ISBN 0-315-54939-4

AN INVESTIGATION OF THE MECHANISM OF RELAXATION OF CANINE AIRWAY SMOOTH MUSCLE BY THE POTASSIUM-SPARING DIURETIC AMILORIDE

A Thesis

presented to the

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

by

Ingrid Kim Krampetz

Department of Pharmacology and Therapeutics

Faculty of Medicine

Winnipeg, Manitoba

AN INVESTIGATION OF THE MECHANISM OF RELAXATION OF CANINE AIRWAY SMOOTH MUSCLE BY THE POTASSIUM-SPARING DIURETIC AMILORIDE

BY

INGRID KIM KRAMPETZ

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Dedicated to my parents,

Monika and Adolf Krampetz,

who, above all, have shown me that wisdom and integrity do not come from a piece of parchment.

"Nur dem kann ich raten, Kunstler zu werden der aus reiner Liebe zu Kunst selbst, aus innerem Drang und Bedurfnis - unbekummert um glanzenden Erfolg - ihr sein Leben weihen will und darin sein Genugen und sein Gluck findet."

Speeman, 1909

Goldenes Buch der Musik

"Only that person can I advise to become an artist, who out of pure love of Art itself, out of inner desire and need - unconcerned with spectacular success - devote his life to it and through it find his satisfaction and happiness."

ABSTRACT

The characteristics and mechanism of relaxation of canine airway smooth muscle induced by the K^+-sparing diuretic amiloride were investigated. Amiloride relaxed isometrically contracted canine tracheal smooth muscle strips, independent of the original stimulus for contraction. During high potassium (80 mM)-induced contractions, amiloride caused a slow monophasic, dose-dependent relaxation (IC_{50} = 12.3 uM). In carbachol (1 uM)-contracted strips, 1 and 10 uM amiloride also induced a slow monophasic relaxation. Exposure to concentrations of 35 to 250 uM amiloride added another initial rapid phase (IC_{50} = 75.5 uM), which was superimposed onto the slow phase (IC_{50} = 23.5 uM), resulting in a biphasic relaxation. The rate of amiloride induced relaxation was decreased with increasing external [Na^+] regardless of stimulus, suggesting competitive inhibition of a sodium dependent process, possibly the Na^+/H^+ antiporter. The presence and importance of this transport process in intracellular pH regulation of canine trachealis was supported by studies using the pH sensitive fluoroprobe BCECF. Resting pH was measured at 7.08 $\pm$ 0.08. This value was reduced upon exposure to amiloride (100 uM) or sodium-free medium (by 0.21 $\pm$ 0.07 and 0.53 $\pm$ 0.05 U, respectively). Amiloride also markedly altered the ability of this tissue to regulate pH_i over a wide range of external proton concentrations, supporting its previously documented properties as a Na^+/H^+ antiport inhibitor. Stimulation of the tissue with high K^+ or carbachol resulted in a simultaneous intracellular acidification of 0.29 $\pm$ 0.07 and 0.40 $\pm$ 0.08 U, respectively. Relaxation of K^+ or carbachol contracted muscle with amiloride (100 uM) coincided with further acidification of the cell interior and suggested an inhibitory effect of excess protons on contracted tissue. Intracellular acidification imposed by treatment with acetic acid also relaxed this tissue and simultaneously reduced intracellular calcium concentration as measured using the Ca^{2+}-sensitive fluoroprobe Fura-2. Because amiloride fluorescence interfered with the Fura-2 spectrum, the effect of amiloride on calcium release and entry was assessed indirectly. Amiloride significantly reduced the force of contraction elicited in calcium-free medium. When calcium was subsequently added to the bathing medium, the initial rate of

contraction was also significantly reduced. These suggested that amiloride reduced both intracellular calcium release and influx. Based on these data, it appears that amiloride relaxes canine airway smooth muscle by decreasing pHi and reducing the amount of or sensitivity to activator calcium. The ability of amiloride to reduce bronchoconstriction in anaesthetized dogs was also assessed. Aerosol delivery of 10 uM amiloride solution over a four minute period significantly reduced peak bronchoconstriction in response to carbachol challenge from 7.78 $\pm$ 2.07 cm H_2O/l/s to 2.53$\pm$ 0.35 cm H_2O/l/s. Mean plateau airway resistance after carbachol challenge was 2.27 $\pm$ 0.40 H_2O/l/s. Subsequent treatment with aerosolized amiloride decreased this plateau to a level not significantly different from control (1.24 $\pm$ 0.18 *vs.* 0.62$\pm$0.07 H_2O/l/s). This indicates that amiloride reduced the bronchoconstrictive effect of carbachol in anaesthetized dogs and suggests possible future therapetic use for this drug in the treatment of bronchospastic disease.

ACKNOWLEDGEMENTS

I would like to acknowledge the contributions of the following individuals in the development if this thesis:

Dr. R. Bose;

Dr. D. Bose; for helpful advice - especially with computer programs and equipment.

Dr. P.I. Polimeni; who taught me what "real science" was about.

Dr. S. Mink; for patience and encouragement.

Dr. I. Bihler; who, by example, showed me that there is honor and dignity in science.

Mike, Ron, and Subah; for friendship and encouragement in good times and bad, and for just being there.

Peggy Merrick; whose smile brought sunshine to the cloudy days.

The Manitoba Lung Association; for continual and generous support of undergraduate research.

Dr. T. Streleski; whose act of courage is an inspiration to us all.

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

PREAMBLE

This thesis is divided into four major sections, each of which written in the form of a manuscript. Part 1 has been published in abridged form (co-authored with Dr. Ratna Bose) in the Journal of Pharmacology and Experimental Therapeutics (246: 641-648, 1988). The major portion of the data included in Part 2 of the thesis is currently in press in the Frontiers in Smooth Muscle Research Syposium proceedings (held May 1989, in Columbus, Ohio), and was also co-authored with Dr. Ratna Bose. Part 3 is currently submitted for review to the Journal of Pharmacology and Experimental Therapeutics (co-authored with Dr. Ratna Bose). The final section, Part 4, is currently in preparation for publication (co-authored with Dr. Ratna Bose, Dr. Steve N. Mink, Dr. Arturo Gomez and Mr. Jim Eng). I am indebted to them for their valuable help and advice.

INTRODUCTION

Amiloride

(i) Historical background

The organic compound referred to as 3,5,-diamino-N-(aminoiminomethyl)-6-chloropyrazinecarboxamide or N-amidino-3,5-diamino-6-chloropyrazine carboxamide is one of a large group of substituted guanidines originally synthesized by E.J. Cragoe Jr. for Merck & Co., Inc. (West Point, U.S.A.) in the early 1960's. It was patented by the same in 1964 under Belgian patent no. **639,386** (Kramer, 1965) and has alternatively been identified as MK-870, amipramizine, amipramidin, and - most commonly - amiloride (Merck Index, 9th ed.).

The structure of amiloride (mol. weight = 229) is that of a pyrazine ring with an acylguanidine group located at ring position 2 (Figure 1). This structure imparts a number of unique and characteristic properties. Amiloride is a weak base, a property which resides in the guanidinium group rather than the amino groups of the ring. Its pK_a has been measured at 8.7 in a 30% ethanol and water solution of zero ionic strength (Smith *et al.*, 1979). Under physiological conditions (pH = 7.4), it seems likely that amiloride would have a single positive charge. While Benos *et al.* (1976) have previously attempted to measure the pK_a of this compound in physiological (Ringer's) solution, these efforts were confounded by other properties of this compound. Specifically, these are: amiloride's limited solubility in aqueous solutions, and the lack of measurable differences in the spectral properties of the protonated and non-protonated form of the drug. The maximum solubility of amiloride is approximately 16 mM in distilled water (Benos, 1982), and this decreases with increasing ionic strength. In addition, while amiloride is highly fluorescent (ultraviolet absorption peaks at 215, 288, and 360 nm and an emission peak at 420 nm), the characteristic spectra of the uncharged and cationic species are identical (Benos *et al.*, 1976).

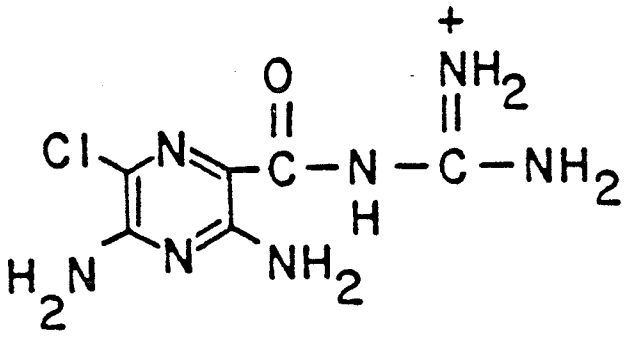

Figure 1: The chemical structure of 3,5,-diamino-N-(aminoiminomethyl)-6-chloropyrazinecarboxamide (amiloride).

Among the first pharmacological properties of amiloride revealed in its screening as a therapeutic agent was its ability to promote diuresis in test animals. In 1966, Glitzer and Steelman from the Merck Institute for Therapeutic Research reported that oral administration of amiloride would enhance sodium excretion (natriuresis) and urine volume in both intact and adrenalectomized rats. Moreover, amiloride inhibited the loss of potassium. This ability to inhibit kaluresis remained even when amiloride was administered along with a thiazide diuretic. Based on these first studies, it was concluded that amiloride acted on the kidney through a unique mechanism apparently unrelated to inhibition of peripheral aldosterone release or activity. Because amiloride could additively *increase* sodium excretion when administered along with a thiazide diuretic yet simultaneously *eliminate* the potassium loss characteristic of thiazide treatment, it was also concluded that amiloride operated by a different mode of action on the kidney.

In 1967, Baer *et al.* reported similar effects of amiloride in the dog. Here, amiloride increased sodium concentrations, and decreased potassium concentrations in urine collected from the distal tubules. Baba and co-workers (1968) examined this drug's activity in normal human subjects and adrenalectomized rats. Amiloride's diuretic and potassium conserving effects were confirmed in both systems. Because of structural similarities between amiloride and another K^+-sparing diuretic - triamterene - these authors hypothesized that their modes of action may also be similar in these models. Since triamterene had been shown previously to block sodium transport across high resistance or "tight" epithelia, they also examined the effect of amiloride on sodium transport in isolated amphibian epithelium. Amiloride was found to reversibly inhibit the sodium-mediated short circuit current through this tissue. In fact, this effect had previously been demonstrated in amphibian skin by Eigler *et al.* in 1967 (as cited in Benos, 1982). Based on the results of these studies, it was concluded that amiloride probably acted by blocking sodium entry through sodium-transporting mechanisms on the luminal surface of the renal epithelium in the distal tubule.

Because of its distinct characteristics, amiloride hydrochloride (manufactured by Merck, Sharp and Dohme under the tradename "Midamor") is currently prescribed alone or in combination with a thiazide diuretic ("Moduret") for the treatment of ascites and edema associated with liver cirrhosis, edema of cardiac origin, or essential hypertension (Weiner and Mudge, 1985). In each of these disorders, the primary advantage of this drug over other diuretics is its ability to reduce fluid retention without promoting the loss of potassium, thereby preserving normal serum potassium levels.

(ii) Effect on epithelial sodium channels

In addition to its clinical use, amiloride is also utilized as a research compound to elucidate physiological mechanisms of sodium transport (Kleyman and Cragoe, 1988). As previously indicated, one of the first described in vitro properties of amiloride was its ability to inhibit the movement of sodium through "tight" or high resistance epithelia. Since the original observation by Eigler *et al.* (1967, as cited in Benos, 1982) in amphibian epithelium, this effect has been demonstrated in many other epithelial preparations, including toad and turtle urinary bladder, rabbit renal collecting duct, pig colon and human kidney (Benos, 1982). Inhibition of the movement of sodium across high resistance epithelia by amiloride occurs at the first step of the transport process, apparently by blocking the apical sodium channels on the external face of the membrane. Entry of sodium through this channel follows the electrochemical gradient and therefore is not directly ATP-dependent. (It is however, dependent on the sodium gradient created by the Na^+/K^+ ATPase.) Sodium entry via this pathway is characteristically inhibited by low concentrations of amiloride (less than 1 uM). To date, these amiloride-sensitive channels have only been described in epithelial cells.

The actual mechanism of epithelial sodium channel blockade has been investigated, however there appears to be some variability depending on the species or the preparation used. Kinetics of inhibition by amiloride have been variously described as competitive, mixed, and non-competitive with external sodium (Benos, 1982). However, two models are currently favoured. Cuthbert and Shum (1976)have proposed that amiloride inhibits the channel by competing with the sodium ion, and physically blocking the channel opening (akin to the blockade of fast sodium channels by tetrodotoxin). Benos (1982) suggests that amiloride acts on a modifier site within the channel protein to inhibit sodium entry, and adds that variations in this modifier site between species may account for the different kinetics observed.

Other important characteristics of amiloride are the pH sensitivity of it's action and an apparent dependence on external calcium ions. Studies of frog skin epithelial sodium transport under conditions of varying external pH indicate that the protonated (and thus positively charged) species is the effective form of the drug (Benos, 1976). In addition, there appears to be a dependence on the presence of external calcium ions, such that the concentration of amiloride required to reduce the short circuit current in frog skin by 50% was increased from 0.24 uM in 1 mM Ca^{2+} to 100 uM in a calcium-free (5 mM EGTA) Ringers solution (Cuthbert and Wong, 1972). The origin of this calcium effect is unclear, however.

(iii) Effect on sodium - hydrogen exchange

In addition to its inhibitory effects on epithelial sodium channels, amiloride interferes with another sodium transport process, the sodium-proton exchanger. This membrane-dependent process for the electroneutral exchange of sodium ions for protons has been described in all vertebrate tissue thus far examined, including canine smooth muscle (Kahn *et al.*, 1986). The stoichiometry of the antiporter is apparently 1:1 (or 2:2, etc.) and lithium and ammonium ions can be transported in addition to sodium and hydrogen. Typically, intracellular protons are removed

from the cell in exchange for extracellular sodium ions, following the sodium gradient. Although it is a passive process, it is secondarily dependent on the sodium-potassium ATPase.

By following the existing gradients, this exchange of sodium and hydrogen ions appears to contribute towards intracellular pH regulation and cell volume regulation (Grinstein *et al.*, 1983). Although the exchange process is passive, its activity can be increased by two known mechanisms. Intracellular acidification has been demonstrated to increase antiporter activity, probably by protonation of a modifier on the cytoplasmic surface of the exchange protein (Aronson *et al.*, 1982). In addition, hormones and mitogens appear to activate the sodium-hydrogen exchange process, leading to intracellular alkalinization. [This event is also apparently linked to so-called "fertilization acid" production during cell fertilization, where export of protons from the cell interior results in a reduction in external pH (Mahnensmith and Aronson, 1983)]. Activation through this latter mechanism may be linked to stimulation of protein kinase C, since stimulation by phorbol esters (which directly activate protein kinase C) can increase the activity of the antiporter (Besterman and Cuatrecasas, 1984).

Inhibition of the Na^+/H^+ antiporter by amiloride requires concentrations of greater than 1 uM; however, IC_{50} values reported range from uM to almost millimolar concentrations (Benos, 1982; Mahnensmith and Aronson, 1985). In the great majority of studies, amiloride inhibition of the antiporter has been characterized as purely competitive with respect to external sodium ions. (Presumably this implies that amiloride acts at or very near the external transport site). In light of this competitive interaction, variations in medium sodium concentration between these studies might account for the wide variability in the IC_{50} values. Indeed, Mahnensmith and Aronson (1985) have suggested that amiloride is not a highly effective inhibitor of the antiporter at normal physiological sodium concentrations. While a number of studies would appear to dispute this conclusion, it is clear that the ability to inhibit this process is increased under low sodium conditions (Grinstein *et al.*, 1983).

(iv) Effect on sodium - calcium exchange

To date amiloride has been shown to inhibit sodium-calcium exchange in a number of preparations, including cardiac sarcolemmal vesicles (Debetto *et al.*, 1987; Siegl *et al.*, 1984), rat cerebral vesicles (Schellenberg *et al.*, 1983), and erythroleukemia cells (Smith *et al.*, 1982). In each case, inhibition of sodium-dependent calcium movement (by 50 % or more) required either solution concentrations in excess of 1 mM amiloride, or prolonged incubation coupled with active accumulation of the drug such that intracellular concentration approximated mM amounts (Smith *et al.*, 1982). Under these conditions, inhibition of the sodium-hydrogen exchanger would also occur, making this drug a poor probe for assessing the functional significance of this process alone.

(v) Effect on the sodium-potassium pump

There is limited evidence that amiloride inhibits the sodium-potassium ATPase. Soltoff and Mandel (1983) have demonstrated that at concentrations of 1 mM amiloride can inhibit the sodium pump in renal epithelial cells. This effect was increased under low sodium conditions. Knauf *et al.* (1976) showed in rabbit kidney proximal tubules, however, that the effect of amiloride on sodium transport was completely explained by its inhibitory effect on passive sodium influx rather than on the sodium-potassium ATPase. These investigators observed that under physiological sodium concentrations, 50 uM amiloride did not alter the activity of this enzyme, and at 500 uM activity was reduced by only 10%. This suggests that the interaction of amiloride with the Na^+/K^+ ATPase is not highly specific.

(vi) Other effects

In addition to the above named ion transport processes, amiloride has been suggested to alter other cellular functions. One study has shown that amiloride competes with rauwolscine, prazosin, and iodocyanopindolol for renal adrenergic alpha and beta binding sites with IC_{50} values ranging from 14 to 84 uM. (Howard *et al.*, 1987). A recent demonstration by Tang *et al.* (1988) using the patch-clamp technique suggests that amiloride selectively blocks T-type calcium channels in mouse neuroblastoma and chick dorsal root ganglion neurons with an apparent K_D of 30 uM. In addition, amiloride has been reported to inhibit kinases (Davis *et al.*, 1985; Ozaki *et al.*, 1987), including protein kinase C (Besterman, 1985), and cAMP-dependent protein kinases (types one and two; Ralph *et al.*, 1982). IC_{50} values for kinase inhibition are approximately 1 mM. Amiloride may alter membrane charge characteristics (Benos *et al.*, 1976), and has been shown to collapse pH gradients across membrane vesicles as a permeant weak base in its unprotonated form (Dubinsky *et al.*, 1983). This latter effect is, however, dependent on a high permeability of the vesicles to amiloride.

Airway smooth muscle

(i) Function

Although smooth muscle is present throughout the conductive structures of the lungs, including the trachea, bronchi and bronchioles, it true function is not well understood. Widdicombe and Nadel (1963) have suggested that contraction of this tissue modulates airway calibre with changing physiological conditions, in order to optimize the relationships between airway resistance and dead space within the lung. They indicate that in this manner, the mechanical work and mean inspiratory force of breathing is minimized. Alternatively, airway smooth muscle tone may serve to broaden the range of critical closing and opening pressures within the lung by "stiffening" the airways, in addition to reducing airway calibre (Macklem and Engel, 1975). Both of these hypotheses, however, have been disputed by Williams (1981) who has suggested that airway smooth muscle constriction may serve as a signal of and defence against hazardous ventilatory conditions, including impure or very cold air. Hyperresponsiveness of the airways to such conditions, Williams contends, would therefore manifest itself as asthma.

Whatever the physiological function of this tissue, there is little doubt that hyperresponsiveness of airway smooth muscle contributes to the pathogenesis of asthma. While other factors including excessive mucus production and airway wall thickening (James *et al.*, 1989) may exacerbate airway narrowing in this disorder, the effectiveness of bronchodilators which act directly on smooth muscle (i.e.,beta agonists and anticholinergics) highlight the contribution of airway smooth muscle shortening towards bronchospasm (Nelson, 1986; Maxwell, 1987). The continued investigation of new airway smooth muscle relaxants would therefore ultimately serve to broaden the pharmacological armamentarium used in the treatment of asthma.

