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Tetrodotoxin-sensitive, K^+-induced relaxation of guinea-pig isolated trachealis in the presence of Ca^{2+}-entry blocking drugs, Ca^{2+}-free solution and after polyamine exposure

Elliott W. Chideckel, Jeffrey S. Fedan* & Pamela Mike

Department of Medicine, West Virginia University Morgantown, West Virginia, 26506 and Division of Respiratory Disease Studies*, National Institute for Occupational Safety and Health, Morgantown, West Virginia, 26505, U.S.A.

1 We have previously observed a paradoxical relaxant effect of K^+ on guinea-pig isolated trachealis after exposure to polyamines. The purpose of the present study was to evaluate whether the relaxation involved a reduction in the entry of extracellular Ca^{2+}. We therefore investigated the effect of K^+ in the presence of Ca^{2+}-entry blocking drugs and in the presence of Ca^{2+}-free solution.

2 In the presence of nifedipine (10^{-5} M), verapamil (10^{-5} M) or diltiazem (10^{-5} M), K^+ (30 mM) induced relaxation of the trachealis muscle. The relaxation to K^+ was not blocked by ouabain (10^{-6} M), propranolol (10^{-6} M), or indomethacin (10^{-6} M).

3 A relaxation in response to K^+ was also observed in Ca^{2+}-free solution, (with tone induced by methacholine), an effect not blocked by propranolol or ouabain.

4 Tetraethylammonium (30 mM) (TEA), which ordinarily evokes contractile responses, induced trachealis relaxation in the presence of verapamil or nifedipine. The relaxation was unaltered by ouabain or propranolol.

5 Tetrodotoxin (10^{-6} M) (TTX) blocked 65% of the K^+-induced relaxation in the presence of nifedipine and 100% of K^+-induced relaxation either in a Ca^{2+}-free solution or after polyamine exposure. TTX was without effect on TEA-induced relaxation after Ca^{2+}-entry blocking drugs.

6 Atropine (10^{-6} M) or hexamethonium (10^{-6} M) did not affect K^+-induced relaxation after polyamine exposure.

7 The concentration-response curve for K^+-induced contraction in normal modified Krebs-Henseleit solution was shifted to the left by TTX.

8 It is concluded: (a) K^+ has a direct effect on the trachealis causing contraction and an indirect effect, mediated by neurotransmitter release, causing relaxation. This latter effect is exposed when the direct effect is inhibited by Ca^{2+}-entry blocking drugs, Ca^{2+}-free solution or polyamine exposure; the indirect effect is non-adrenergic, non-cholinergic and not via ganglionic transmission; (b) the TEA-induced relaxation and a component of the K^+-induced relaxation after Ca^{2+} blocking drugs cannot be explained by neurotransmitter release; (c) polyamines may act as naturally occurring Ca^{2+} antagonists.

Introduction

During an investigation of the relaxant effects of polyamines on guinea-pig respiratory tract smooth muscle, we observed an unusual interaction between these compounds and K^+ (Chideckel *et al.*, 1985a). If isolated guinea-pig trachealis were exposed transiently to the polyamine, spermidine, then K^+ (30 mM) induced a relaxation. This response was shown not to be secondary to stimulation of the Na^+, K^+-pump or β-adrenoceptor, or to the production of cyclo-oxygenase products. Ordinarily K^+ causes contraction of the tissue. This paradoxical relaxation to K^+ does not appear to be consistent with a mechanism involving

membrane depolarization, the opening of voltage-sensitive Ca^{2+} channels, and an increase in Ca^{2+} permeability.

