical responses of the tissue to TEA were fully restored (Figure 5 and Table 2).

Measurement of the lanthanum-resistant calcium fraction

Values of the lanthanum-resistant calcium fraction of trachea are presented in Table 3. Both concentrations of TEA examined caused a significant ($P < 0.05$) increase in the lanthanum-resistant calcium fraction of the muscle containing tissue strips.

The lanthanum-resistant calcium fraction of tissue taken from the tracheal wall diametrically opposite the trachealis muscle was greater than that of the muscle-containing tissue. TEA 8 mmol/l failed to cause any significant change in the lanthanum-resistant calcium fraction of tissues devoid of trachealis muscle (Table 3).

Discussion

The biphasic response of guinea-pig trachealis to field stimulation results from the activation of cholinergic excitatory neurones and two types of inhibitory neurones (Coburn & Tomita, 1973; Clark, 1978). Since the concentration of tetrodotoxin used in the present experiments virtually abolished such responses yet left the spasm evoked by TEA or acetylcholine unaffected, we conclude that the actions of TEA and acetylcholine do not require the discharge of Na^+-dependent nerve action potentials.

A concentration of atropine causing profound antagonism of the action of acetylcholine did not sig-

Table 2 The influence of Ca^{2+}-free Krebs solution on electrical and mechanical responses of guinea-pig trachealis to tetraethylammonium 2 mmol/l

	Initial value in normal Krebs solution	*40 min incubation in Ca^{2+}-free Krebs solution*	*40 min after restoration of normal Krebs solution*
Tension evoked as % of initial value	100	5.03 ± 3.5	112.2 ± 6.9
Maximal amplitude of slow waves (μV)	24 ± 3	0	30 ± 5
Slow wave frequency (Hz)	1.23 ± 0.07	0	1.43 ± 0.09

Data represent means ± s.e. of values from tissues from six animals.

nificantly modify the action of TEA. Similarly, a concentration of mepyramine effective against histamine did not antagonize TEA. These observations suggest that the action of TEA did not involve the release of acetylcholine or histamine as mediators. While we have not employed inhibitors or antagonists to test for the release of other substances such as prostaglandins or slow reacting substance of anaphylaxis (SRS-A), the reproducibility of the TEA log concentration–effect curve (Figure 2) argues against the involvement of mediator substances. An agent acting by releasing a mediator might be expected to exhibit some tachyphylaxis. This was not the case with TEA and all our observations are consistent with the drug acting directly on the smooth muscle cells.

Cerrina, Renier, Floch, Duroux & Advenier (1982) observed that TEA-induced spasm of guinea-pig trachealis is markedly suppressed in a Ca^{2+}-free medium. In contrast the spasm evoked by acetylcholine is relatively resistant to such treatment (Creese & Denborough, 1981; Cerrina *et al.*, 1982). The results of our tissue bath experiments confirm these earlier findings. The differential sensitivity of TEA- and acetylcholine-induced spasm to Ca^{2+}-free media may suggest that these two drugs use different sources of Ca^{2+} in order to activate the contractile proteins. That TEA and acetylcholine cause tracheal contraction by differing mechanisms is also suggested by the observation (Small, 1982) that co-ordinated electrical slow waves accompany the spasm evoked by TEA but are not necessary for spasm evoked by acetylcholine.

Changes in the lanthanum-resistance calcium fraction provide an estimate of changes in transmembrane calcium flux (Daniel *et al.*, 1979). The fact that TEA increased the lanthanum-resistant calcium fraction of muscle-containing strips of trachea is indicative of the cellular influx of Ca^{2+} during the action of TEA.

Table 3 The effects of tetraethylammonium (TEA) on the lanthanum-resistant calcium fraction of guinea-pig trachea

	Control	*TEA* 2 mmol/l	*TEA* 8 mmol/l
Muscle containing tissue	0.083 ± 0.009	0.142 ± 0.020	0.148 ± 0.015
Tissue devoid of trachealis muscle	0.326 ± 0.077		0.236 ± 0.049

Data indicate mean tissue : medium ratio (ml/g) for $^{45}Ca^{2+}$ ± s.e. ($n = 6$).

TEA 8 mmol/l did not significantly change the lanthanum-resistant calcium fraction of tissue taken from a point diametrically opposite the trachealis muscle. Here the tracheal wall is devoid of trachealis muscle but contains mucosa, connective tissue and cartilage (Dixon & Small, unpublished observations). We can assume therefore that the influx of Ca^{2+} observed in the muscle-containing strips involved the smooth muscle cells rather than mucosa, connective tissue and cartilage. This assumption is supported by the fact that the rise in the lanthanum-resistant calcium fraction was stimulated by concentrations and contact times of TEA shown to evoke spasm of the trachealis muscle.

Mechanical responses to TEA exhibited a clear concentration-dependency. In view of this it is interesting to note that the two concentrations of TEA used in the lanthanum experiments yielded values of the lanthanum-resistant calcium fraction which did not significantly differ (Table 3). An explanation for this is not easy to provide, but one possibility is that low concentrations of TEA may act largely to promote Ca^{2+} influx while higher concentrations might additionally liberate Ca^{2+} from intracellular stores. The intracellular release of Ca^{2+} would not have been detectable by the lanthanum technique.

Our observation that the lanthanum-resistant calcium fraction of tissue containing the trachealis muscle was lower than that of tracheal wall diametrically opposite the muscle was not anticipated. The difference almost certainly reflects differences in the cartilage content of the two tissues, though why cartilage should bind Ca^{2+} in a lanthanum-resistant manner we cannot yet explain.

Very little is currently known of the ionic basis of the spontaneous slow waves of guinea-pig trachealis muscle. However, two pieces of evidence now suggest that these slow waves are Ca^{2+}-dependent. The slow waves can be abolished by the Ca^{2+} antagonist D600 (Small, 1982) or by treatment with Ca^{2+}-free media (present study). To equate this Ca^{2+}-dependency with the cellular influx of Ca^{2+} during a slow wave may be premature for several reasons. Firstly, it is possible that Ca^{2+}-dependent processes have a permissive rather than a direct role in the production of a slow wave. Secondly, the experimental observations of the Ca^{2+}-dependency of the slow waves were both made using an extracellular recording technique (where it is possible that the disappearance of recorded slow waves might represent a desynchronization of their discharge rather than their abolition in individual cells). Thirdly, the dependency of the slow waves on the extracellular concentration of other ions (e.g. Na^{+}) has not yet been examined.

The present experiments have shown that, like spontaneous slow waves, TEA-promoted waves are abolished in Ca^{2+}-free Krebs solution. This suggests that similar mechanisms underlie the drug-induced and spontaneous waves. If the rising phase of the slow waves is due to the influx of Ca^{2+} then the observed increase in the lanthanum-resistant Ca^{2+} fraction may partly be the result of slow wave promotion by TEA. However, TEA not only induces slow waves but also an overall depolarization of the smooth muscle cells (Small, 1982; Dixon & Small, 1983). It is possible, therefore, that Ca^{2+} influx is additionally increased by the membrane permeability changes which underlie the fall in resting membrane potential or by voltage-dependent Ca^{2+} channels opened by the overall depolarization.

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