(ii) The tracheal smooth muscle model

Because of the difficulty in obtaining clean isolated preparations from the smaller airways, tracheal smooth muscle strips have long been utilized as a model in the investigation of airway smooth muscle function. The easy accessibility and large amount of smooth muscle obtainable from the large bronchi and trachea have made these the preparations of choice. Our model of airway smooth muscle, the canine *muscularis trachealis transversus*, is a thick, well-defined, band of smooth muscle located on the dorsal aspect of the trachea between the ends of the "C"-shaped cartilagenous rings. The attached cartilage and the lumenal and serosal layers of loose connective tissue which envelop it are easily dissected away, leaving a preparation essentially free of non-muscle components. Thin strips are cut from this muscle (in the plane of the cartilagenous rings) which can then be maintained in vitro under physiological conditions for periods of hours without decrement in contractile activity. The validity of this tissue as a model for airway smooth muscle located in the remainder of the respiratory tract has been supported by the work of Russel (1978), who compared the *in vitro* contractility of canine tracheal smooth muscle strips and spiral strips from smaller airways (5 mm and 1.5 mm diameter). These preparations exhibited both qualitatively similar length-tension relationships and similar dose-response curves upon exposure to exogenous acetylcholine.

Ultrastructural studies of canine tracheal smooth muscle have revealed that within this tissue, the muscle cells are aligned in parallel bundles with their long axes parallel to the tracheal rings (Nathaniel, 1981), presumably oriented for maximum shortening and/or stiffening ability. Innervation of this muscle is primarily cholinergic, with a few adrenergic fibres present (Suzuki *et al.*, 1976; Kannan and Daniel, 1980; Nathaniel, 1981). Contraction elicited during electrical field stimulation is almost entirely atropine sensitive, and is therefore primarily due to release of acetylcholine from the cholinergic nerve terminals (Suzuki *et al.*, 1976; Kannan and Daniel, 1980). In the presence of atropine, field stimulation causes a relaxation which is blocked by propranolol

(Suzuki *et al.*, 1976; Kannan and Daniel, 1980), suggesting the presence of beta adrenoceptors. Barnes *et al.* (1983) have demonstrated that in canine trachealis, 80% of the beta receptors are of the beta-2 variety, with the remainder of the beta-1 type. According to these investigators, both mediate relaxation. A small alpha -adrenergic excitatory response is also present (Suzuki *et al.*, 1976). Unlike other species, including the cat and guinea pig (Gabella, 1987), non-cholinergic non-adrenergic inhibitory fibres (probably VIP-ergic) are not present in the dog (Suzuki *et al.*, 1976). Low resistance pathways (gap junctions or nexi) are present between cells (Kannan and Daniel, 1978; Nathaniel, 1981), and the space constant of this tissue is many times the cell length (Kroeger and Stephens, 1975; Suzuki *et al.*, 1976). This indicates that electrical coupling exists between the individual smooth muscle cells.

Functionally, tracheal smooth muscle is neither characterized as single unit or multi-unit. Its relatively dense innervation (characteristic of multi-unit smooth muscle) and close cell to cell coupling (typical of single unit muscle) place it within an intermediate class (Stephens, 1987). Electrophysiological studies have demonstrated that no spontaneous membrane activity exists in the dog trachealis (Kroeger and Stephens, 1975; Suzuki *et al.*, 1976), which may be due to the high resting permeability of these cells to potassium (Kroeger and Stephens, 1975; Kannan and Daniels, 1978). Action potentials do not normally occur (Bose and Bose, 1977), and the muscle cells respond to stimulation with a graded depolarization.

(iii) Excitation-contraction coupling: Electromechanical versus pharmacomechanical coupling.

The events which link tracheal smooth muscle stimulation and eventual contraction appear to be similar to those observed in other "graded" smooth muscles. To understand the current thinking on this process it is important to examine it in a historical context. Originally, it was believed that in smooth muscle, as in striated muscle, contraction was elicited exclusively by electrical means through depolarization of the smooth muscle membrane. However, the studies of

Evans and Schild and Evans *et al.* in the late 1950's suggested that certain drugs - acetylcholine, histamine, oxytocin and serotonin - could activate the contractile elements of a variety of smooth muscles even after the membrane had been depolarized in a high potassium bathing solution. This indicated that the contractile elements could be activated by some additional process over and above the contraction elicited by a depolarizing stimulus. Based on this observation, it was concluded that there was a "possibility that the action of the drugs on the membrane resulted in the liberation of a chemical agent in the interior of the fibre which activates the contractile elements" (Evans et al., 1958).

At this time, there was growing evidence that the movement of inorganic ions, especially the calcium ion, was important in the process of smooth muscle contraction. The investigations of Briggs (1962) showed that potassium-elicited contraction of vascular smooth muscle induced calcium influx. In 1965, Filo *et al.* demonstrated the importance of *intracellular* calcium ion concentration in smooth muscle contraction using a glycerinated (permeablized) hog carotid artery preparation. In the same year, Hinke observed that a contractile response could be elicited by norepinephrine in the absence of extracellular calcium - even after the response to potassium induced depolarization had disappeared.

These differences in the characteristics of depolarization-induced and drug-elicited contractions led Somlyo and Somlyo (1968) to coin the terms electromechanical and pharmacomechanical coupling to describe the phenomenon of contraction which was largely independent of changes in membrane potential. These investigators observed that in quiescent, non-spontaneously active smooth muscles, small graded depolarizations were produced by exposure to pharmacological agents. The magnitude of these depolarizations was roughly proportional to the mechanical response elicited. However, they questioned the "cause and effect" relationship between these two observations, since the magnitude of the contractile response to norepinephrine, angiotensin, and histamine were similar in depolarized and polarized muscle. In addition, they

observed that caffeine could induce contraction without a change in membrane potential. From these and subsequent studies (Hudgins and Weiss, 1967; Casteels and Raeymaekers, 1979), it became clear that pharmacological agents were able to mobilize a calcium store not depletable by calcium-free medium and apparently unavailable for high potassium-induced contraction. This store is thought to be represented by the smooth muscle sarcoplasmic retiulum (Somlyo , 1985.)

Airway smooth muscle, like all other muscles, is dependent on calcium for contraction, and the sources of calcium used for contraction in this tissue are dependent on the original form of stimulation. Studies of the dependence of canine tracheal smooth muscle on different calcium sources have been performed (Kannan *et al.* 1987 ; Weiss *et al.*, 1985) and indicate the primary sources of calcium utilized during pharmacological versus depolarization -induced stimulation are different.

In canine tracheal smooth muscle, stimulation by a high potassium solution results in a graded depolarization of the membrane (Coburn and Yamaguchi, 1975; Coburn, 1977, 1979) and concurrent increase in calcium influx (Weiss et al, 1985). Lanthanum, verapamil, or D-600 will block calcium entry (Coburn, 1977; Weiss et al., 1985), without altering membrane depolarization (Coburn, 1977). Repolarization of the membrane to resting Em (using anodal current), however, almost entirely eliminated high K^+-induced contraction (Coburn, 1979). This indicates that depolarization is essential for calcium influx during this form of stimulation. It has been proposed that calcium entry during membrane depolarization is mediated by the opening of "voltage activated" or "potential sensitive" calcium channels (Bolton, 1979), which can be blocked by the above mentioned compounds.

Membrane potential changes appear to be less important during stimulation with pharmacological agents. In contrast to K^+-induced contraction of canine trachealis, repolarization of the sarcolemma during serotonin or acetylcholine contraction do not eliminate contraction

(Coburn and Yamaguchi, 1975; Coburn, 1979). Coburn and Yamaguchi (1975) showed that serotonin contractions were reduced by only 20-40 %. Paradoxically, application of anodal current during acetylcholine stimulation produced further contraction (Coburn, 1979). Unlike K^+-induced contractions, acetylcholine contractions were also unaffected by La^{3+}, verapamil or D-600, suggesting that membrane potential dependent calcium transport mechanisms were not important during this form of stimulation. These agonists are believed to act on specific receptors which admit calcium through receptor operated channels (Bolton and Kitamura, 1983). In addition, they can stimulate the release of calcium from intracellular sites, as indicated from calcium efflux experiments (Farley and Miles, 1978).

The exact mechanism of intracellular calcium release during pharmacological stimulation of smooth muscle is not known, however, a strong candidate for second messenger of calcium release is inositol-1,4,5-trisphosphate (IP_3). This compound is a hydrolysis product of membrane phosphoinositides located in the inner leaflet of the lipid bilayer. Stimulation of the phosphatidylinostitol bisphosphate phosphodiesterase (also known as phospholipase C) results in the formation of phosphatidyl inositol bisphosphate. The breakdown products of this compound are diacylglycerol (DAG) and IP_3 (Berridge and Irvine, 1984). Hydrolysis is calcium dependent and has been shown to be activated by muscarinic agonists in smooth muscle (Akhtar and Abdel-Latif, 1980), including bovine airway smooth muscle (Baron and Coburn, 1984). Suematsu et al. (1984) have shown that IP_3 can release calcium from an intracellular non-mitochondrial storage site in permeablized (saponin-treated) single smooth muscle cells of the porcine coronary artery. More recently, Walker *et al.* (1987) demonstrated in permeablized rabbit main pulmonary artery, that IP_3 contracts this tissue at a similar rate and concentration to that observed in vivo during norepinephrine-induced contraction. These investigators avoided the major weakness of the Suematsu group study - diffusion limitation - by incubating the tissue in a biologically inactive photolabile precursor of IP_3 ("caged" IP_3) which was then rapidly hydrolysed by exposure to light. The exact mechanism by which IP_3 may promote calcium release from the SR is unknown,

however, it has been recently shown that IP_3 (at physiologically relevant concentrations) can induce opening of channels in planar lipid bilayers enriched with vesicles from aortic smooth muscle sarcoplasmic reticulum (Erlich and Watras, 1988). It is possible that these channels may represent a calcium permeation pathway from the SR.

Indirect evidence suggests that this pathway may also be important in the release of intracellular calcium stores from airway smooth muscle. In support of this, Meurs and coworkers (1988) have observed a direct correlation between the IC_{50} values for muscarinic contraction of bovine tracheal smooth muscle and IP_3 release. Although not conclusive, this data would suggest an important role for IP_3 release during this form of contraction, and imply that SR calcium release may be mediated in this fashion.

The importance and specific contribution of different calcium sources to smooth muscle contraction, particularly airway smooth muscle, is becoming increasingly clear, however, it is interesting to note that only after 30 years of continuous research, can the prophetic nature of Evans' and Schild's conclusions be fully appreciated.

References:

Akhtar, R.A. and Abdel-Latif, A.A. Requirement for calcium ions in acetylcholine-stimulated phosphodiesteratic cleavage of phosphatydyl-*myo*-inositol 4,5,bisphosphate in rabbit iris smooth muscle. Biochem. J. **192**: 783-791, 1980.

Aronson, P.S., Nee, J., and Suhm, M.A. Modifier role of internal H^+ in activating the Na^+-H^+ exchanger in renal microvillus membrane vesicles. Nature (Lond.) **299**: 161-163, 1982.

Baba, W.I., Lant, A.F., Smith, A.J., Townshend, M.M., and Wilson, G.M. Pharmacological effects in animals and normal human subjects of the diuretic amiloride hydrochloride (MK-870). Clin. Pharm. Therap. **9**:318-327, 1968.

Baer, J.E., Jones, C.B., Spitzer, S.A., and Russo, H.F. The potassium sparing and natriuretic activity of N-amidino-3,5-diamino-6-chloropyrazinecarboxamide hydrochloride dihydrate (amiloride hydrochloride). J. Pharm. Exp. Therap. **157**: 472-485, 1967.

Barnes, P.J., Nadel, J.A., Skoogh, B.-E., and Roberts J.M. Characterization of beta-adrenergic subtypes in canine tracheal smooth muscle by radioligand binding and physiologic responses. J. Pharm. Exp. Therap. **225**: 456-461, 1983.

Baron, C.B. and Coburn, R.F. "Synthesis of inositol phospholipids during carbachol-induced contraction." *In* Regulation and contraction of smooth muscle. M.J. Seigman, A.P. Somlyo, and N.L. Stephens, eds. Alan R. Liss Publ., New York, 1984, pp. 449-450.

Benos, D.J., Simon, S.A., Mandel, L.J., and Cala, P.M. Effect of amiloride and some of its analogues on cation transport in isolated frog skin and thin lipid membranes. J.Gen. Physiol. **68**: 43-63, 1976.

Benos, D.J. Amiloride: a molecular probe of sodium transport in tissues and cells. Am. J. Physiol. **242**: C131-C145, 1982.

Berridge, M.J. and Irvine, R.F. Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature **312**: 315-321, 1984.

Besterman, J.M. and Cuatrecasas, P. Phorbol esters rapidly stimulate amiloride-sensitive Na^+/H^+ exchange in a human leukemic cell line. J. Cell. Biol. **99**:340-343, 1984.

Besterman, J.M., May, W.S., Jr., Le Vine, H., III, Cragoe, E.J., Jr. and Cuatrecasas, P. Amiloride inhibits phorbol ester-stimulated Na^+/H^+ exchange and protein kinase C. An amiloride analog selectively inhibits Na^+/H^+ exchange. J. Biol. Chem. **260**: 1155-1159, 1985.

Bolton, T.B. Mechanisms of action of transmitters and other substances on smooth muscle. Physiol. Rev. **59**: 606-718, 1978.

Bolton, T.B. and Kitamura, K. Evidence that ionic channels associated with the muscarinic receptor may admit calcium. Br. J. Pharmacol. **78**: 405-416, 1983.

Bose, R. and Bose, D. Excitation-contraction coupling in multiunit tracheal smooth muscle during metabolic depletion: induction of rhythmicity. Am. J. Physiol. **233**: C8-C13, 1977.

Briggs, A.H. Calcium movement during potassium contracture in isolated rabbit aortic strips. Am. J. Physiol. **203**: 849-852, 1962.

Coburn, R.F. and Yamaguchi, T. J. Membrane potential dependent and independent tension in the canine tracheal muscle. Pharm. Exp. Therap. **201**: 276, 1977.

Coburn, R.F. Electromechanical coupling in canine trachealis muscle: acetylcholine contractions. Am. J. Physiol. **263**: C177, 1979.

Cuthbert, A.W., and Shum, W.K. Characteristics of the entry process for sodium in transporting epithelia as revealed with amiloride. J. Physiol. (Lond.) **255**: 587-604, 1976.

Cuthbert, A.W., and Wong, P.Y.D. The role of calcium ions in the interaction of amiloride with membrane receptors. Mol. Pharmacol. **8**: 222-229, 1972.

Davis, R.J. and Czech, M.P. Amiloride directly inhibits growth factor receptor tyrosine kinase activity. J. Biol. Chem. **260**: 2543-2551, 1985.

Dubinsky, W.P., and Frizzel, R.A. A novel effect of amiloride on H^+ dependent Na^+ transport. Am. J. Physiol. **245**: C157-C159, 1983.

Debetto, P., Floreani, M., Carpendo, F., and Luciani,S. Inhibition of the Na^+/Ca^{2+} exchange in cardiac sarcolemmal vesicles by amiloride. Life Sci. **40**: 1523-1530, 1987.

Ehrlich, B.E. and Watras, J. Inositol 1,4,5-trisphosphate activates a channel from smooth muscle sarcoplasmic reticulum. Nature **336**: 583-586, 1988.

Evans, D.H.L. and Schild, H.O. Mechanism of contraction of smooth muscle by drugs. Nature (Lond.) **180**: 341-342, 1957.

Evans, D.H.L., Schild, H.O., and Thesleff, S. Effects of drugs on depolarized plain muscle. J. Physiol. (Lond.) **143**: 474-485, 1958.

Filo, R.S., Bohr, D.F. and Ruegg, J.C. Glycerinated skeletal and smooth muscle: calcium and magnesium dependence. Science **147**: 1581-1583, 1965.

Gabella, G. Innervation of airway smooth muscle: fine structure. Annu. Rev. Physiol.**49**:583-594, 1987.

Glitzer, M.S. and Steelman, S.L. N-amidino-3,5-diamino-6-chloropyrazine-carboxamide: an active diuretic in the carboxamide series. Nature (Lond.) **212**: 191-193, 1966.

Grinstein, S., Clarke, C.A., Rothstein, A. Activation of the Na^+/H^+ exchange in lymphocytes by osmotically induced volume changes and by cytoplasmic acidification. J. Gen. Physiol. **82**: 619-657, 1983.

Hinke, J.A.M. Calcium requirements for noradrenaline and high potassium ion contraction in arterial smooth muscle. In Muscle, ed. by W.M. Paul, E.E. Daniel, C.M. Kay and G. Monckton, pp. 269-285, Pergammon Press, New York, 1965.

Howard, M.J., Mullen, M.D., and Insel, P.A. Amiloride interacts with renal alpha and beta adrenergic receptors. Am. J. Physiol. **253**: F21-F25, 1987.

Hudgins, P.M. and Weiss, G.B. Differential effects of calcium removal upon vascular smooth muscle contraction induced by norepinephrine, histamine and potassium. J. Pharm. Exp. Therap. **159**: 91-97, 1967.

James, A.L., Pare, P.D. and Hogg, J.C. The mechanics of airway narrowing in asthma. Am. Rev. Respir. Dis. **138**: 242-246, 1989.

Kahn, A.M., Shelat, H. and Allen, J.C. Na^+/H^+ is present in sarcolemmal vesicles from dog superior mesenteric artery. Am. J. Physiol. **250**: H313-H319, 1986.

Kannan, M.S. and Daniel, E.E. Formation of gap junctions by treatment in vitro with potassium conductance blockers. J. Cell. Biol. **78**: 348-358, 1978.

Kannan, M.S. and Daniel, E.E. Structural and functional study of control of canine tracheal smooth muscle. Am.J. Physiol. **238**: C27-C33, 1980.

Kannan, M.S., Davis, C., Ladenius, A.R.C., and Kannan, L. Agonist interactions at the calcium pools in skinned and unskinned canine tracheal smooth muscle. Can. J. Physiol. Pharmacol. **65**: 1780-1787, 1987.

Kleyman, T.R. and Cragoe, E.J. Jr. Amiloride and its analogs as tools in the study of ion transport. J. Memb. Biol. **105**: 1-21, 1988.

Knauf, H., Simon, B., and Wais, U. Non-specific inhibition of membrane ATPase by amiloride: a comparative in vivo and in vitro study with ouabain. Naunyn-Schmeideberg's Arch. Pharmacol. **292**:189-192, 1976.

Kramer, J.M. Substituted guanidines. Chemical Abstracts **62**: 14698f, 1965.

Kroeger, E.A. and Stephens N.L. Effect of tetraethylammonium ion on tonic airway smooth muscle: initiation of phasic electrical activity. Am. J. Physiol. **228**: 633-636, 1975.

Macklem, P.T. and Engel, L.A. The physiological implications of airway smooth muscle constriction. Post. Grad. Med. J. **51**(suppl. 7): 45-50, 1975.

Mahnensmith, R.L. and Aronson, P.S. The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. Circ. Res. **56**: 773-788, 1985.

Maxwell, D. Drug therapy of asthma. Drug Protocol **2**: 9-15, 1987.