Polyamines have been reported to produce a number of effects related to membrane Ca^{2+} fluxes and cellular Ca^{2+} handling. In heart and kidney slices, putrescine induces a rapid influx of $^{45}Ca^{2+}$ across the cell membrane and an efflux of $^{45}Ca^{2+}$ from mitochondria (Koenig *et al.*, 1983; Fan *et al.*, 1985). The result is a net increase in cytosolic Ca^{2+}. In the uterus, spermine inhibits spontaneous contraction (Hashimoto *et al.*, 1973), an effect which can be counteracted by increasing the external Ca^{2+} concentration (Hashimoto *et al.*, 1973), suggesting an apparent antagonism between spermine and Ca^{2+}. In smooth muscle of the gut (Demeis, 1967), uterus (Hashimoto *et al.*, 1973), respiratory tract (Chideckel *et al.*, 1985a) and vasculature (Chideckel *et al.*, 1985b), spermine and spermidine have a relaxant effect. It seems possible that polyamines cause relaxation by decreasing the entry of extracellular Ca^{2+}, perhaps in a manner analagous to that for the organic Ca^{2+}-entry blocking drugs, nifedipine, verapamil and diltiazem. If so, the effect of the polyamines and the Ca^{2+}-entry blockers on guinea-pig smooth muscles may be similar, in so far as responses to K^+ are concerned. Likewise, information on the manner in which polyamines cause relaxation of the tissue in response to K^+ could be obtained from a comparison between the effects of Ca^{2+}-entry blockers, Ca^{2+}-free solutions and polyamines on the K^+-induced relaxation response. It was to compare the effects of polyamines and Ca^{2+}-entry blockers and to determine the mechanism of the K^+-induced relaxation that the series of studies described in this paper were initiated.

Methods

Experiments were performed on guinea-pig isolated trachealis. Male guinea-pigs (500 g average weight; Hilltop Laboratory Animals, Scottsdale, PA, U.S.A.) were killed by cervical dislocation. For the trachealis muscle preparations, the trachea was removed, cleaned and slit along the long axis opposite the muscle. Units consisting of two muscle-cartilage segments were prepared, and ligatures were tied to the cartilage at each end. The tissues were mounted in water-jacketed (37°C) organ chambers containing modified Krebs-Henseleit (MKH) solution containing (mM): NaCl 113, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 5.7, gassed with 95% O_2:5% CO_2. Resting load was adjusted to the optimum level of 1 g with the muscle attached to a force-displacement transducer for the measurement of isometric tension responses. The tissues were equilibrated in MKH under resting tension for 60 min. KCl (100 mM) was then added to induce a reference contraction in order to test the ability of each tissue to contract before exposure to other agents. The tissues were then washed with MKH solution three times, over a 1 h period, before other procedures were started. Different tissues in each animal were employed for control and experimental treatments.

Three types of studies were performed. The timing and concentrations of the various agents employed were as follows:

Ca^{2+}-entry blocking drugs

A number of experiments were performed: (1) seventy minutes after a Ca^{2+}-entry blocking drug (10^{-6} M) was added to the baths, KCl was introduced. Ouabain (10^{-6} M) or propranolol hydrochloride (10^{-6} M) was added 10 min after the Ca^{2+}-entry blocker; when employed, tetrodotoxin (10^{-6} M) or atropine sulphate (10^{-6} M) was added 40 min after the Ca^{2+}-entry blocker. (2) Indomethacin (10^{-6} M) was introduced to the baths; immediately thereafter, methacholine chloride (3×10^{-6} M) was added to restore tone. Upon stabilization (15 min), nifedipine was added and 30 min later, KCl. In this latter experiment, the control tissues were treated similarly, except that 0.1% ethanol, the vehicle for indomethacin, replaced the indomethacin. (3) When responses to tetraethylammonium hydroxide (TEA) were elicited, TEA (30 mM) was added 45 min after the Ca^{2+}-entry blocker; ouabain and propranolol (together) or tetrodotoxin were added 10 min after the Ca^{2+}-entry blockers.

Studies with polyamines

The tissue was exposed to spermidine chloride (20 mM) for 45 min and then washed with polyamine-free MKH solution three times over 20 min; KCl was then added. Atropine sulphate (10^{-6} M), hexamethonium bromide (10^{-6} M), or tetrodotoxin (10^{-6} M) were added during the last two washes and remained present when KCl was added.