Meurs, H., Roffel, A.F., Postema, J.B., Timmermans, A., Elzinga, C.R.S., Kaufman, H.F., and Zaagsma, J. Evidence for a direct relationship between phosphoinositide metabolism and airway smooth muscle contraction induced by muscarinic agonists. Eur. J. Pharmacol. **156**: 271-274, 1988.

Nathaniel, V.E.C. A quantitative ultrastructural study of isolated canine tracheal smooth muscle and its innervation. M.Sc. Thesis, University of Manitoba, 1981.

Nelson, H.S. Adrenergic therapy of bronchial asthma. J. Allergy Clin. Immunol. **77**: 771-785, 1986.

Ozaki, H., Kojima, T., Moriyama, T., Karaki, H., Urakawa, N., Kohama, K., and Nonomura, Y. Inhibition by amiloride of contractile elements in smooth muscle of guinea pig taenia cecum and chicken gizzard. J. Pharm. Exp. Therap. **243**: 370-377, 1987.

Russell, J.A. Responses of isolated canine airways to electrical stimulation and and acetylcholine. J. Appl. Physiol.: Respirat. Environ. Exercise Physiol. **45**: 690-698, 1978.

Ralph, R.K., Smart, J., Wojik, S.M., McQuillan, J. Inhibition of mouse mastocytoma protein kinases by amiloride. Biochem. Biophys. Res. Commun. **104**: 1054-1059, 1982.

Schellenberg, G.D., Anderson, L., and Swanson, P.D. Inhibition of Na^+-Ca^{2+} exchange in rat brain by amiloride. Mol. Pharm. **24**: 251-258, 1983.

Siegl, P.K.S., Cragoe, E.J.Jr., Trumble, M.J., and Kaczorowski, G.J. Inhibition of Na^+/Ca^{2+} exchange in membrane vesicles and papillary muscle preparations from guinea pig heart by analogs of amiloride. Proc. Natl. Acad. Sci. USA **81**: 3238-3242, 1984.

Smith, R.L., Cochran, D.W., Gund, P. and E.J. Cragoe, Jr. Proton, carbon-13, and nitrogen-15 nuclear magnetic resonance and CNDO/2 studies on the tautaumerism and conformation of amiloride, a novel acylguanidine. J. Am. Chem. Soc. **101**:191-201, 1979.

Smith, R.L., Macara, I.G., Levenson, R., Housman, D., and Cantley, L. Evidence that a Na^+/Ca^{2+} anitport system regulates murine erythroluekemia cell differentiation. Jour. Biol. Chem. **257**: 773-780, 1982.

Soltoff, S.P., and Mandel, L.J. Amiloride directly inhibits the Na, K-ATPase activity of rabbit kidney proximal tubules. Science **220**: 957-959, 1983.

Somlyo, A.V. and Aomlyo, A.P. Electromechanical pharmacomechanical coupling in vascular smooth muscle. J. Pharm. Exp. Therap. **159**: 129-145, 1968.

Somlyo, A.P. Excitation contraction coupling and the ultrastructure of smooth muscle. Circ. Res. **57**: 497-507, 1985.

Stephens, N.L. Airway smooth muscle. Am. Rev. Respir. Dis. **135**: 960-975, 1987.

Suematzu, E., Hirata, M., Hashimoto, T. and Kuryama, H. Inositol 1,4,5-trisphosphate releases calcium from intracellular storage sites in skinned single cells or porcine coronary artery. Biochem. Biophys. Res. Comm. **120**: 481-485, 1984.

Suzuki, H., Morita, K., and Kuriyama, H. Innervation and properties of the smooth muscle of the dog trachea. Jap. J. Physiol. **26**: 303-320, 1976.

Tang, C.-M., Presser, F., and Morad, M. Amiloride selectively blocks the low threshold (T) calcium channel. Science **240**: 213-215, 1988

Walker, J.W., Somlyo, A.V., Goldman, Y.E., Somlyo, A.P. and Trentham, D.R. Kinetics of smooth and skeletal muscle activation by laser pulse phosphorylation of caged inositol 1,4,5-trisphosphate. Nature **327**: 249-252, 1987.

Weiner, I.M. and Mudge, G.H. "Diuretics and other agents employed in the mobilization of edema fluid." *in* The Pharmacological Basis of Therapeutics, 7th ed. (A. Goodman Gilman, L.S. Goodman, T.W. Rall, and F. Murad, eds.) Mcmillan Publishing Co., New York, 1985, pp. 887-907.

Weiss, G.B., Pang, I.-H., and Goodman, F.R. Relationship between ^{45}Ca movements, different calcium components and responses to acetylcholine and potassium in tracheal smooth muscle. J. Pharm. Exp. Therap. **233**: 389-394, 1985.

Williams, M.H. Jr. Why do the airways contain smooth muscle? Lung **159**: 291-293, 1981.

Widdicombe, J.G. and Nadel, J.A. Airway volume, airway resistance and the work and force of breathing: theory. J. Appl. Physiol. **18**: 863-868, 1963.

THESIS OBJECTIVES

No research begins in a vacuum. The original idea to examine the effect of amiloride on canine airway smooth muscle arose primarily for Dr. Bose's longstanding interest in ion transport abnormalities associated with genetic hypertension, and my own limited background in airway smooth muscle physiology. An original observation by Haddy *et al.* (1985) suggested that amiloride - a potassium-sparing diuretic and putative Na^+/H^+ exchange inhibitor - had potent vasodilatory effects in the dog and in the rat. Based on these observations, we performed a preliminary study of the effect of amiloride on contraction of isolated canine trachealis strips, to determine if the drug would effect the contraction of non-vascular smooth muscle. It soon became apparent that amiloride also had potent relaxant effects in airway smooth muscle. A systematic study of the actions of amiloride on the canine trachealis was therefore initiated. The initial objective of this study was to document the effect of amiloride on the contraction of isolated canine airway smooth muscle in vitro, followed by an examination of the mechanism of amiloride's relaxant actions. Since amiloride is known to be an inhibitor of the plasmalemmal Na^+/H^+ exchanger, a process which contributes towards intracellular pH regulation in smooth muscle, it was necessary to design a method for the measurement of intracellular pH. To correlate any effect of intracellular pH disturbances on muscle function, however, it was necessary to be able to measure contractile activity simultaneously. Thus, the method described in Part II of the thesis was developed. The final objective of this work was to determine whether the relaxant effect of amiloride in vitro would be carried over in vivo. The study outlined in Part IV was designed to examine the possibility that amiloride - when applied as an aerosol - could relax an induced bronchoconstriction in the anaesthetized dog, and thus establish in a limited way, whether amiloride possesses therapeutic potential as a bronchodilatory drug.

PART I: THE RELAXANT EFFECT OF AMILORIDE ON CANINE TRACHEAL SMOOTH MUSCLE

ABSTRACT

Amiloride, a K+-sparing diuretic, relaxed canine tracheal smooth muscle strips contracted isometrically with high potassium (KCl), carbachol, serotonin and histamine. This indicated that relaxation was not linked to an interaction with an agonist specific receptor. Amiloride-induced relaxation was also not mediated through the production of relaxant prostaglandins, or by the endogenous release of catecholamines. During potassium contractions, amiloride addition produced a slow monophasic, dose-dependent relaxation (IC_{50} = 12.3 uM). In carbachol contracted strips, 1 and 10 uM amiloride induced a slow monophasic relaxation. With 35 to 250 uM, an initial rapid phase (IC_{50} = 75.5 uM) was superimposed onto this slow phase (IC_{50} = 23.5 uM), producing a biphasic relaxation. The rates of relaxation decreased with increased external [Na^+] regardless of stimulus, suggesting possible competitive inhibition of a sodium-dependent process. Exposure of carbachol contracted strips to increased external [H^+] caused a rapid decline in tension followed by a recovery phase. Tension maintenance during potassium contraction transiently decreased to a much lesser extent upon the addition of acid. Amiloride (100 uM) depressed tension recovery after acid exposure in both cases. Based on the known actions of this drug, inhibition of the Na^+-H^+ antiporter appears to be consistent with these data. This suggests amiloride may well belong to a new class of smooth muscle relaxants.

INTRODUCTION

Until recently, the clinical usefulness of amiloride as an antihypertensive drug has been entirely attributed to its properties as a K^+-sparing diuretic. The proposed mechanism of diuresis and natriuresis involves the inhibition of electrogenic Na^+ entry by this compound, via blockade of specific epithelial Na^+ channels present on specialized cells of the distal tubules (Benos, 1982). While diuresis may still play a primary role in the antihypertensive actions of this drug, there is increasing evidence which suggests that amiloride may also have a direct vasodilatory effect. This has been demonstrated *in vivo* by Haddy *et al.* (1985), who have observed that intra-arterial bolus injection of amiloride (100 to 400 ug) into the dog forelimb resulted in a "prompt, transient, dose dependent decrease in perfusion pressure" under constant flow conditions. These authors suggest that this may be due to blockade of putative sodium channels in vascular smooth muscle, although this is difficult to assess in such a complex system.

Vasorelaxant effects have also been observed in isolated vascular preparations. Palaty (1986) has shown that amiloride relaxes isolated rat caudal artery strips contracted by exposure to the alkaloid veratridine, an effect which he attributes to possible alpha-antagonist activity of amiloride. Pinon *et al.* (1985) have demonstrated that amiloride reduced contraction of a rat aortic strip preparation stimulated with a depolarizing potassium solution in a concentration dependent manner. These authors suggest that inhibition of Na^+-Ca^{2+} exchange by amiloride might be responsible for the observed effect.

Demonstration of the relaxant effect of amiloride has not been limited to vascular preparations alone. Akhtar-Khavari *et al.* (1981) showed that the presence of this compound decreased the response of rat vas deferens to electrical stimulation, citing inhibition of noradrenaline release via sodium channel blockade as a probable explanation. Amiloride also affects the contraction of guinea pig airway smooth muscle, as observed by Souhrada *et al.* (1985). These authors, who also utilized this compound as a sodium channel blocker, indicated

that amiloride could prevent contraction to ovalbumin in an *in vitro* sensitized tracheal preparation.

A thorough investigation of the effect of amiloride on airway smooth muscle contraction is of obvious practical interest, specifically as it relates to a possible therapeutic use in the treatment of bronchospastic diseases. Therefore, one purpose of our study was to document the effect of amiloride on the contraction of canine tracheal smooth muscle. An additional rationale for such an investigation is also apparent. The observations cited above, when taken together, appear to indicate that amiloride has a consistent relaxant effect on smooth muscle tissue. Yet the proposed mechanisms of this relaxation understandably lack such consistency because each study has employed a different form of stimulation in a different tissue preparation. The fact that amiloride relaxes various smooth muscle types in diverse species with dissimilar forms of stimulation, suggested to us that this drug may be having a broader effect on the process of smooth muscle contraction, possibly by inhibiting a common event in excitation contraction coupling. In order to elucidate this possibility, we studied the effects of this compound on contractions elicited by different modes of excitation-contraction coupling in a single tissue. It was therefore the purpose of the current study to document and characterize the previously undescribed non-specific relaxant effect of amiloride on canine respiratory smooth muscle. Some of these data have been presented in preliminary form (Krampetz and Bose, 1987*a* and 1987*b*).

MATERIALS AND METHODS

Tissue dissection and preparation

Tracheae were removed from dogs of either sex immediately after lethal KCl injection to the heart, and were quickly placed in cold Krebs-Henseleit solution. (The animals had been previously anaesthetized with pentobarbital.) Smooth muscle, dissected free of epithelium, cartilage and connective tissue, was cut into strips (approximately 1-2 mm x 1mm x 10 mm dimensions). These strips were then placed in identical 10 ml capacity muscle baths containing oxygenated Krebs-Henseleit solution (pH =7.41, 37° C, aerated with 95% O_2-5% CO_2, minimum PO_2= 400 Torr, PCO_2= 40±5 Torr). Tissue strips were suspended vertically by attachment to a surgical steel wire loop at one end and to a mechanical force transducer (Grass FT03) at the other via 5.0 silk thread. Signals were amplified and recorded with a Gould 260 6-channel chart recorder. Preload was initially adjusted to approximately 3 g, which resulted in a passive tension of approximately 1-2 g after the 1 hour equilibration period.

Experimental Solutions

The composition of the Krebs-Henseleit solution was (in mM): NaCl, 118; KH_2PO_4, 1.4; $MgCl_2$, 1.2; Glucose, 11; KCl, 4.7; $NaHCO_3$, 25; $CaCl_2$, 2.5.

Compounds were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. Stock solutions were prepared from the following chemicals (concentrations in parentheses): amiloride hydrochloride (10 mM), atropine sulphate (0.1mM), carbamyl choline hydrochloride (0.1 mM), histamine hydrochloride (20 mM), propranolol hydrochloride (1 mM), serotonin hydrochloride (1 mM), indomethacin (10 mM, Merck, Sharp, & Dohme, Pointe Claire, Canada), sotalol hydrochloride (1 mM, Bristol-Meyers, Ottawa, Canada), and potassium chloride (4 M, Fisher Scientific, Winnipeg, Canada). All solutions were prepared using distilled deionized water, except indomethacin, which was suspended in ethanol.

Experimental Procedures

1. Addition of amiloride to contracted muscle.

Muscle strips were stimulated by the addition of exogenous agonists (final concentrations in parentheses), specifically potassium chloride (80mM)*, carbachol (CCh, 1 uM), histamine (10 uM), or serotonin (10 uM). Atropine (1 uM) was included in the bathing solution of the potassium-, histamine-, and serotonin-stimulated muscle strips to prevent contraction due to acetylcholine released from cholinergic nerve endings. For potassium- and carbachol-induced contractions, muscle strips were contracted isometrically until stable and reproducible tensions were produced. Amiloride (1 to 150 uM) was added during peak contraction. For histamine and serotonin-induced contractions, such stable and reproducible tensions could not be achieved, therefore amiloride was added within 10 minutes of onset of contraction. In the initial series of experiments, propranolol or sotalol (10 uM), and indomethacin (1 uM) were included in the bathing solution, in order to eliminate the possibility that amiloride-induced relaxation was mediated via beta-adrenergic agonist release or through the production of relaxant prostaglandins. As it became obvious that inclusion of these compounds had no effect, they were subsequently omitted from the bathing medium.

2. Dose-response study.

The ability of amiloride to relax both potassium and carbachol contracted muscle strips was studied in a dose related fashion using the following concentrations of the drug: 1, 4, 6, 10, 15, 35, 50, 75, 100, 150 uM, and 250 uM. A different concentration of amiloride was added to each of 6 strips (taken adjacently from the same tracheal tissue) and the subsequent relaxation

* Note that the NaCl concentration of the medium was not altered to accomodate the additional KCl. Physiological sodium concentrations were maintained throughout unless specified since the response to amiloride was affected by the concentration of Na^+ in the medium.

was recorded. The drug and stimulant were washed out, and the muscle strip was repeatedly washed and contracted until original tension was recovered. Original tension traces of amiloride-induced relaxations were digitized using a Houston Instruments digitizing tablet (HIPAD^{TM}). Each of the data points obtained was transformed to a logarithmic value, resulting in a log tension versus time plot of relaxation. The lines of best fit through these points were determined using the "ASYST" computer program (MacmillanSoftware Co., NY) on a MIND II-XT, IBM^{TM} compatible computer (Mind Computers, Winnipeg, Canada). The slopes of these transformed data points (expressed as log tension units per minute) were also calculated.

3. Simultaneous addition of amiloride with KCl or carbachol.

In another series of experiments the same range of amiloride doses previously described were added simultaneously with the contractile stimulus, either potassium (80 mM) or carbachol (1 uM), in order to assess the effect on tension development. Peak response was measured as percentage of maximum control tension.

4. Elevation of extracellular sodium ion concentration

The effect of increased external sodium concentration on amiloride-induced relaxation was investigated in muscle strips contracted with potassium (plus 1 uM atropine) or carbachol. At plateau tension, an additional 30mM NaCl was added to the bathing medium. This was followed by the addition of either 10 or 100 uM amiloride. Relaxation rates were analyzed as previously described and were compared with control values obtained from the same strips in the presence of normal sodium concentration (143 mM).

5. Elevation of extracellular hydrogen ion concentration

The effect of decreased external pH on canine tracheal smooth muscle contraction initiated by potassium or carbachol was investigated. This was accomplished with the addition of a 10ul aliquot of glacial acetic acid to the bathing medium (10 ml volume) during peak contraction, resulting in a final pH of 6.6±0.1.

In another experimental series amiloride (10 uM or 100uM) was added simultaneously with the acid. Analysis of these data consisted of digitization of the traces and computation of the area under the tension-time curve for a 25 minute period after acid addition. Treatment values were expressed as percent of control area.

Statistical Procedures

Data were analyzed statistically using the Student's t-test or Duncan's New Multiple Range (NMR) test, as indicated in the figure legends. Significant differences were considered at p values of less than or equal to 0.05. All results are expressed as mean ± SEM.

RESULTS

1. Addition of amiloride to contracted muscle.

Addition of amiloride (1 to 250 uM) relaxed the tracheal smooth muscle contracted with carbachol, potassium chloride, serotonin, or histamine (Figure 2). To assess whether this observed relaxation was mediated through the production of relaxant prostaglandins, or by the release of beta adrenergic agonists we examined the effect of the inclusion of indomethacin (1 uM) and the beta-blocking drugs, propranolol (1 uM) or sotalol (10 uM), on amiloride-induced relaxation. There was no observed difference in the response of this tissue to amiloride (data not shown).

2. Dose-response study.

While amiloride relaxed the strips regardless of the form of stimulation, the time course of amiloride-induced relaxation appeared to be different for each agonist. The greatest contrast in this relaxation appeared between the carbachol and potassium contracted strips. As these agonists have been shown to initiate contraction via different modes (electromechanical versus primarily pharmacomechanical coupling; Farley and Miles, 1977 and 1978), they were chosen for subsequent dose response studies. In addition, these contractions -- unlike those produced with serotonin or histamine -- are highly reproducible and uncomplicated by decay, thereby allowing for a measurement of the rate of relaxation due solely to amiloride addition.

In order to examine whether the observed differences in relaxation between these two forms of stimulation were related to the dose of amiloride added to the bathing medium (thus implying a difference of sensitivity to this compound between these contractions), we studied the concentration dependence of this relaxant effect for potassium- and carbachol-contracted muscles (Figures 3 and 4).