Studies with Ca^{2+}-free MKH

Thirty min after substituting Ca^{2+}-free MKH solution for the MKH solution, methacholine (3×10^{-6} M) was added to the baths. Fifteen min later (when a stable baseline was present) KCl was added. Tetrodotoxin or propranolol and ouabain in combination were added 10 min after the Ca^{2+}-free solution.

Concentration-response studies for the relaxant effect of K^+ were not cumulative. Each tissue was exposed to only one concentration of K^+ and the concentration-response curves were obtained by pooling the responses to a given concentration of K^+ obtained with the tissues from several animals. The

reason cumulative responses were not examined in detail was that in pilot studies, relaxation responses to lower and gradually increasing concentrations of K^+, diminished subsequent responses. The studies with nifedipine were carried out in a darkened room.

In each type of experiment (except the concentration-responses to K^+ in MKH with and without TTX), the contact time with K^+ before measurement of a response was dictated by the time to peak relaxation in the treatment group. This contact time was then constant for all control and treatment studies in that type of experiment. These times may be derived from Figures 1 and 3.

Except for the concentration-response studies, each experiment was performed in duplicate or triplicate in each animal. The number of animals (n) in each experiment was 4 to 6. The results are expressed as means ± s.e.mean. The data were evaluated for differences using Student's (two-tailed) t test for paired samples. Drugs used were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

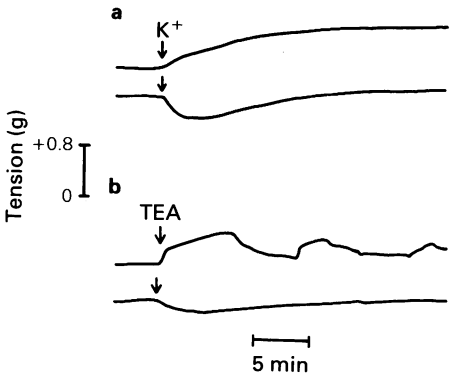

Figure 1 The effect of verapamil (10^{-5} M) on the response of trachealis to 30 mM K^+ (arrows, a), and 30 mM tetraethylammonium (TEA, arrows, b). The upper tracing in each panel represents the response to KCl or TEA in the absence of verapamil; the lower tracing, in its presence. KCl and TEA induced relaxation in the presence of verapamil. Similar results were obtained with diltiazem (10^{-5} M) and nifedipine (10^{-5} M).

Results

In the absence of Ca^{2+}-entry blockers, K^+ (30 mM) caused contraction of isolated trachealis muscle. However, after 70 min exposure to verapamil (10^{-5} M), diltiazem (10^{-5} M) or nifedipine (10^{-5} M), K^+ (30 mM) caused relaxation of the tissues (Figure 1, Table 1). Similar results were obtained over a range of K^+ concentrations (Figure 2). This paradoxical relaxant response in the presence of Ca^{2+}-entry blockers did not occur when methacholine (3×10^{-6} M) or

Table 1 Responses to K^+ (30 mM) or tetraethylammonium (TEA, 30 mM) of guinea-pig trachealis after various treatments

Stimulant	*Ca^{2+} entry blocker*	*Treatment*	*Response (g)*
K^+	Nifedipine (10^{-5} M)	Control	−0.21 ± 0.02
		Propranolol (10^{-6} M)	−0.10 ± 0.03
		Control	−0.21 ± 0.02
		Ouabain (10^{-6} M)	−0.20 ± 0.05
	Verapamil (10^{-5} M)	Control	−0.14 ± 0.05
		Propranolol	−0.12 ± 0.02
		Control	−0.12 ± 0.04
		Ouabain	−0.16 ± 0.05
	Diltiazem (10^{-5} M)	Control	−0.16 ± 0.04
		Propranolol	−0.14 ± 0.04
		Control	−0.14 ± 0.05
		Ouabain	−0.16 ± 0.04
	Nifedipine	†Control	−0.13 ± 0.04
		†Indomethacin (10^{-6} M)	−0.11 ± 0.05
TEA	Control		+0.15 ± 0.11
	Verapamil		−0.08 ± 0.01*
	Nifedipine	Control	−0.08 ± 0.01
		Propranolol plus ouabain	−0.07 ± 0.01

†Tone was maintained with methacholine 3×10^{-6} M.
*Significantly different from control ($P < 0.01$).
+ indicates contraction; − indicates relaxation. Results are means ± s.e.mean.