The digitization and manipulation of the relaxation traces resulting from amiloride addition to these contractions showed that they were indeed quite different. In the potassium

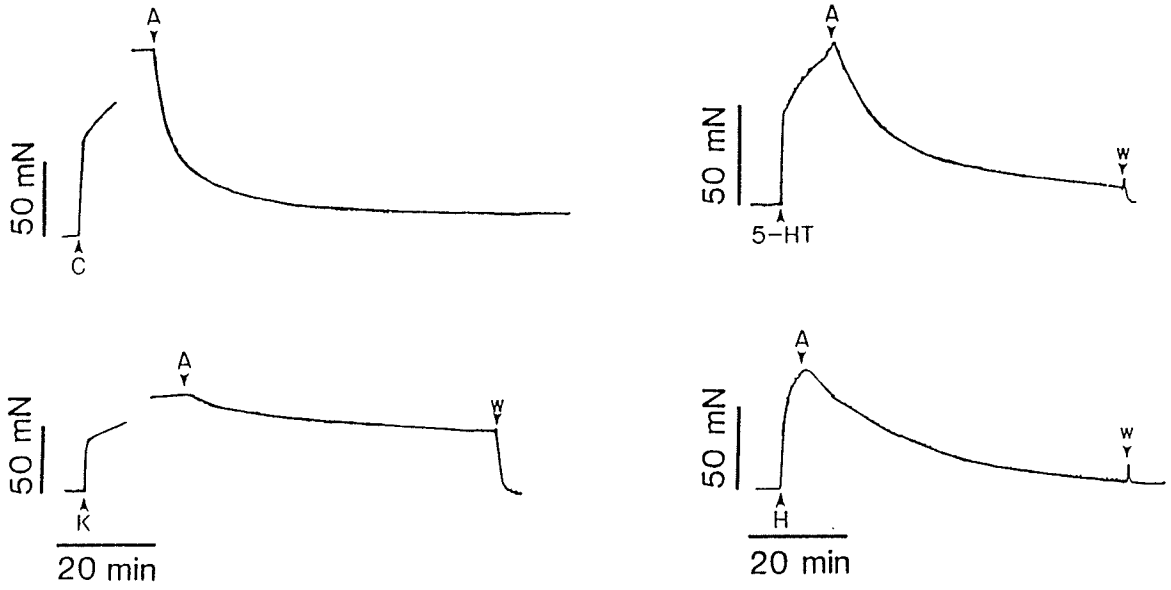

Figure 2: Isometric tension-time traces for tracheal smooth muscle strips contracted with the following agonists: 1 uM carbachol (C), 80 mM KCl (K), 10 uM serotonin (5-HT), or 10 uM histamine (H). All but the carbachol-stimulated strip were contracted in the presence of 1 uM atropine. Amiloride was added where indicated by A to a final concentration of 150 uM.

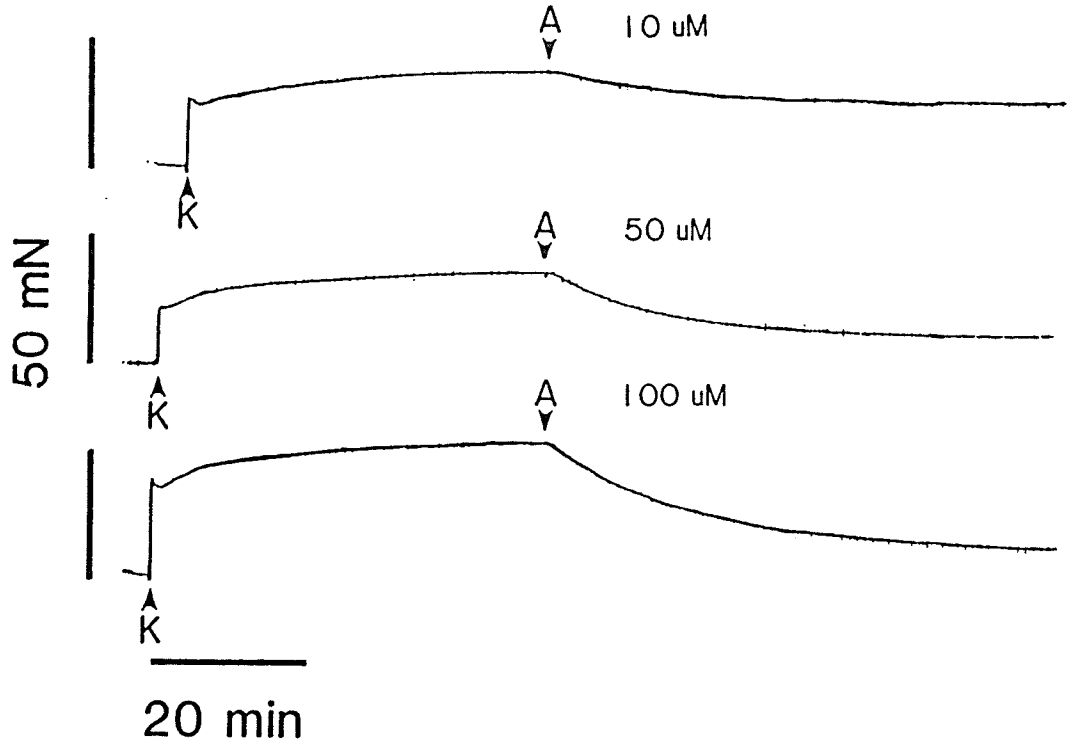

Figure 3: Isometric tension-time traces for 80 mM potassium (K)-induced contractions to which 10, 50, and 100 uM amiloride were added (A).

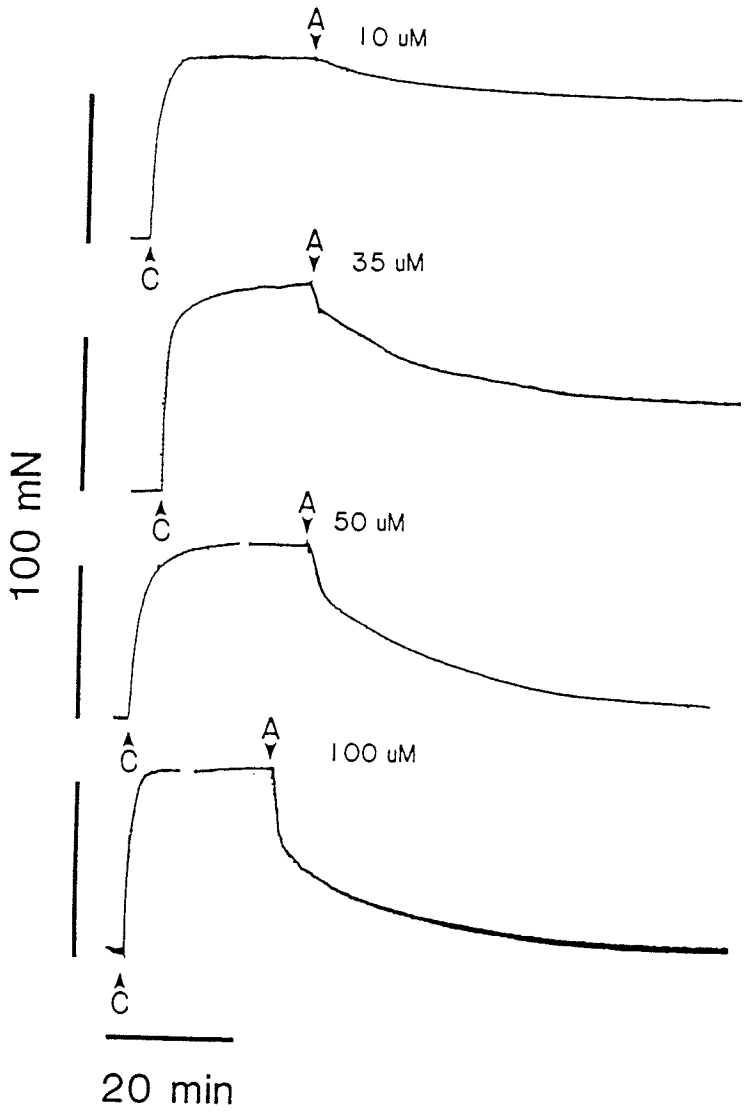

Figure 4: Isometric tension-time trace of tracheal muscle contracted with 1 uM carbachol (C), and relaxation responses to addition of 10, 35, 50 and 100 uM amiloride (A).

contracted muscle strips, semilogarithmic conversion of data points from the relaxation trace produced a linear plot, indicating an exponential function (Figure 5). The slope of this line, the rate of relaxation, was found to increase with greater concentration of the drug. Only one component of relaxation was apparent in the amiloride-induced relaxation of a potassium contraction. This was noted for all concentrations of drug tested (up to 150 uM). The time course of amiloride-induced relaxation in carbachol contracted muscles was considerably different from that observed in those muscles contracted with potassium (compare Figures 3 and 4). At low concentrations of amiloride (1 and 10 uM), an exponential monophasic decrease in tension was observed. However, at higher concentrations of amiloride an additional component of relaxation appeared, resulting in a biphasic relaxation characterized by an initial rapid drop in tension followed by a slow rate of tension decay (Figure 3). Semilogarithmic transformation and curve peeling of these data showed that this relaxation was consistently described by two linear components with distinct slopes (Figure 6). These were subsequently designated as the "fast" and "slow" phases of amiloride-induced relaxation.

The relationship between concentration of amiloride and rates of amiloride-induced relaxation during contraction with potassium, and rates of both phases found in carbachol-contracted muscle are summarized in Figure 7. The rates of relaxation observed during a potassium-induced contraction were not significantly different from those of the slow phase of relaxation of a carbachol contraction obtained with the same concentration of amiloride ($p<0.05$, two-sided Student t-test). This suggests that amiloride may be acting in these two modes of contraction via inhibition of a similar process. The rates of relaxation of the fast phase were significantly different from both the slow phase of the carbachol contraction and the rate of relaxation in potassium contracted muscle at amiloride concentrations greater than 75 uM ($p<0.05$, Duncan's NMR Test).

A logit analysis {*i.e.* log (%max/100-%max) versus log concentration} of these relaxation rates yielded IC_{50} concentrations of 12.3 uM amiloride for relaxation of potassium contractions and 23.5 uM for the slow phase relaxation of a carbachol contraction. The IC_{50}

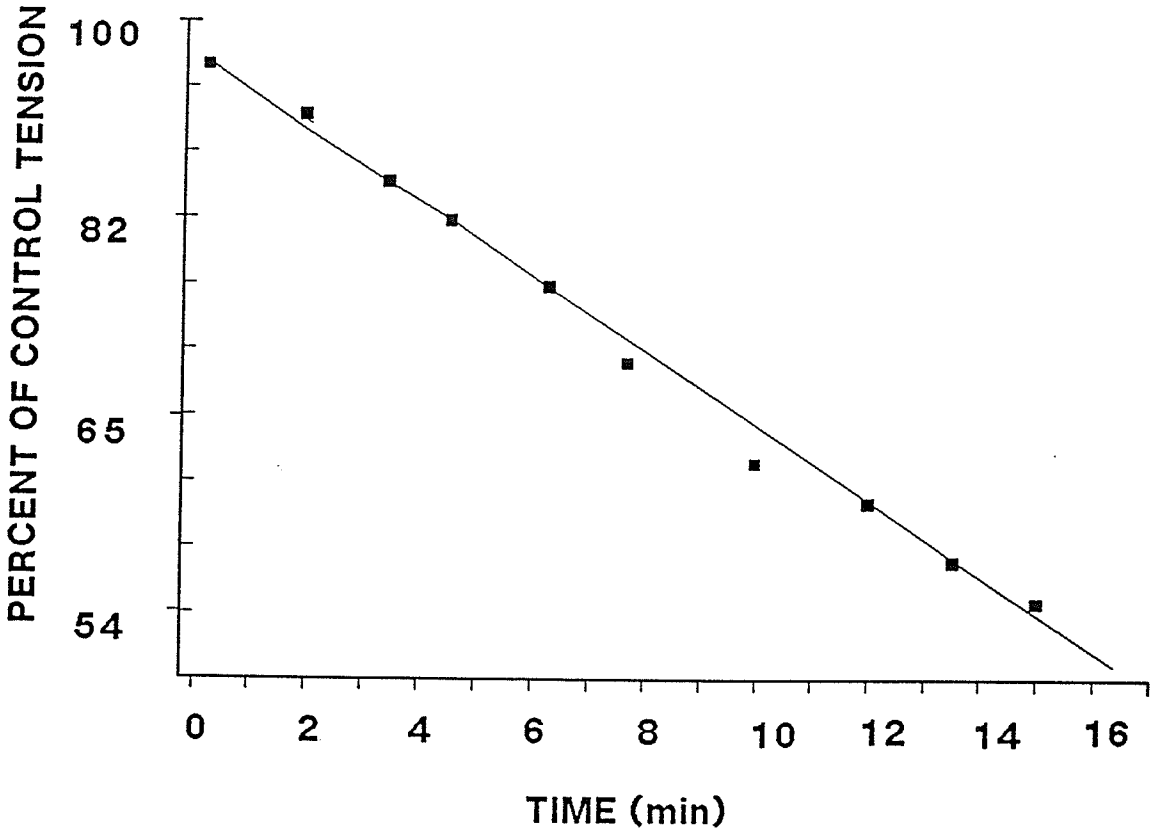

Figure 5: Semilogarithmic plot of digitized relaxation data obtained after addition of 50 uM amiloride to a KCl-contracted muscle. The decay is described by a single component.

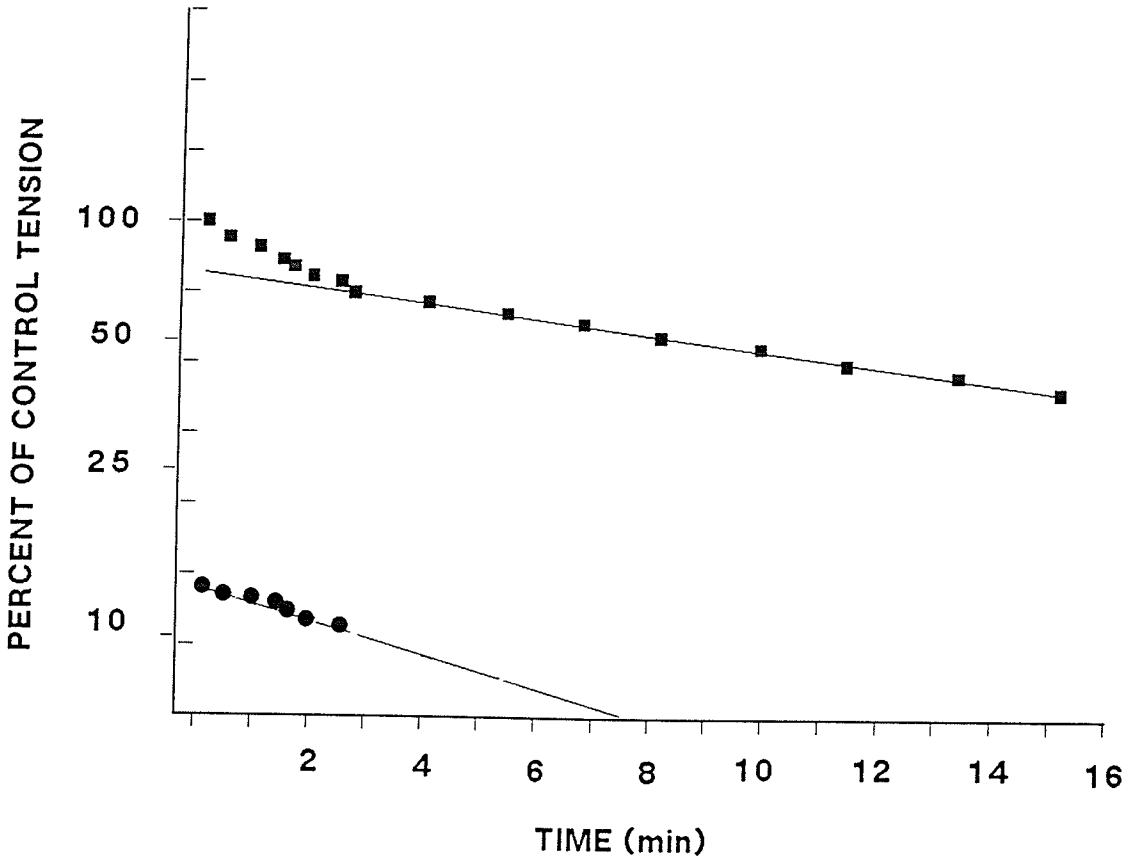

Figure 6: Semilogarithmic plot of digitized relaxation trace obtained after addition of 50 uM amiloride during a carbachol contraction. The tension decay is described by two components with distinct slopes. Upper line denotes line of best fit for "slow" relaxation phase (all points are shown). Lower line represents line of best fit for "fast" phase of relaxation ("peeled" points only).

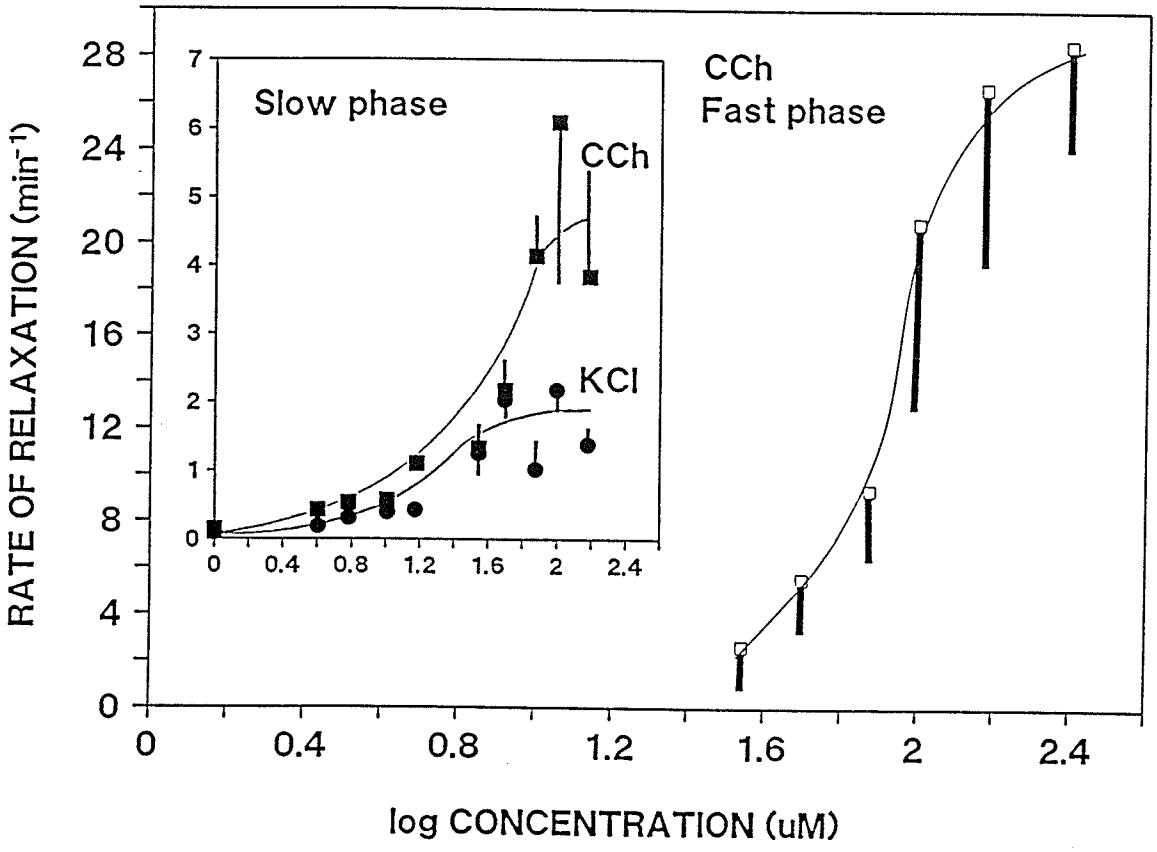

Figure 7: Effect of amiloride concentration on the rate of relaxation, expressed here as percent decrease in tension at any one minute interval. Solid circles denote relaxation rates in potassium contractions (inset). Number of dogs (n) within each group (in ascending order) were: 7, 6, 6, 6, 6, 7, 4, 6, 8, and 5. Solid squares denote data from slow phase relaxation observed in carbachol contracted strips in response to amiloride (inset). Number of dogs (n) = 5, 4, 6, 5, 5, 6, 7, 7, 7 and 6 for each group. Open squares represent data from fast phase of relaxation in carbachol contracted muscles (n = 3, 7, 7, 7, 6, and 3).

concentration for the fast relaxation phase of a carbachol contracted muscle was calculated to be 75.5 uM.