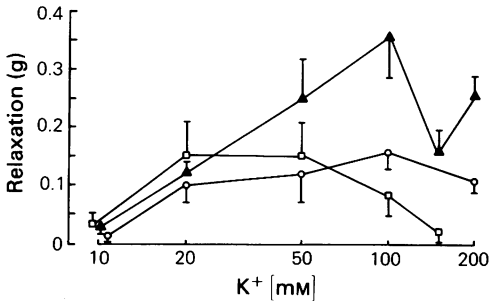

Figure 2 Concentration-response curves of the trachealis to K^+: in Ca^{2+}-free solution (○); after 45 min exposure to spermidine (20 mM) followed by 3 spermidine-free washes over 20 min (□); and after 70 min exposure to nifedipine (10^{-5} M) (▲). Each point represents the mean for 4–6 animals with s.e.mean indicated by vertical line.

histamine (2×10^{-5} M) was added to the tissue following exposure for 70 min to verapamil; only contractile responses were produced.

TEA inhibits K^+ conductance and depolarizes smooth muscle (Kirkpatrick, 1975). When TEA was added to the trachealis 45 min after verapamil (10^{-5} M), a paradoxical relaxation similar to that induced by K^+ occurred (Figure 1, Table 1). In the absence of verapamil, TEA caused contraction and phasic activity.

In order to determine whether the K^+-induced relaxation of trachealis in the presence of Ca^{2+}-entry blockers was due to the effect of released noradrenaline, or mediated by cyclo-oxygenase products, the effects of propranolol or indomethacin on the K^+-induced relaxation responses were tested (Table 1). Propranolol (10^{-6} M), added 15 min after the Ca^{2+}-entry blockers, but 60 min before K^+ (30 mM), was without effect on the K^+-induced relaxation. Indomethacin (10^{-6} M) caused an initial loss of tone, thereby preventing an evaluation of further relaxation responses. Therefore, shortly after adding indomethacin to the baths, methacholine (3×10^{-6} M) was added to restore tone. After stabilization of the response to methacholine, nifedipine (10^{-5} M) was added. Forty five min later the response to K^+ (30 mM) was evaluated. Under these conditions, indomethacin did not affect the K^+-induced relaxation (Table 1).

To determine whether stimulation of the Na^+,K^+-pump is responsible for the K^+-induced relaxation, ouabain (10^{-6} M) was added 5 min after the Ca^{2+}-entry blockers and 45 min before K^+. No inhibitory effect of ouabain on the K^+-induced relaxation was evident (Table 1). It should also be noted that ouabain and propranolol were without effect on TEA-induced relaxation. (The concentration of ouabain used was demonstrated in separate experiments ($n = 4$) to block totally K^+-induced relaxation of the tissue in the presence of reduced K^+ bathing solutions; results not shown).

We have previously demonstrated that after exposure of trachealis to spermidine, K^+ induces relaxation, and as is the case with the Ca^{2+}-entry blocking drugs, the relaxation is not affected by propranolol, ouabain or indomethacin (Chideckel *et al.*, 1985a). We decided to determine if the paradoxical relaxation of trachealis to K^+ seen in the presence of Ca^{2+}-entry blocking drugs and after spermidine could be due to a reduction in the entry of extracellular Ca^{2+}. When a Ca^{2+}-free bath was substituted for the MKH, a loss of tone ensued. In order to establish enough tone to allow a further relaxation response, methacholine (3×10^{-6} M) was added. After a steady state contractile response occurred, the tissue was exposed to K^+. Relaxation resulted (Figure 2, Table 2). This K^+-induced relaxation was unaffected by ouabain and propranolol (Table 2).