3. Simultaneous addition of amiloride with KCl or carbachol.

Since amiloride could relax a contracted muscle, it was important to assess if amiloride would reduce tension development in tracheal smooth muscle if present before stimulation. Again, we employed both potassium and carbachol as stimulants to evaluate whether differences existed. It was found that amiloride (in concentrations greater than 10 uM) significantly reduced the percentage of peak tension achieved for both potassium and carbachol-stimulated muscle strips when compared to control strips ($p<0.05$, Duncan's NMR Test). These data are summarized in Figure 8. However, at high concentrations of amiloride (100 and 150 uM) the muscle strips contracted with carbachol displayed a significantly greater percentage decrease in tension than the potassium-stimulated muscle strips ($p<0.05$). Upon examination of the original traces of this treatment, it could also be seen that at amiloride concentrations greater than 100 uM reduced the ability of the strips contracted with carbachol to maintain peak tension, while such an effect was never seen in potassium contracted strips (Figure 9).

4. Elevation of external sodium ion concentration

Most actions of amiloride reported to date implicate inhibition of sodium related processes. Since amiloride exists at physiological pH as a positive cation ($pK_a=8.7$), there is some evidence that it may inhibit these transport processes by a mechanism of competitive inhibition (as reviewed by Benos, 1982). In order to evaluate this possibility, the effect of increased external concentration of sodium on amiloride-induced relaxation was investigated. Figure 10 illustrates the results of this study. It was found that increased external concentration of this ion (173 mM versus 143 mM) significantly decreased the rate of amiloride-induced

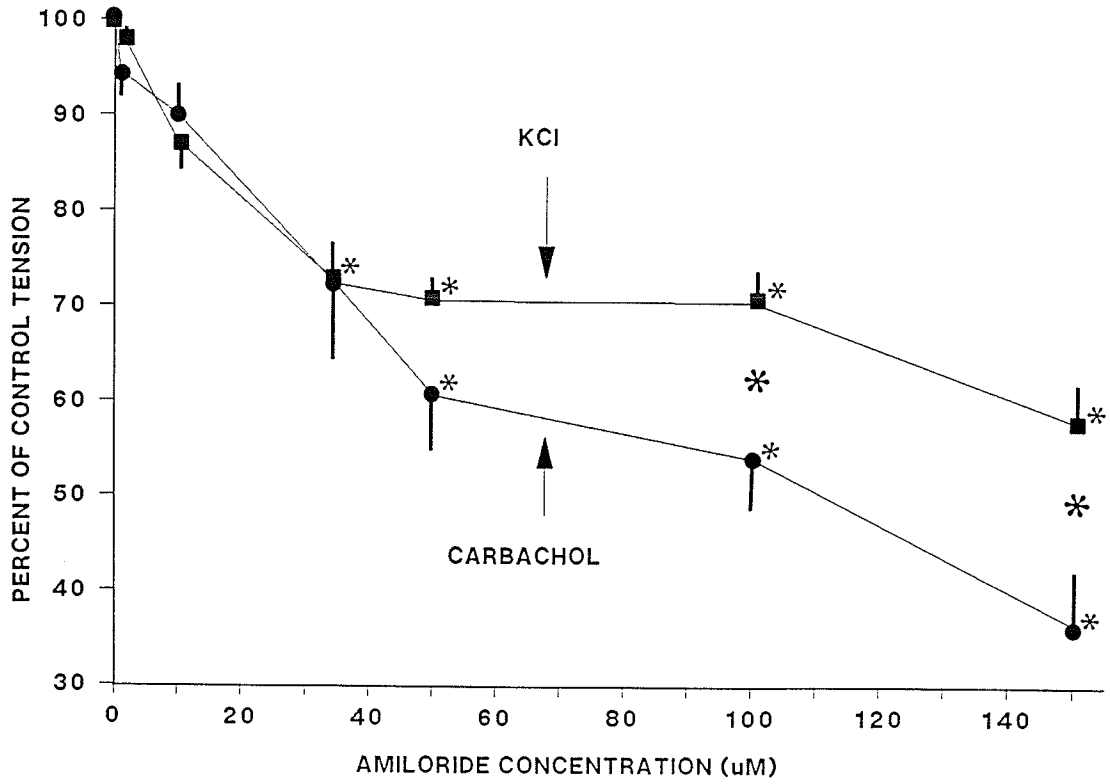

Figure 8: Percent maximal contraction achieved with stimulation in the presence of increasing amiloride concentration. A significant decrease from control values for both carbachol and KCl contractions was noted at concentrations of amiloride greater than 10 uM (small asterisks, $p<0.05$, Duncan's NMR test). Large asterisks represent significant differences between carbachol and potassium contracted muscle strips ($p<0.05$, unpaired t-test).

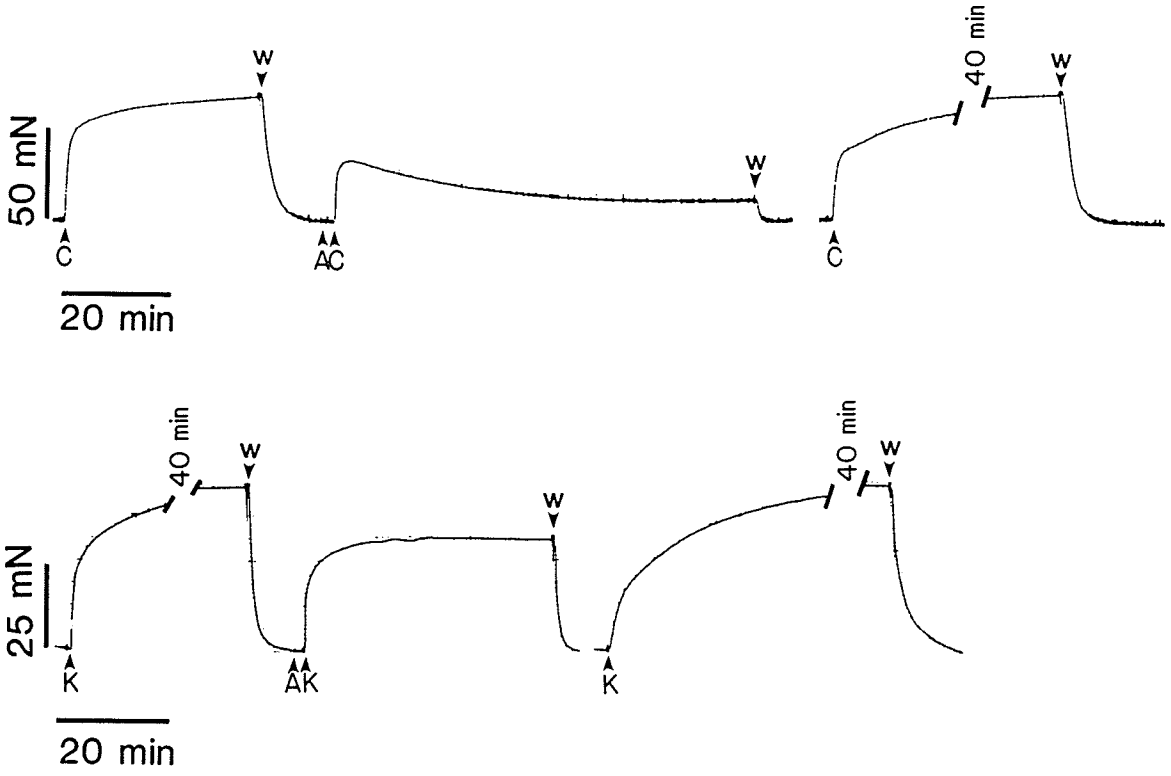

Figure 9: Tension-time trace showing the effect of the presence of amiloride on subsequent contraction. Upper trace shows a control carbachol contraction (C) followed by a contraction in the presence of 150 uM amiloride (A) and recovery of original tension with wash out of compound and restimulation. The lower trace follows a similar protocol for a muscle strip contracted with potassium (K). Note that in the presence of amiloride, the carbachol-stimulated strip was unable to maintain peak tension.

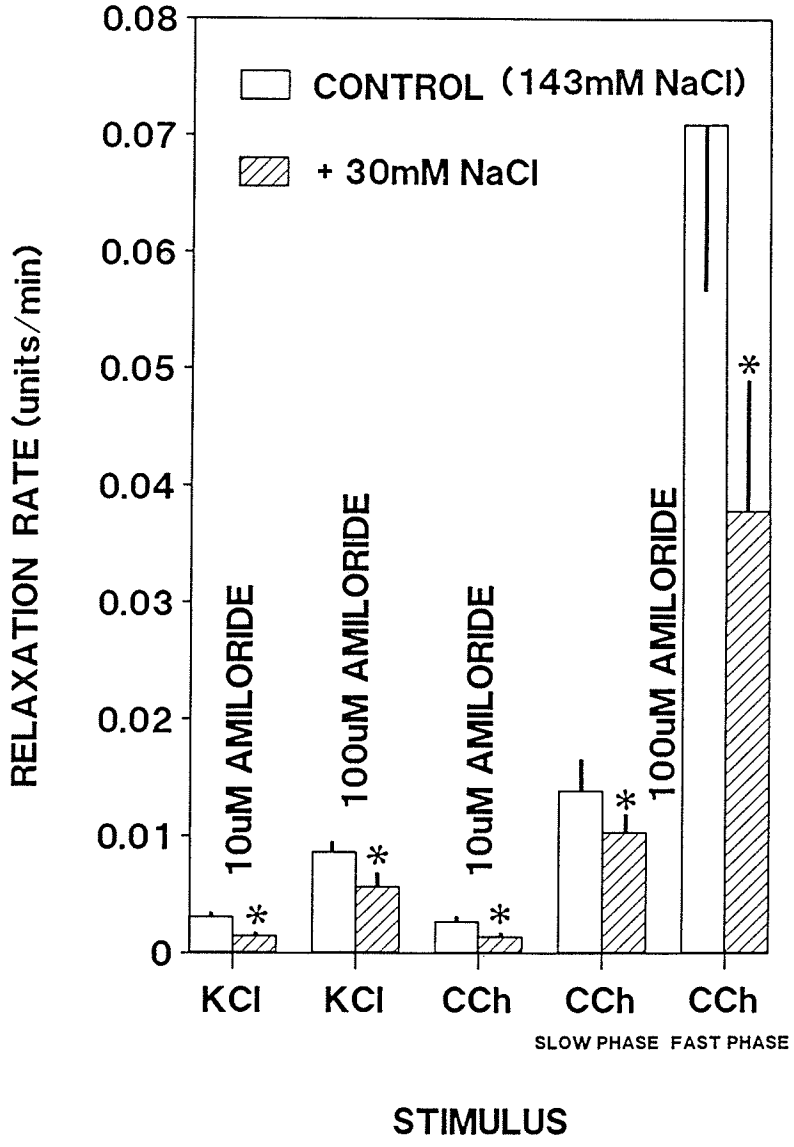

Figure 10: Effect of increased external sodium concentration on rate of amiloride-induced relaxation in 80 mM potassium-and 1 uM carbachol-stimulated muscle strips. Rates are shown for 10 and 100 uM amiloride concentrations. Asterisks denote significant difference from control values ($p<0.05$, one-sided paired t-test). Number of individuals per group were: 5 (KCl, 10 uM amiloride), 6 (KCl, 100 uM amiloride), 5 (CCh, 10 uM amiloride), and 7 (CCh, 100 uM amiloride for both fast and slow phases).

relaxation. (It should be noted that the addition of sodium chloride itself caused a small initial decrease in plateau tension, but had no effect on tension maintenance.) The rates of relaxation during potassium contraction and of both the fast and slow components of relaxation found in the carbachol-contracted muscle were attenuated. This occurred with the addition of both high (100 uM) and low (10 uM) concentrations of amiloride. Control treatments in which the addition of sucrose to the same osmolar concentration followed by the addition of amiloride did not produce these effects.

5. Elevation of extracellular hydrogen ion concentration

Inhibition of the Na^+-H^+ exchange process should also occur when the tracheal smooth muscle strips are exposed to to excess extracellular hydrogen ion concentrations. This would reduce or eliminate the normal outward gradient, and thereby discourage efflux via this pathway. Such an effect has been documented for the renal Na^+-H^+ antiporter (Aronson, 1985).

We have observed that the addition of acid to the external medium (pH_{ext} = 6.6±0.1) reduced the ability of both KCl and carbachol-contracted muscle strips to maintain tension. (This effect was proton specific, as treatment of equimolar, neutral acetate did not promote relaxation.) However, carbachol-contracted muscle strips were more profoundly affected. In these strips, the presence of excess extracellular protons produced an immediate and rapid drop in tension which was followed by a recovery phase in spite of continued low external pH (Figure 11). Analysis of the area under the tension-time trace for a 25 minute period after acid addition showed that it was approximately 60% that of control (Figure 12). Similar treatment of potassium-stimulated muscle showed that these contractions are much less sensitive to a decrease in external pH, with an area under the tension time curve corresponding to approximately 95% of control (Figure 12). Acid addition to KCl contractions, while producing a

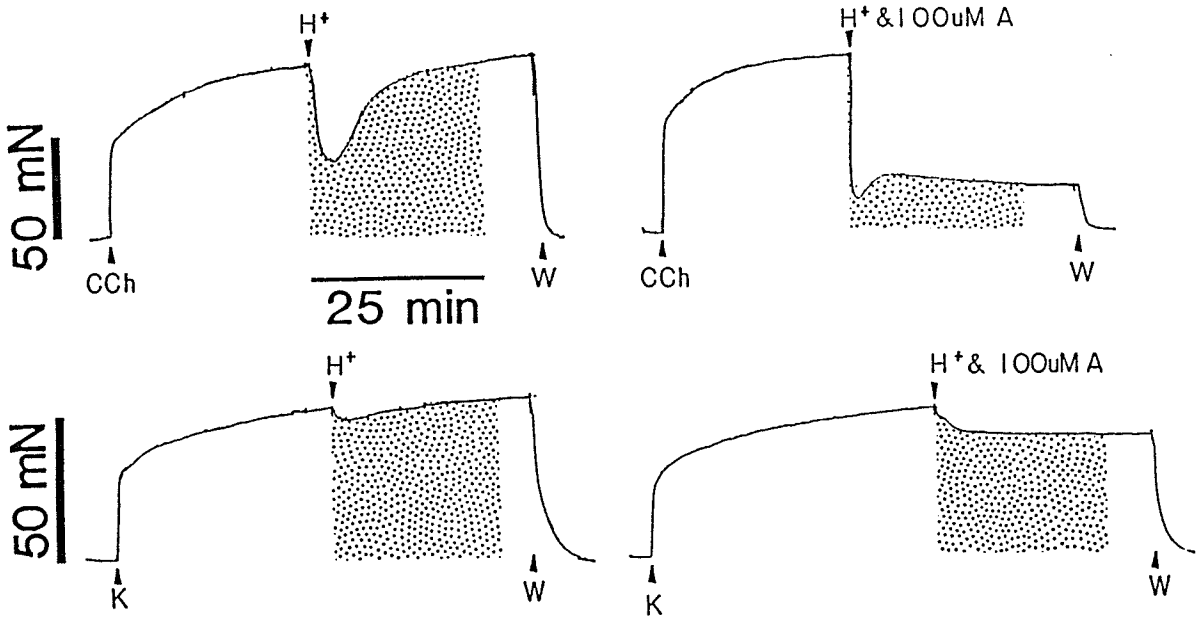

Figure 11: *Upper trace* The effect of acid addition (H^+) on tension maintenance in carbachol-stimulated muscle strips (left) and the effect of amiloride (100 uM) on recovery from the addition of acid in the same strips (right). Areas under the contraction profile which have been compared are shaded. *Lower trace* The effect of the addition of acid (H^+) on tension maintenance in potassium-stimulated muscle strips (left) and the effect of amiloride (100 uM) on recovery from acid addition in the same strips (right). Areas under the contraction profile which have been compared are shaded.

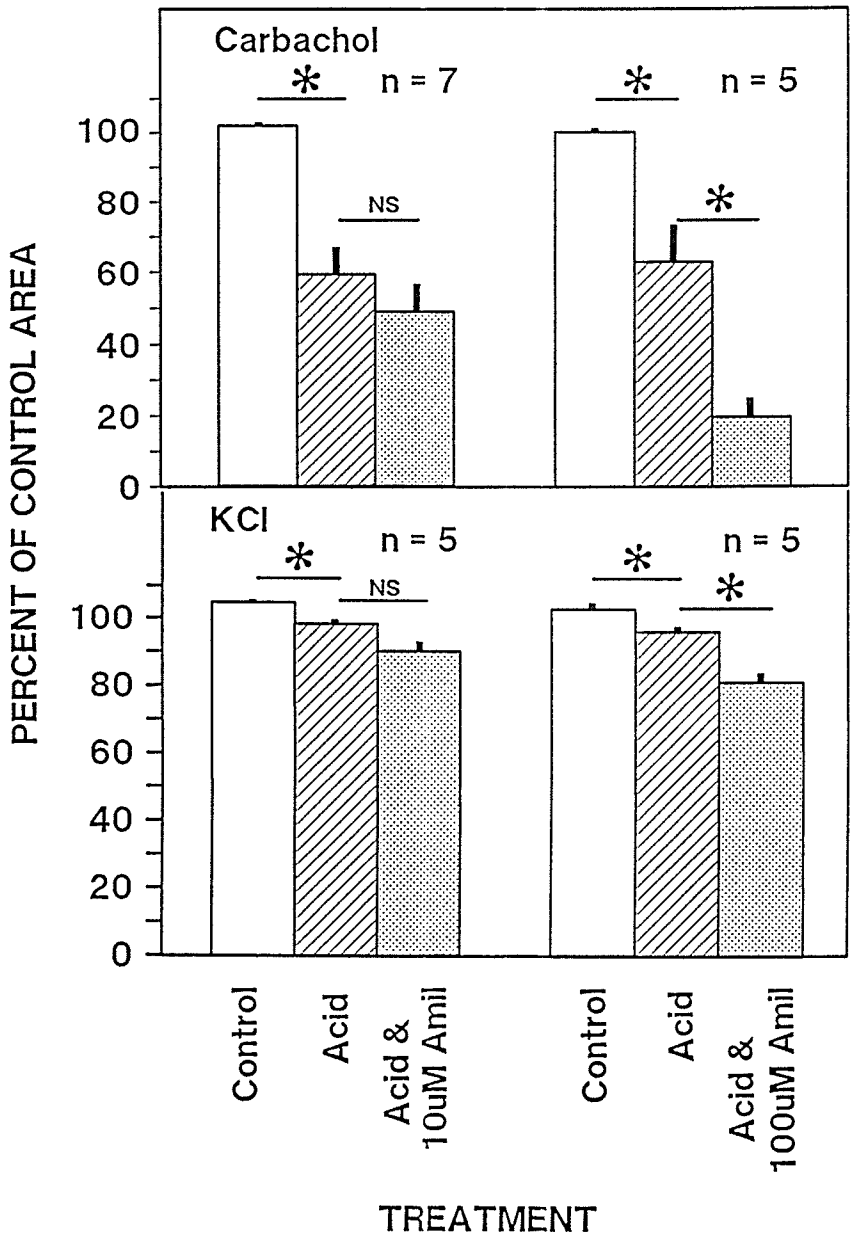

Figure 12: Summary and comparison of areas under the curve as represented in Figure 11, from strips contracted with carbachol (upper bars) and potassium (lower bars). Controls are expressed as percentage of ideal area of contraction profile for a 25 minute period. Statistical analysis employed Duncan's NMR test.

small decrease in tension, was also followed by recovery to original or slightly higher than original levels.