We examined the effect of the sodium channel blocker, tetrodotoxin (TTX) on K^+-induced relaxation responses obtained (a) in the presence of Ca^{2+}-entry blocking drugs, (b) in a Ca^{2+}-free solution and (c) after spermidine exposure. TTX blocked 65% of

Table 2 The effect of various agents on the response of trachealis to K^+ (30 mM) or tetraethylammonium (TEA 30 mM)

Treatment	*Agent*	*Response (g)*
Nifedipine (10^{-5} M),K^+	Control	-0.20 ± 0.03
	Tetrodotoxin (10^{-6} M)	-0.07 ± 0.01*
Nifedipine, K^+	Control	-0.14 ± 0.02
	Atropine (10^{-6} M)	-0.16 ± 0.04
Nifedipine, TEA	Control	-0.03 ± 0.01
	Tetrodotoxin	-0.04 ± 0.01
Spermidine (20 mM), K^+	Control	-0.15 ± 0.03
	Tetrodotoxin	$+0.18 \pm 0.04$
	Control	-0.19 ± 0.09
	Atropine	-0.24 ± 0.04
	Control	-0.07 ± 0.03
	Hexamethonium (10^{-6} M)	-0.09 ± 0.04
Ca^{2+}-free MKH, methacholine (3×10^{-6} M), K^+	Control	-0.04 ± 0.01
	Tetrodotoxin	$+0.08 \pm 0.01$*
	Control	-0.05 ± 0.02
	Propranolol (10^{-6} M) plus ouabain (10^{-6} M)	-0.04 ± 0.01

*Significantly different from control ($P < 0.005$).
+ indicates contraction; − indicates relaxation.
Results are mean ± s.e.mean.

the relaxation to K^+ in the presence of nifedipine (Figure 3, Table 2). It blocked 100% of the relaxation in Ca^{2+}-free solution and after spermidine exposure (Figure 3, Table 2).

It was of interest that the relaxation response to TEA after nifedipine was unaffected by TTX (Table 2). We also tested TEA to determine whether it was able to produce relaxation, as did K^+, after exposure of tissues to spermidine. TEA caused only a contraction ($n = 4$, data not shown).

After spermidine exposure, atropine or hexamethonium was without effect on the K^+-induced relaxation (Table 2). Atropine also was without effect on the K^+-induced relaxation after nifedipine (Table 2).

Non-cumulative concentration-response curves for K^+ in the presence and absence of TTX were compared to determine whether the TTX-sensitive K^+-reduced relaxation would influence sensitivity to K^+ in a normal MKH solution (Figure 4). At 20 mM K^+, TTX caused a decrease in the level of response ($P < 0.05$); however, at 50 mM responses in the presence of TTX were augmented ($P < 0.005$). The EC_{50} for K^+ in the absence of TTX was 16.3 mM (95% confidence limits, 10.4–25.2 mM) and in its presence, 22.6 mM (14.3–35.8 mM). The difference in EC_{50}s was significant ($P < 0.005$). Thus, TTX while itself without an effect on basal tone, shifted the K^+ concentration-response curve to the left.

Discussion

These studies demonstrate that in the presence of Ca^{2+}-entry blocking drugs, trachealis smooth muscle responds to elevations in extracellular K^+ by relaxation. This paradoxical effect is not secondary to β-adrenoceptor stimulation, stimulation of the Na^+,K^+-pump, or mediated by cyclo-oxygenase products. The effect is specific for K^+ and TEA in so far as it did not occur during responses to methacholine and histamine.

A similar relaxation occurs if K^+ is added to (methacholine-contracted) preparations in Ca^{2+}-free solution. K^+-induced relaxation also occurs in tissues that have been transiently exposed to polyamines (Chideckel *et al.*, 1985a). None of these relaxations was affected by propranolol or ouabain. All of the K^+-induced relaxations were partially or completely in-