The recovery phase observed in these contractions may result from a compensatory activation of the Na^+-H^+ antiporter. Since amiloride inhibits the antiporter, we studied the effect of this compound on recovery from acid exposure. Amiloride was added simultaneously with the acid, at a concentration of either 10 uM or 100 uM. In both carbachol- and potassium-contracted muscle strips, it was found that the inclusion of 10 uM amiloride had no significant effect on the area under the recovery curve, indicating little or no inhibition of the recovery process at this concentration (Figure 12).

Addition of 100 uM amiloride with acid had a somewhat more marked effect. In the potassium contracted muscle, this treatment resulted in a small drop in tension, followed by a plateau at this new lower level (Figure 11). Comparisons of areas under the tension-time trace (Figure 12) showed that inclusion of 100 uM amiloride resulted in a significant reduction in area when compared to the strips treated with acid alone (80% of control versus 95%). Although tension did not recover under these conditions, as had been observed with acid treatment alone or with the inclusion of 10 uM amiloride, it should be noted that in the potassium contracted muscle strips only a small component of tension was eliminated.

The effect of addition of 100 uM amiloride along with the acid during a carbachol contraction showed a similar pattern to that seen in a potassium contraction, but with a more marked effect on tension (Figure 12.). Analysis of areas under the curve showed that the presence of this concentration of amiloride significantly decreased the extent of recovery from 63% with acid alone to 20% of control area with acid and 100 uM amiloride addition (Figure 12). In two out of five cases, amiloride inclusion completely abolished redevelopment of tension after acid exposure (data not shown).

DISCUSSION

The studies outlined in this paper suggest that amiloride relaxes canine tracheal smooth muscle via two different processes which have distinct IC_{50} values. The demonstration of these sites was dependent on the type of stimulation, with "high affinity" sites present during both potassium and carbachol contractions (IC_{50} = 12.3 and 23.5uM, respectively) and the "low affinity" amiloride-sensitive site appearing only with addition of high amiloride concentrations to carbachol-contracted muscle strips (IC_{50} = 75.5uM). Further support for an additional site in carbachol-stimulated muscle is provided by the pretreatment data, where amiloride was shown to limit these contractions to a greater extent. As in those strips treated with amiloride at plateau tension, it may be that the superimposition of an additional inhibitory process accounts for the greater effect on peak tension development in strips contracted with carbachol.

The attenuation of amiloride-induced relaxation in both KCl- and carbachol-contracted strips observed under conditions of increased external sodium concentration indicates that both of the aforementioned sites are sensitive to external sodium concentrations. This supports our contention that amiloride is interacting with one or more sodium dependent processes. This contention is not without basis, as there is considerable experimental evidence from a variety of cell and tissue preparations which demonstrates that amiloride is a selective inhibitor of passive sodium influx pathways (Benos, 1982).

As previously mentioned, the origin of the diuretic action of amiloride has been proposed to result from the blockade of high affinity sodium channels in specialized cells of the distal tubules. These channels are characterized by their extreme sensitivity to amiloride (K_i = 0.5 uM; Palmer, 1986). Similar high affinity epithelial type sodium channels have been described in other locales, including cells of the urinary bladder, descending colon, and in the ducts of the salivary and sweat glands (Benos, 1982). Their presence has not been clearly

demonstrated in mammalian smooth muscle. For this reason, and because inhibition of these channels by amiloride occurs at such low concentrations, we suggest that they are of minor importance in the relaxation phenomenon which we have described.

Additional amiloride-sensitive sites unique from the above sodium channels have been shown to be present in various tissues. The best documented amongst these is the sodium-hydrogen antiporter, which has been described in hepatocytes (Fuchs *et al.*, 1986), cells of the gastric gland (Paradiso *et al.*, 1984), neutrophils (Wright *et al.*, 1986), fibroblasts (Pouysegur *et al.*, 1982), and skeletal muscle (Klip *et al.*, 1986) to name but a few (reviewed in Mahnensmith and Aronson, 1985). In addition, inhibition of the sodium-calcium antiporter by amiloride has been demonstrated in at least three preparations: erythroleukemia cells (Smith *et al.*, 1982), rat brain synaptosomes (Schellenberg *et al.*, 1983), and bovine cardiac sarcolemmal vesicles (Debetto *et al.*, 1987). Sordahl *et al.* (1984) have shown inhibition of the mitochondrial sodium-induced calcium exchanger by this compound.

While Na^+/Ca^{2+} exchange has been repeatedly demonstrated in vascular smooth muscle (Ashida and Blaustein, 1987), the presence and importance sodium-calcium exchange in tracheal smooth muscle is unclear. Kirkpatrick and McDaniel (1976) reported that replacement of extracellular sodium with substitutes other than K^+ caused no appreciable contraction in bovine trachealis. Later, this lack of effect was reported by the same group to be dependent on the medium buffer employed, since repetition of this experiment in the presence of bicarbonate buffer (rather than Tris) did produce a small contracture (Bullock *et al.*, 1981). In light of these results, it is difficult to ascribe any effect of amiloride to inhibition of this process in canine tracheal smooth muscle.

Only the Na^+/H^+ exchange mechanism has been clearly shown in a canine smooth muscle preparation. Kahn *et al.* (1986) have demonstrated an amiloride sensitive, pH gradient-

stimulated, ^{22}Na uptake in sarcolemmal vesicles from dog superior mesenteric artery. Thus, it seems likely that such a mechanism could also be present in canine respiratory smooth muscle. Blockade of this process would lead to a decrease in sodium influx and an increase in intracellular hydrogen ion concentration. A reduction in intracellular pH could also reduce tension through a number of different mechanisms.

The effects of proton concentration on contraction-related processes in smooth muscle have been documented by a number of investigators. For example, Mrwa *et al.* (1974) demonstrated a strong pH dependence of tension development using chemically "skinned" (glycerinated) hog carotid artery. More specifically, it was shown that a decrease in one pH unit (from 7.0 to 6.0) resulted in a hundredfold decrease in calcium sensitivity. A similar result was obtained while measuring Mg^{2+}-activated ATPase activity of actomyosin. While this may be a rather large and unphysiological shift in hydrogen ion concentration, a smaller decrease in pH would nonetheless tend to decrease the tension producing ability of the contractile apparatus if calcium levels remained unchanged. Other calcium related processes are also affected by alterations in proton concentration. Grover *et al.* (1983) have shown that passive calcium binding to isolated plasmalemmal vesicles from rat myometrium decreases to approximately one tenth from a pH of 7.07 to 6.27. A threefold *increase* in oxalate-stimulated (active) calcium uptake from ER enriched membrane vesicles of porcine coronary arterial smooth muscle has been demonstrated with a decrease in pH from 7.6 to 6.8 units (Grover and Samson, 1986). These effects would decrease the ability of the muscle to contract by altering calcium availability.

By inhibiting the Na^+/H^+ antiporter using amiloride and through exposure of contracted strips to low extracellular pH, we have observed that the method of stimulation determines the importance of this process during contraction. While both potassium- and carbachol-contracted strips were relaxed through inhibition of this process, carbachol

contractions were obviously dependent to a much greater degree on its proper function. This greater dependence seems most clearly demonstrated by the marked effect of acid addition during a carbachol contraction, as compared to the relatively small perturbation in tension seen during a potassium-induced contraction. This may reflect a greater requirement for regulation of intracellular pH and/or sodium in this mode of contraction.

It is interesting to note that in spite of continued high external [H^+], most strips were able to redevelop tension to pre-acid values. It seems apparent that mechanisms exist within these cells which allow them to overcome the effects of an excessive extracellular concentration of protons. One of these mechanisms may be the increased activation of the Na^+-H^+ antiporter via intracellular acidification (Aronson *et al.*, 1982). This activation is thought to occur through the interaction of protons with a site remote from the transport site itself. By promoting efflux, antiporter activation would serve to reduce the net influx of protons incurred by the inward gradient and thus help to maintain a stable intracellular pH. The blockade of this recovery phase seen in the presence of 100uM amiloride would seem to indicate that this is the case.

In order to explain the distinct effects of amiloride in carbachol- and potassium-induced contractions, the basic differences in contraction between these two agents in dog tracheal smooth muscle must be stated, particularly with respect to their dependence on different calcium sources. Potassium induces contraction in the trachealis through membrane depolarization and subsequent activation of voltage dependent channels, which allow the influx of calcium from extracellular sites and thus permit contraction. These contractions are inhibited by verapamil, indicating complete dependence on extracellular calcium sources (Coburn, 1977). Stimulation with cholinergic agonists is thought to activate receptor operated channels in addition to these voltage activated channels (Farley and Miles, 1977), and promote release of calcium from intracellular stores as well (Farley and Miles, 1978). It may be that the activation

of this additional source of calcium, introduces another site sensitive to changes in hydrogen ion concentration (the SR calcium pump, for example), or to sodium concentration.

Bund *et al.* (1987) have shown that human resistance arterioles contracted with norepinephrine are dose dependently relaxed by amiloride and ethylisopropyl amiloride (a more specific blocker of Na^+/H^+ exchange). The sensitivity of norepinephrine-induced contraction to amiloride in this tissue might also reflect the dependance of this mode of contraction on intracellular calcium stores. Because high potassium (125 mM) contractions were not affected in their preparation, these investigators concluded that only phosphoinositide mediated contraction (as induced by norepinephrine) is dependent on Na^+/H^+ exchange. Activation of the inositide pathway during muscarinic stimulation may present another amiloride sensitive site. We speculate that the "low" affinity site for amiloride observed exclusively in carbachol contraction may represented a direct or indirect interaction with these processes.

Recently, Ozaki *et al.* (1987) have reported that amiloride inhibited contractions induced by high potassium and carbachol in guinea pig cecum when present during stimulation (IC_{50} of 41 uM). They ascribe this to direct inhibition of myosin light chain kinase by amiloride, which they demonstrated using isolated chicken gizzard native actomyosin. Direct addition of 100uM amiloride to this system in the presence of 5 mM and 1 mM ATP reduced phosphorylation by approximately 10 and 30%, respectively. Although we have not investigated this, it may be possible that such inhibition could have occurred in our system, particularly at high concentrations. However, since myosin light chain phosphorylation is required for both carbachol and KCl-induced contractions, it would be difficult to assess whether such an action corresponded to the "high" or "low" affinity sites which we have described.

We have established that amiloride relaxes canine tracheal smooth muscle whether it has been stimulated by electromechanical or pharmacomechanical means. It is clear from our

analysis of relaxation kinetics that there are at least two components of amiloride-induced relaxation. One component probably represents an interference with sodium-hydrogen exchange function, suggesting an important role for this process in tracheal smooth muscle excitation contraction coupling. The relaxant effect of amiloride on respiratory smooth muscle may also indicate a possible therapeutic potential for this drug. Amiloride is currently prescribed as a K^+-sparing diuretic in the treatment of mild hypertension. However, in light of this study, it is conceivable that the relaxant properties of this compound might also be utilized in the treatment of bronchoconstrictive disorders such as asthma.

REFERENCES

Akhtar - Khavari, F., Khoyi, M.A., and Rezaei, E. Effects of amiloride on contractions and the release of tritium from rat vas deferens preloaded with [^{3}H] - noradrenaline. Br. J. Pharmac. **74:** 123-127, 1981.

Aronson, P.S. Properties of the renal Na^+-H^+ exchanger. Ann. NY. Acad. Sci. **456**: 220-228, 1985.

Aronson, P.S., Nee, J., and Suhm, M.A. Modifier role of internal H^+ in activating the Na^+-H^+ exchanger in renal microvillus membrane vesicles. Nature (Lond.) 299: 161-163, 1982.

Ashida, T., and Blaustein, M.P. Regulation of cell calcium and contractility in mammalian arterial smooth muscle: the role of sodium-calcium exchange. J. Physiol. (Lond.) **392**: 617-635, 1987.

Benos, D.J. Amiloride: a molecular probe of sodium transport in tissues and cells. Am. J. Physiol. **242**: C131-C145, 1982.

Bullock, C.G., Fettes, J.J.F. and Kirkpatrick, C.T. Tracheal smooth muscle - second thoughts on sodium-calcium exchange. J. Physiol. (Lond.) **318**: 46*P*, 1981.

Bund, S.J., Heagerty, A.M., Issard, A.S., and Swales, J.D. Amiloride-induced relaxation of human resistance arterioles: evidence for a central role of Na+/H+ exchange in sustained smooth muscle contraction. J. Physiol. (Lond.) **391**:25P, 1987.

Coburn, R.F. The airway smooth muscle cell. Fed. Proc. **36**: 2692-2697, 1977.

Debetto, P., Floreani, M., Carpendo, F., and Luciani, S. Inhibition of the Na^+/Ca^{2+} exchange in cardiac sarcolemmal vesicles by amiloride. Life Sciences **40**:1523-1530, 1987.

Farley, J.M. and Miles, P.R. Role of depolarization in acetylcholine-induced contractions of dog trachealis muscle. J. Pharmacol. Exp. Ther. **201**:199-205, 1977.

Farley, J.M. and Miles, P.R. The sources of calcium for acetylcholine-induced contractions of dog tracheal smooth muscle. J. Pharmacol. Exp. Ther. **207**:340-346, 1978.

Fuchs, R., Thalhammer, T., Peterlik, M. and Graf, J. Electrical and molecular coupling between sodium and proton fluxes in basolateral membrane vesicles of the rat liver. Pflugers Arch. **406**:430-432, 1986.

Grover, A.K., Kwan, C.Y., and Daniel, E.E. High affinity pH-dependent passive Ca binding by myometrial plasma membrane vesicles. Am. J. Physiol. **244**: C61-C67, 1983.

Grover, A.K. and Samson, S.E. Pig coronary artery smooth muscle: substrate and pH dependence of the two calcium pumps. Am.J. Physiol. **251**: C529-534, 1986.

Haddy, F.J., Pamnani, M.B., Swindall, B.T., Johnston, J., and Cragoe, E.J. Jr. Sodium channel blockers are vasodilators as well as natriuretic and diuretic agents. Hypertension 7(Suppl.I): I-121--I-126, 1985.

Kahn, A.M., Shelat, H., and Allen, J. C. Na^+/H^+ exchange is present in sarcolemmal vesicles from dog superior mesenteric artery. Am. J. Physiol., **250**: H313-H319, 1986.

Kirkpatrick, C.T. and McDaniel, D.G. Tracheal smooth muscle - failure to demonstrate sodium-calcium exchange. Pflug. Arch. **361**: 301-302, 1976.

Klip, A., Ramlal, T., and Cragoe, E.J. Jr. Insulin-induced cytoplasmic alkalinization and glucose transport in muscle cells. Am. J. Physiol. **250**:C720-C728, 1986.

Krampetz, I.K. and Bose R. Amiloride relaxes tracheal smooth muscle. Fed. Proc. **46**: 651, 1987*a*.

Krampetz, I.K. and Bose, R. Amiloride: a diuretic with bronchodilatory properties. Clin. Invest. Med., **10**(suppl.): B120, 1987*b*.

Mahnensmith, R.L. and Aronson, P.S. The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. Circ. Research, **56**:773-788, 1985.

Mrwa, U., Achtig, I., and Ruegg, J.C. Influences of calcium concentration and pH on tension development and ATPase activity of the arterial actomyosin contractile system. Blood Vessels **11**:277-286, 1974.

Ozaki, H., Kojima, T., Moriyama, T., Karaki, H., Urakawa, N., Kohama, K., Nonomura, Y. Inhibition by amiloride of contractile elements in smooth muscle of guinea pig cecum and chicken gizzard. J. Pharmacol. Exp. Ther. **243**: 370-377, 1987.

Palaty, V. Amiloride acts as an alpha-adrenergic antagonist in the isolated rat tail artery. Can. J. Physiol. Pharmacol. **64**: 931-933, 1986.

Palmer L.G. The epithelial sodium channel. New horizons in therapeutics. Editors: G. Poste and S.T. Crooke. Publishers: Plenum press.pp. 327-344, 1986.

Paradiso, A.M., Tsien, R.Y., and Machen, T.E. Na^+-H^+ exchange in gastric glands as measured with a cytoplasmic-trapped, fluorescent indicator. Proc. Natl. Acad. Sci. U.S.A. **81**:7436-7440, 1984.

Pinon, J.F. and Fabre, J. Inhibition of potassium-induced contracture of the isolated rat aorta by amiloride. Arzneim.-Forsch./ Drug Res. **35**: 421-423, 1985.

Pouysegur, J., Chambard, J.C., Franchi, A., Paris, S., and Van Oberghen-Shilling, E. Growth factor activation of an amiloride-sensitive Na^+/H^+ exchange system in quiescent fibroblasts: Coupling to ribosomal protein S6 phosphorylation. Proc. Natl. Acad. Sci. U.S.A. **79**: 3935-3939, 1982.

Schellenberg, G.D., Anderson, L., and Swanson, P. D. Inhibition of Na^+-Ca^{2+} exchange in rat brain by amiloride. Mol. Pharm. **24**: 251-259, 1983.

Smith, R.L., Macara, I.G., Levenson, R., Housman, D., and Cantly, L. Evidence that a Na^+-Ca^{2+} antiport system regulates murine erythroleukemia cell differentiation. J. Biol. Chem. **257**:773-780, 1982.

Sordahl, L.A., Labelle, E.F., and Rex, K.A. Amiloride and diltiazem inhibition of microsomal and mitochondrial Na^+ and Ca^{2+} transport. Am. J. Physiol. **246**: C172-C176, 1984.

Souhrada, M. and Souhrada, J.F. Sensitization-induced sodium influx in airway smooth muscle cells of guinea pigs. Respiration Physiol. **60**: 157-168, 1985.

Wright, J., Schwartz, J.H., Olson, R., Kosowsky, J.M., and Tauber, A.I. Proton secretion by the sodium-hydrogen ion antiporter in the human neutrophil. J. Clin. Invest. **77**: 782-788, 1986.

PART II: SIMULTANEOUS MEASUREMENT OF INTRACELLULAR pH AND ISOMETRIC TENSION IN CANINE TRACHEAL SMOOTH MUSCLE STRIPS USING THE pH-SENSITIVE FLUORESCENT DYE, BCECF.

ABSTRACT

Intracellular pH and isometric tension were monitored simultaneously in isolated tracheal smooth muscle strips using the pH-sensitive fluoroprobe, BCECF [2'7'-bis-(2-carboxyethyl)-5(and-6)carboxyfluorescein]. Tissue strips were incubated for 30 minutes under physiological conditions in Krebs-Henseleit solution containing 2 uM of BCECF-AM (a permeant tetraacetoxymetyl ester). Fluorescence signal of the hydrolysed dye (BCECF) was measured at 503 nm (excitation wavelength) and 530 nm (emission wavelength) using a Perkin Elmer spectrophotofluorimeter. Autofluorescence of the tissue corresponded to less than 10% of the total signal. In Krebs-Henseleit bicarbonate-buffered medium (pH_o = 7.41), resting muscle strips maintained an intracellular pH of 7.08 $\pm$ 0.08 U (n = 5). Activation with carbachol (1 uM) coincided with a rapid acidification (0.40 $\pm$ 0.08 U, n = 8), as did depolarization using 80 mM KCl (0.29 $\pm$ 0.07 U, n = 9). Amiloride (100uM) markedly acidified the cell interior (0.21 $\pm$ 0.07 U, n = 6) and altered the ability of the tissue to regulate pH_i over a wide range of external proton concentrations. Exposure to sodium deficient medium also promoted intracellular acidification (0.52 $\pm$ 0.04 U, n = 4). This effect could be reversed by sodium addition. These properties indicate that intracellular pH changes with contractile state of the tissue and suggest that sodium-hydrogen exchange is important in the regulation of proton concentration within the airway smooth muscle cell.