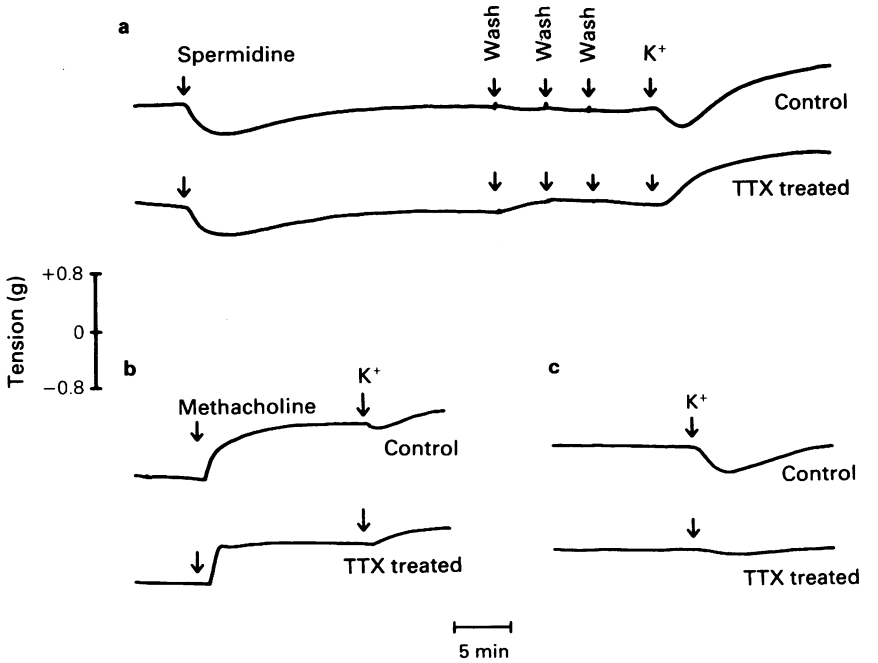

Figure 3 The effect of tetrodotoxin (TTX) on K^+-induced relaxation after exposure to spermidine (a), in a Ca^{2+}-free solution (b), and in the presence of nifedipine (c). Methacholine (3×10^{-6} M) was added to the Ca^{2+}-free solution to restore tone. TTX completely blocked K^+-induced relaxation after polyamine exposure and in the presence of Ca^{2+}-free solution. It blocked 65% of the K^+-induced relaxation in the presence of nifedipine.

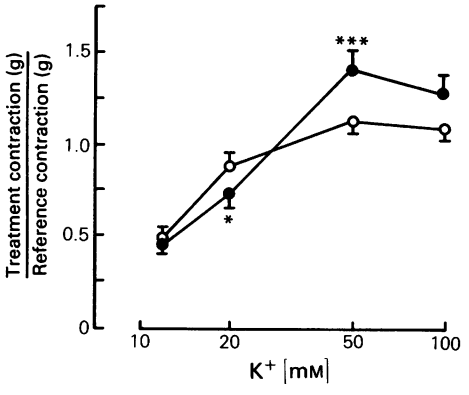

Figure 4 The effect of tetrodotoxin (TTX) on the response of trachealis to K^+ in MKH solution. The data are non-cumulative. The tissue in each bath was initially exposed to K^+ (100 mM); the response at 20 min is the reference contraction. The K^+ was washed out and 1 h later the tissue was re-exposed to K^+ at different concentrations, in the presence (●) or absence (○) of tetrodotoxin; the response at 20 min is the treatment contraction. Responses at 20 min were evaluated because at this time degree of contraction was becoming stable. Each point represents the mean for 5–6 animals with s.e.mean shown by vertical line. Statistics by paired *t* test. $*P<0.01$; $***P<0.005$. At 20 mM K^+, TTX decreases the contractile response; however at 50 mM it is associated with an increased response. The EC_{50} without TTX = 16.3 mM (95% confidence limits, 10.4–25.2), with TTX = 22.6 mM (14.3–35.8). Though the effect of TTX is complex, it unmasks a relaxant action of K^+ in MKH solution.

hibited by TTX, suggesting that they were mediated indirectly by released inhibitory neurotransmitter(s).

We and others (Foster *et al.*, 1983a) have observed that a very transient relaxation of the guinea-pig trachealis to K^+ occurs when the tissues are in MKH solution, but this occurrence is too infrequent to allow systematic study. Our present data showing a leftward shift in the concentration-response curve for K^+ in the presence of TTX, indicates a neurotransmitter-induced relaxation is part of the response of the trachealis to K^+.