INTRODUCTION

The pH sensitivity of many enzymes indicates that fundamental biochemical processes could be affected by changes in intracellular proton concentration. This observation, coupled with the now widely accepted view that protons do not distribute passively across the plasma membrane, has led to a widening interest in the importance and mechanisms of regulation of intracellular pH (Roos and Boron, 1981; Mahnensmith and Aronson, 1985; Busa and Nuccitelli, 1984). At present, two major ion transport processes are thought to be responsible for regulating intracellular pH in mammalian cells. These are the Na^+/H^+ antiporter and the $HC0_3^-/Cl^-$ exchanger.

We have recently observed that experimental maneuvers which modify the activity of the Na^+/H^+ antiporter, such as altering external proton concentration or treatment with the drug amiloride also affect the contraction of canine tracheal smooth muscle (Krampetz and Bose, 1988). This suggested that intracellular pH regulation by the antiporter was important in airway smooth muscle function. However, to correlate the observed changes in tension with actual changes in intracellular pH, a method was required for the simultaneous measurement of intracellular proton concentration and isometric tension in this tissue.

Successful measurements of intracellular pH have been made in smooth muscle using a variety of techniques (Wray, 1988*a*), including pH sensitive microelectrodes (Aickin, 1984), distribution of labelled weak acid (Stephens *et al.*, 1977), nuclear magnetic resonance (Vermue and Nicolay, 1983; Hellstrand and Vogel, 1985; Spurway and Wray, 1987), and most recently pH sensitive intracellular fluorescent dyes (Weisberg *et al.*, 1987; Berk *et al.*, 1987*a* and *b*; Hatori *et al.*, 1988; Korbmacher *et al.*, 1988). Only the latter method has the immediate potential to allow for the continuous measurement of intracellular proton concentration and tension development. However, the above cited studies have used only cultured and isolated smooth muscle cells.

Unfortunately, contractile tension cannot be measured in these preparations. In this report, a method for the simultaneous measurement of isometric tension and cytosolic pH using the fluorescent probe 2'7'-bis-(2-carboxyethyl)-5(and-6) carboxyfluorescein tetraacetoxymetyl ester (BCECF) in intact tracheal smooth muscle strips is described. The results of the study suggest that intracellular pH is dependent on the contractile state of the tissue and that its regulation is dependent on an intact Na^+/H^+ exchanger.

METHODS

Tissue preparation and dissection

Mongrel dogs of either sex were anaesthetized with 30 mg per kg sodium pentabarbital and sacrificed with a KCl injection to the heart. Tracheae were removed and immediately placed in cold (4°C) Krebs-Henseleit solution (KHS). The trachealis muscle was dissected free of cartilage and connective tissue in cold aerated KHS. A thin strip of muscle (1 mm x 1 mm x 6 mm) was cut and knots tied at both ends with fine surgical silk. (These prevented slippage from stainless steel wire clamps used to position the strip in the incubating cuvette).

Experimental apparatus

A special attachment to a Perkin Elmer LS-5 fluorescence spectrophotometer was constructed locally (Figure 13). The cuvette holder consisted of a solid aluminum block with two slits on adjacent sides to allow for the entry and exit of excitation and emission beams. The temperature of this block could be controlled such that the solution in the cuvette was maintained at 37°C. The tissue strip was suspended vertically under 1 g preload in 3 ml of vigorously aerated (95% O_2 - 5% CO_2) KHS using surgical wire clamps attached to a stationary hook at the bottom of the cuvette and to a Grass FT03 force transducer via surgical thread at the top. The entire assembly was enclosed in a light proof box. Three plastic tubes exited this box from the cuvette. The ends of two tubes were placed at the bottom of the cuvette (gas and fluid delivery/withdrawal tube), and the third (also a fluid delivery/withdrawal tube) was held a the 3 ml volume mark inside the cuvette. (This allowed for maintenance of fluid level when a circulating pump was in use.) During most experiments, a flexible glass pH electrode (Model MI-508 and MI-404, Microelectrode Inc., Londonderry, NH) was utilized to measure external

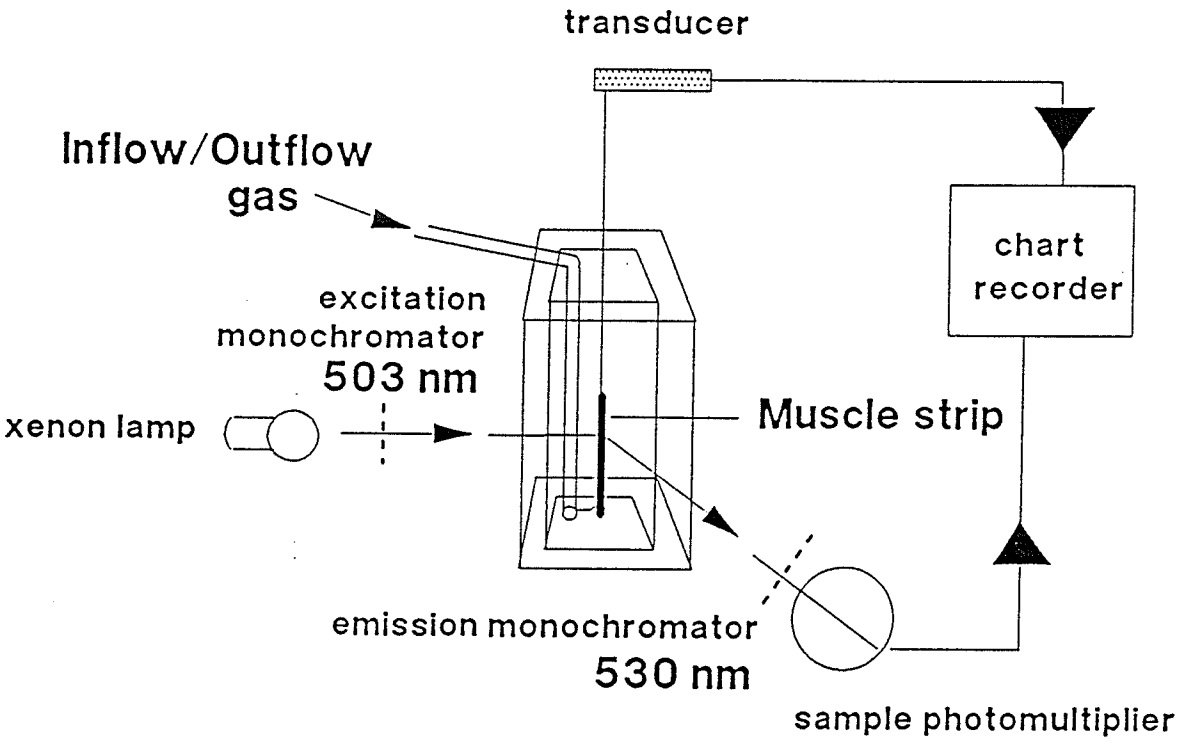

Figure 13: Schematic diagram of experimental apparatus for the simultaneous measurement of fluorescence and isometric tension. The muscle strip was suspended vertically inside the optical cuvette and was attached from its upper end to a force transducer. Although three separate tubes were present, inflow, outflow, and the gas line are shown as a single port for clarity.

pH directly in the cuvette. During experiments where pH_o was changed over a wide range, a standard glass electrode was used to measure external pH from the circulating reservoir.

pH measurement

Once positioned in the light beam, the muscle was stretched to provide one g resting tension and equilibrated for 30 minutes. Tissue autofluorescence was measured at 503 excitation and 530 emission, which had been previously determined to be the maximum wavelengths for BCECF fluorescence inside the tissue. The strip was contracted using 80 mM KCl followed by 1 uM carbachol (or the reverse) and changes in tissue autofluorescence were monitored. The permeable ester form of the dye was dissolved in DMSO (1 mM concentration), and 6 ul of this stock was added to the cuvette and left for 30 minutes (final concentration 2 uM). At the end of the incubation period the extracellular dye was removed with several changes of physiological solution. The tissue strip was allowed to equilibrate for another 30 minutes to ensure complete hydrolysis of the parent compound. Periodically throughout the experiment, fluorescence was measured at an isobestic excitation wavelength (440 nm) as an estimate of dye leakage.

After the fluorescence signal of the resting tissue had stabilized, the strip was stimulated with KCl (80 mM) or carbachol (1 uM). The effect of exposure to amiloride (100 uM) and sodium-free medium were investigated to assess the contribution of the Na^+/H^+ exchanger towards intracellular pH regulation.

At the termination of each experiment, intracellular pH was calibrated using 140 mM K^+ Hepes-buffered salt solutions titrated to a range of approximately 6.7 to 7.4 pH units (using HCl and NH_4OH, respectively) containing 7 uM of the K^+/H^+ ionophore nigericin to equalize

$[H^+]_{in}$ and $[H^+]_{out}$. In the experiments represented in Figures 5 and 6, this calibration range was extended.

Drugs and solutions

The fluorescent pH indicator, 2,7-bis(2-carboxyethyl)- 5, 6 carboxyfluorescein (BCECF) and its pentaacetoxymethyl ester form (BCECF-AM) were obtained from Molecular probes, Inc. (Junction City, OR); dimethylsulfoxide (DMSO) from BDH Chemicals (Canada); carbamylcholine hydrochloride, amiloride hydrochloride and nigericin from Sigma Chemical Co. (St. Louis, MO). Krebs-Henseleit solution contained the following (in mM): sodium chloride 118, potassium chloride 4.7, potassium dihydrogen phosphate 1.4, sodium bicarbonate 25, magnesium sulphate 1.2, calcium chloride 2.5 and glucose 11. Sodium free solution was similar to KHS, with NaCl replaced by 232 mM sucrose, and $NaHCO_3$ by 25 mM HEPES (n-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid). The composition of the pH calibrating solution was as follows: potassium chloride 140 mM, calcium chloride 2.5 mM, magnesium sulphate 0.8 mM, glucose 11 mM, HEPES 10 mM, nigericin 7 uM (as described in Weisberg *et al.*, 1987). HEPES-buffered solutions were aerated with 100% O_2.

RESULTS

Initially, the spectral characteristics of the dye were examined. In solution, the free acid form of BCECF displayed a characteristic emission peak at 530 nm and an excitation peak of 490 nm (Figure 14). A pH insensitive isobestic point was found at 440 nm emission. As expected (Rink et al., 1982), the relationship between changes in fluorescence and solution pH was linear (Figure 16). Once inside the cells, the excitation peak was shifted to 503 nm, while the emission peak remained at 530 nm (Figure 14). The BCECF molecule retained its pH-sensitivity once inside the smooth muscle cells, and fluorescence changes remained linear in the physiological range of 6.6 to 7.6 U when pH_i and pH_o were equalized using the hydrogen/potassium ionophore nigericin (Figure 18).

Other sources of fluorescence were small in comparison to the BCECF signal. The pentaacetoxymethyl ester of the dye BCECF-AM was not fluorescent at these wavelengths (Figure 14). Another source - tissue autofluorescence - contributed to less than 10% of the signal at peak wavelengths, and changes in autofluorescence with contraction made up a maximum of 5% of the total signal change seen upon contraction in BCECF-loaded cells (Figure 14). Leakage of the dye apparently occurred, as suggested by a small but steady reduction in fluorescence signal at 440 nm excitation. However, the effect of this factor was minimized by randomizing the interventions and by limiting the duration of each experiment to an approximate 3 hours maximum.

In the resting muscle pH_i was maintained at 7.08 ± 0.08 (n=5). Contraction was apparently unaffected by exposure to the dye, since similar tension development was observed before and after BCECF treatment. Although external pH remained constant, contraction - whether elicited using carbachol or KCl - was coincident with a rapid and sustained intracellular

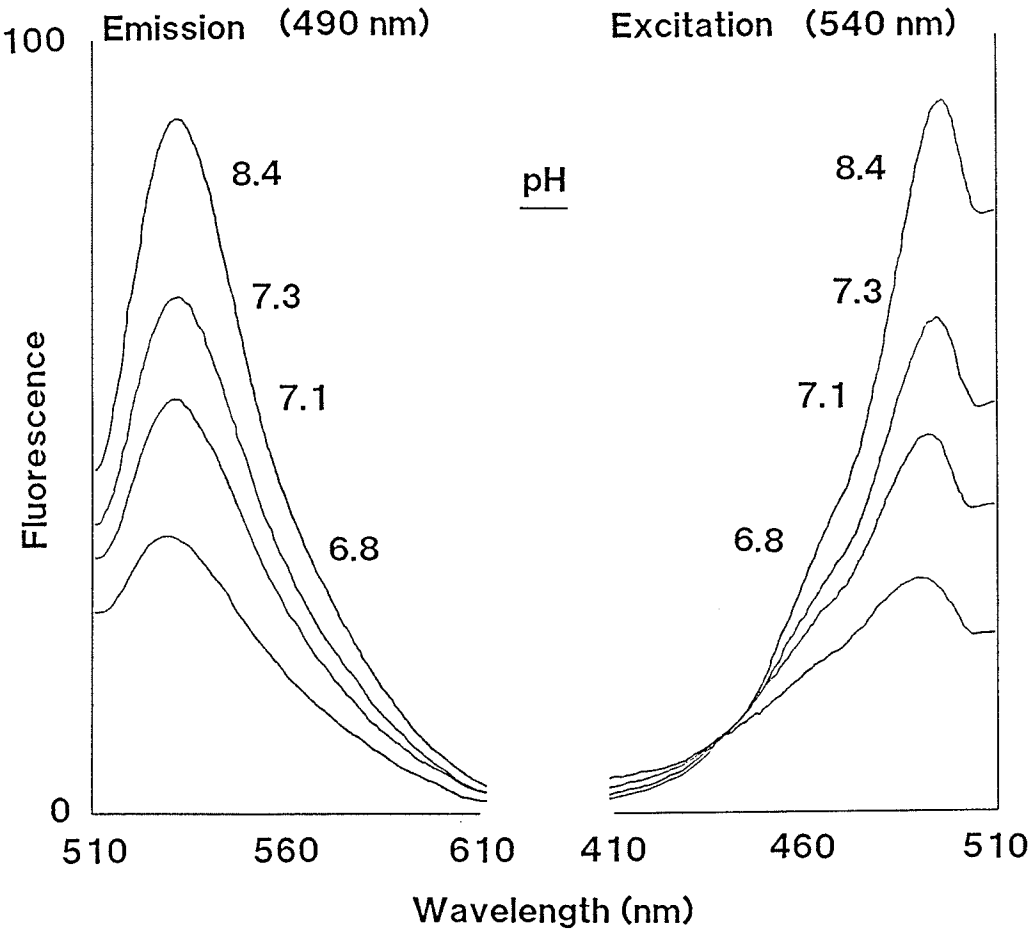

Figure 14: Emission and excitation spectra (measured at 490 and 540 nm, respectively) for free BCECF (70 uM) in calibration solution of different pH values. Fluorescence peaks were located at 530 (emission) and 490 nm (excitation). The pH-insensitive fluorescence (isobestic point) was measured at 440 nm on the excitation spectrum.

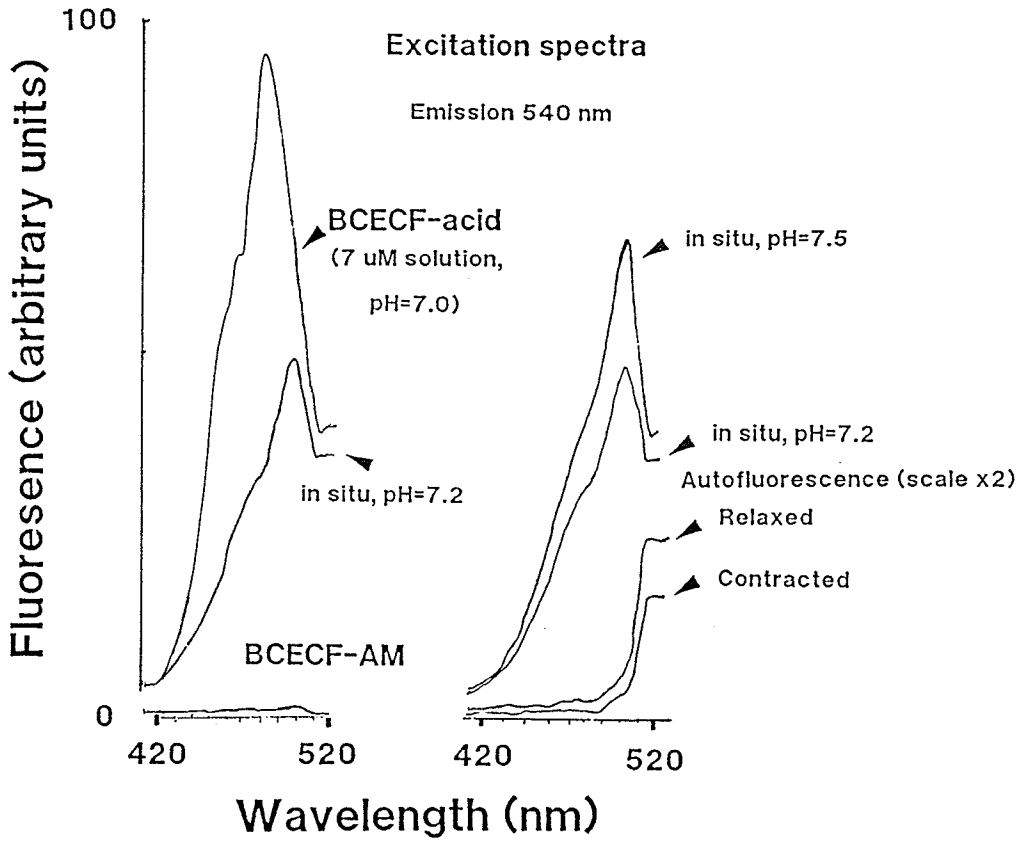

Figure 15: Comparison of excitation spectra (measured at 540 nm emission). *Left panel*: Excitation spectra of BCECF (free acid) in solution and within the tissue strip, and of the parent compound (BCECF-AM). Note the shift in excitation peak once the dye has entered the tissue, and the minimal fluorescence of the parent compound. *Right panel*: Comparison of fluorescence changes with an imposed 0.3 U shift in pH_i in a nigericin treated tissue strip previously loaded with BCECF, and the observed changes in autofluorescence during contraction in an untreated, unloaded tissue strip. Note the relatively small changes observed in autofluorescence when compared to the signal difference resulting from the pH change.