K^+ thus has opposite direct and indirect effects on trachealis muscle. K^+-induced contraction is very dependent on the entry of extracellular Ca^{2+}. This entry has been shown to be blocked by the Ca^{2+}-entry blocking drugs and markedly diminished in Ca^{2+}-free solutions (Kirkpatrick, 1975; Foster *et al.*, 1984). It is probable that the polyamines act in a similar manner, though why multiple washes with polyamine-free solutions are needed to evoke this action is unclear. It seems likely that the Ca^{2+}-entry blocking drugs, the Ca^{2+}-free solutions and polyamine exposure inhibit the direct response of the muscle and allow the indirect response, mediated by an inhibitory neurotransmitter, to be revealed.

The relaxation induced by K^+ in the presence of the Ca^{2+}-entry blocking drugs is not completely prevented by TTX, indicating a non-neurogenic component. Also, the TEA-induced relaxation is not affected at all by TTX. The precise mechanism of this non-neurogenic relaxation is unclear. However, in rat vas deferens and in guinea-pig trachealis muscle, K^+ causes an increase in $^{45}Ca^{2+}$ efflux as well as influx (Hay & Wadsworth, 1984; Bryson *et al.*, 1985). TEA also increases $^{45}Ca^{2+}$ efflux in the trachealis (Bryson *et al.*, 1985). In the vas deferens the K^+-induced influx is blocked by Ca^{2+}-entry blocking drugs, while the efflux is unaffected. It is thus possible that, in the presence of one of these drugs, K^+ or TEA exposure results in a decrease in cytoplasmic Ca^{2+} and, in a tissue with basal or stimulated tone, relaxation. The specificity of the effect for K^+ and TEA may be related to the dependency of responses to these contractile substances on extracellular Ca^{2+}, as opposed to acetylcholine or histamine, contractile responses to which, rely, at least in part, on the release of Ca^{2+} from intracellular stores (Kirkpatrick, 1975; Foster *et al.*, 1983b). A similarity between the non-neurogenic component of K^+-induced relaxation and the totality of TEA relaxation is also suggested by the fact that neither of these occur after polyamine exposure.

It thus appears that most (65%) of the relaxation to K^+ in the presence of Ca^{2+}-entry blockers, and all the relaxation in the presence of a low Ca^{2+} solution and after polyamine treatment, is neurogenically mediated. This relaxation response is not blocked by atropine, and, therefore, not dependent on muscarinic receptors. It is not blocked by propranolol and thus probably does not involve the release of catecholamines and β-adrenoceptor stimulation. It is not affected by hexamethonium, and, thus, ganglionic transmission is not involved. It appears that K^+ may be inducing the release of a non-adrenergic, non-cholinergic, inhibitory neurotransmitter(s).

A non-adrenergic, inhibitory innervation (Burnstock *et al.*, 1964) has been suggested for the guinea-pig trachealis (Coburn & Tomita, 1973). If the direct effect of K^+ on the muscle is blocked, a K^+ effect on release of the inhibitory transmitter may become apparent. Because of the demonstrated shift in the K^+ concentration-response curve by TTX, this inhibitory nervous system would appear to influence the response to K^+ when it is added to normal physiological solution.

We conclude that the data presented suggest: (1) that K^+ stimulates the release of a non-adrenergic, non-cholinergic inhibitory neurotransmitter; (2) that K^+ and TEA also cause a paradoxical relaxation in the presence of Ca^{2+}-entry blocking drugs via another

mechanism; (3) that K^+-induced relaxation is evident only in the presence of agents that prevent a direct contractile response of muscle to K^+; (4) that spermidine shares with the Ca^{2+}-entry blocking drugs and Ca^{2+}-free solutions the ability to inhibit directly the contractile effect of K^+ on trachealis smooth muscle; and (5) that it is possible that polyamines may function as naturally occurring Ca^{2+} antagonists.

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