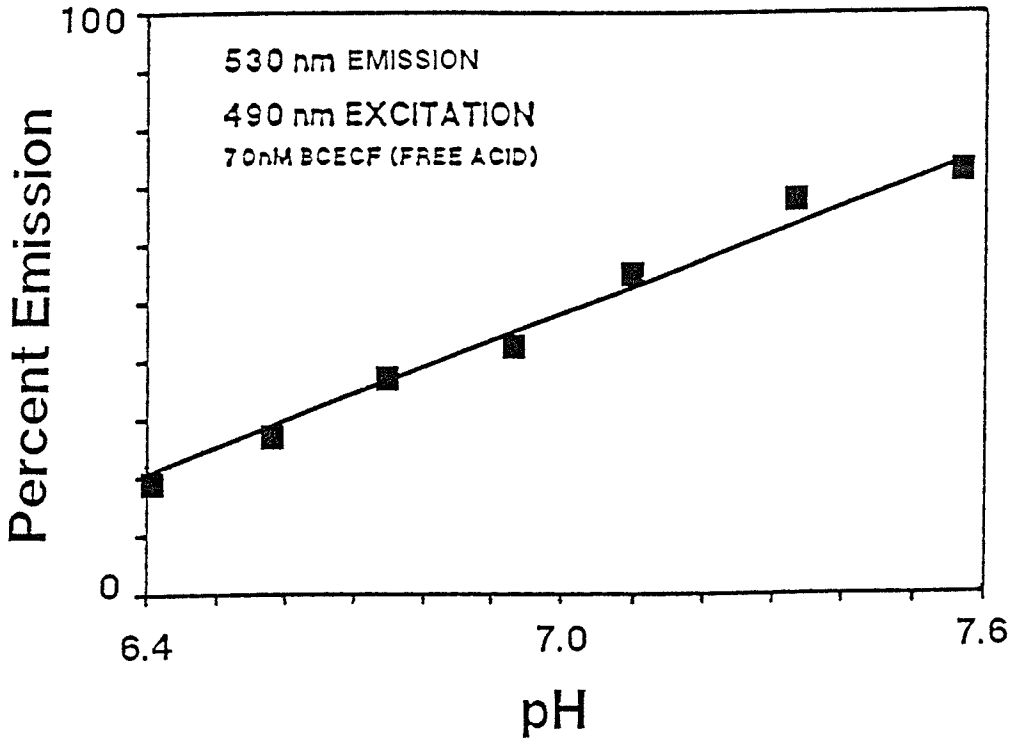

Figure 16: Fluorescence *versus* pH of BCECF (free acid) in solution. Note the linear nature of the relationship.

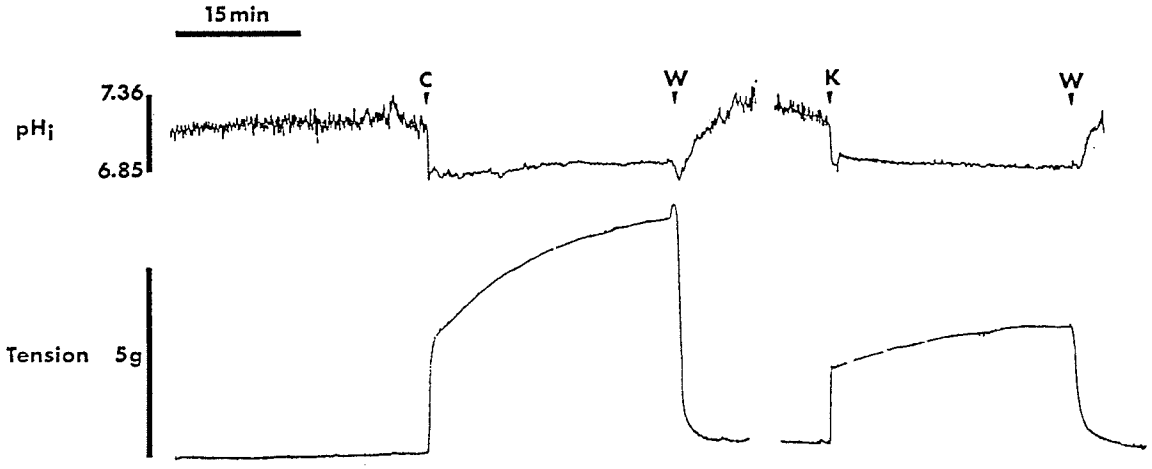

Figure 17: Typical time course of isometric tension development (lower trace) and intracellular pH changes (top trace) during carbachol(C)- and KCl(K)- induced contractions in a tracheal strip. W denotes wash with KHS.

acidification (Figure 17). The magnitude of acidification during carbachol-induced contraction was similar (0.40±0.08, n=8) to that observed during KCl-induced contraction (0.29±0.07, n=9). Once the stimulants were removed, pH_i was restored to precontraction values along with tension (Figure 17).

After these data were established, a comparison of the effect of external pH on intracellular pH was made (Figure 18). In tracheal strips, pH_i did vary with extracellular pH, and the slope of this relationship was visibly reduced from that observed in the same tissue strips after pH_o and pH_i were equalized using nigericin. This would indicate that pH regulating mechanisms of one form or another are operative within these cells.

Since the sodium-hydrogen exchanger has been shown to be a major regulator of intracellular pH, the effect of amiloride (a Na^+/H^+ exchange inhibitor) on intracellular pH was examined. The presence of 100 uM amiloride had a marked effect on the relationship of pH_i *versus* pH_o, as shown in Figure 19. Under all but extreme pH_o conditions, pH_i was considerably reduced.

As predicted from the data in Figure 19, the addition of 100 uM amiloride to a resting tissue strip at pH_o of 7.41, reduced pH_i by 0.25±0.07 U (n=6, Figure 20). Inhibition of the Na^+/H^+ antiporter by removal of sodium from the bathing medium also caused intracellular acidification. Under these conditions the decrease in intracellular pH was 0.52±0.04 U (n=4, Figure 20). This reduction in pH_i was reversed by the reintroduction of sodium to the medium (120 mM as NaCl). Unlike the acidification observed during carbachol- or KCl-induced stimulation, amiloride-induced acidification was not associated with an increase in muscle tension, and exposure to sodium free medium was associated with only a small phasic contraction (data not shown), suggesting that a sustained intracellular acidification was not in itself a stimulus for contraction.

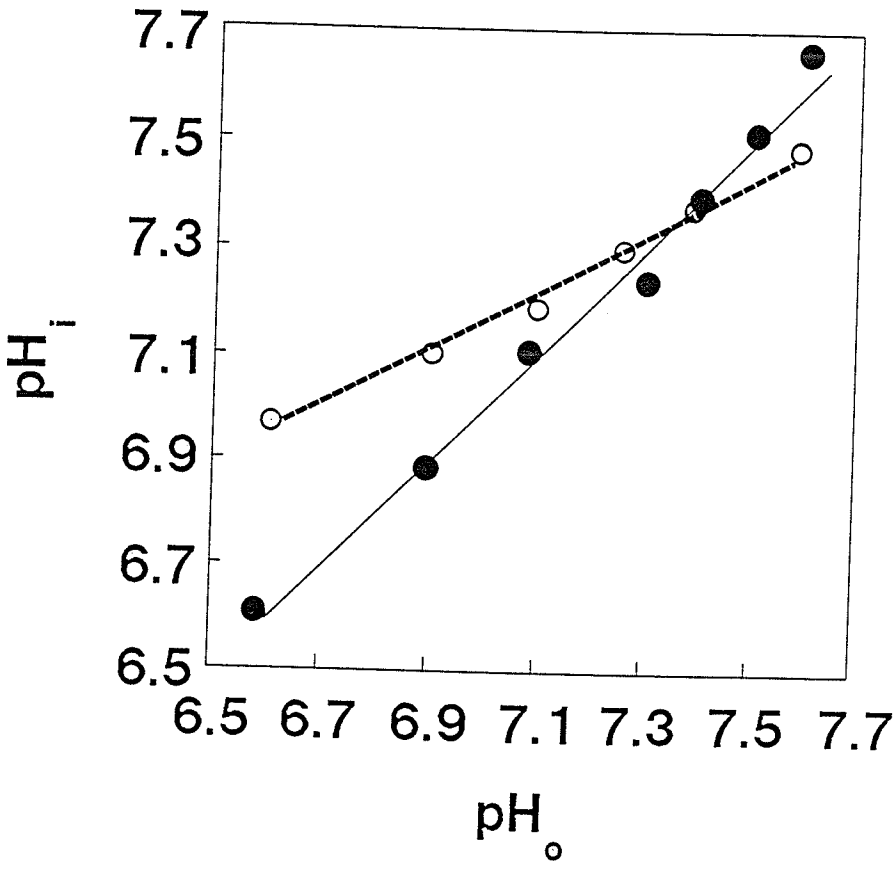

Figure 18: Linearity of pH_i *versus* pH_o in a BCECF-loaded tissue strip prior to (open circles) and after nigericin treatment (solid circles). The slope of this relationship is reduced in the untreated strip, indicating the presence of intracellular pH regulating mechanisms.

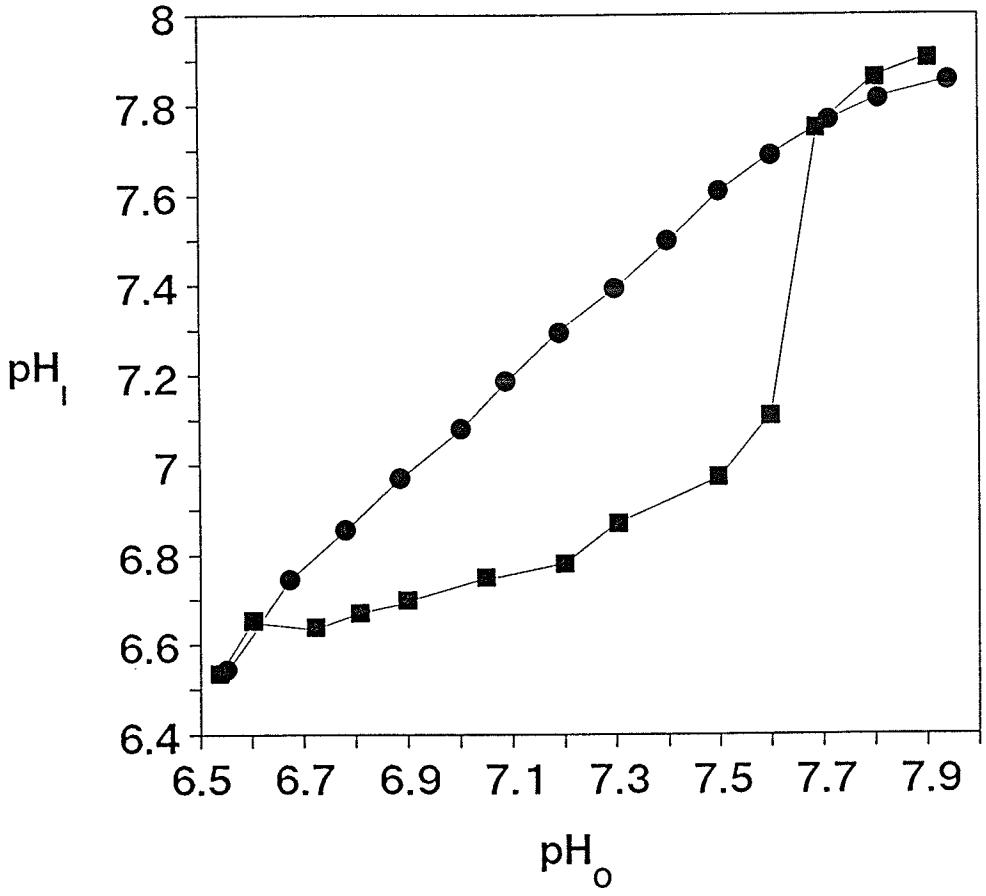

Figure 19: pH_i *versus* pH_o in a BCECF-loaded tissue strip treated with 100 uM amiloride (squares) and subsequently with nigericin solution (circles). Except at extreme pH_o values, intracellular pH was depressed. Contrast the pattern of pH changes with that observed in an untreated strip (Figure 18).

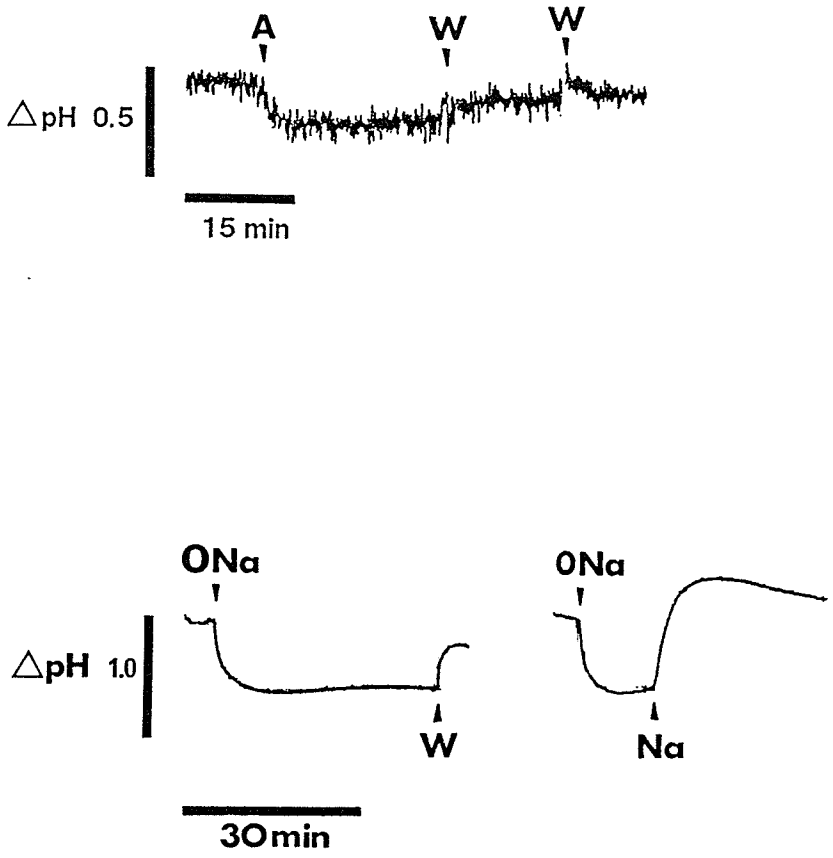

Figure 20: *Upper trace*: The effect of exposure to 100 uM amiloride on pH_i in unstimulated tracheal smooth muscle. (W denotes wash with normal KHS.) *Lower traces*: Effect of treatment with sodium-free HEPES buffered medium and subsequent sodium addition (120 mM as NaCl) on the intracellular pH of tracheal smooth muscle.

DISCUSSION

The results of this study indicate that with this novel technique it is possible to monitor both intracellular pH and isometric tension simultaneously in smooth muscle strips obtained from the canine trachealis. We are confident that the observed signals were a true reflection of changes in intracellular pH for a number of reasons. Dye fluorescence from the tissue strips, while obviously influenced by extracellular pH, was more markedly altered in the presence of the ionophore nigericin. This, coupled with the observed changes in signal in the absence of pH changes in the external bathing medium (such as occurred with exposure to sodium-free medium) suggested an intracellular localization of the BCECF. Although we have not directly measured intracellular distribution of dye in our preparation, we hypothesize that it primarily reflects cytosolic rather than intra-organelle pH. This is based on the observations of Steinberg *et al.* (1987), who demonstrated that carboxyfluorescein stained bovine aortic endothelial cells diffusely in spite of the numerous and prominent mitochondria found in these cells. Commonality in the structures of BCECF and carboxyfluorescein would also suggest similar intracellular distribution characteristics. The possibility that signal changes were merely a result of tissue autofluorescence can also be dismissed since this amounted to only a minor fraction of total dye fluorescence. It appears therefore that with this technique true changes in intracellular pH can be continuously monitored in tracheal smooth muscle strips.

The intracellular pH of unstimulated tracheal muscle was measured at 7.08. A previous study by Stephens *et al.* (1977) also measured intracellular pH in canine trachealis, using the (^{14}C)-DMO (weak acid) distribution technique. Their value of 7.20 was somewhat more alkaline than that which we observed, however, this may be related to the difference in method of measurement. Our value is in closer agreement with those obtained from other smooth muscles also measured using BCECF. Berk *et al.* (1987) and Weissberg *et al.* (1988) have obtained resting pH_i's in vascular smooth muscle of 7.08 and 7.16, respectively. These and our

own value of 7.08 for intracellular pH are not consistent with a passive distribution of protons across the cell membrane. Using the Nernst equation and assuming a resting membrane potential of -59.2 mv for canine tracheal smooth muscle (Suzuki *et al.*, 1976), the predicted pH_i at 37°C would be 6.45. This discrepancy indicates that proton distribution must be modulated in some way. Another observation which supports this that a 1 U change in external pH results in only an approximate 0.5 U change in pH_i in this tissue. Furthermore, the reduced slope of pH_i versus pH_o in untreated tissue compared with that exposed to the H^+ ionophore nigericin also indicated the presence of mechanisms for modulation of intracellular proton concentration. This pattern of pH_i versus pH_o is very similar to that observed by Wray (1988*b*) in rat uterus.

In many mammalian tissues including vascular smooth muscle (Hatori *et al.*, 1988; Berk *et al.*, 1987; Weissberg *et al.*, 1987; Korbmacher *et al.*, 1988), the Na^+/H^+ antiporter has been shown to be an important regulator of intracellular pH. It is apparent that this also holds true for canine airway smooth muscle. This was demonstrated by the marked effect of the sodium-hydrogen exchange blocking compound, amiloride, on the pattern of intracellular pH regulation. The observation that amiloride-exposure increased intracellular proton concentration over the acid and neutral pH_o range indicated that the Na^+/H^+ antiporter is a important mechanism for the extrusion of protons from the cell interior. The concordance of pH_i in the amiloride-treated and ionophore-treated strips under highly alkaline conditions probably reflects a reduced contribution of the antiporter towards regulation of pH_i at high pH_i values. Such modulation of antiporter activity by internal proton concentration was first shown by Aronson *et al.* (1982), who observed a considerable decrease in sodium influx into renal microvillus membrane vesicles at neutral or alkaline pH_i. This characteristic has been attributed by these and subsequent researchers (Grinstein and Rothstein, 1986, for example) to allosteric modulation of the Na^+/H^+ exchanger by protons at a site distinct from the transport moiety.

The importance of the Na^+/H^+ antiporter to pH_i maintenance was further demonstrated by the prominent drop in pH_i which resulted from the removal of extracellular sodium. The complete reversal of this acidification observed upon addition of sodium also highlighted the primary value of this ion in intracellular pH regulation of canine trachealis.

More important and perhaps even controversial is the observation that contraction is simultaneous with intracellular acidification. A decrease in intracellular pH has been observed upon stimulation of isolated smooth muscle although contraction was not simultaneously measured. Vermue and Nicolay (1983) revealed (using ^{31}P-NMR) that stimulation with high K^+ resulted in an intracellular acidification of approximately 0.5 U in guinea-pig taenia caecum. Both Berk *et al.* (1987) and Hatori *et al.* (1988) have demonstrated the effects of angiotensin-stimulation on intracellular pH in isolated rat aortic smooth muscle cells using BCECF. Each observed a biphasic response to stimulation consisting of rapid acidification (of approximately 0.1 units) followed by an alkalinization phase. The maximum degree of acidification was proportional to the concentration of angiotensin employed (Hatori *et al.*, 1988). Although smooth muscle contraction appears to be coincident with at least a transient acidification, it is clear from our own data that intracellular acidification itself cannot be responsible for contraction, since reduction in pH_i using either amiloride or sodium-free medium did not result in contraction of this tissue. This suggests that the observed increase in intracellular proton concentration results from smooth muscle activation.

In addition to angiotensin, Berk *et al.* also examined the effect of other calcium-mobilizing agents, including platelet derived growth factor and bradykinin on pH_i. In each case, the reduction and recovery of pH_i were mirrored by a transient increase in intracellular calcium concentration. Hatori *et al.*(1988) also observed that 10 uM verapamil could virtually eliminate the acidification associated with stimulation by angiotensin II. Both of these observations suggest that the reduction in pH_i is closely related to an increase in intracellular calcium. We

have observed that, unlike that of aortic smooth muscle cells stimulated with angiotensin II, intracellular calcium concentration in canine tracheal smooth muscle strips increases and is sustained during both carbachol- and KCl-stimulated contraction (see Part III, p.104). If intracellular acidification is somehow a reflection of an increase in intracellular free calcium, this may explain the sustained acidosis observed in our own preparation with these two forms of stimulation, and may explain the differences between our data and those of the Berk and Hatori groups.

While the exact origin of the protons which produce intracellular acidification upon stimulation is unknown, there are a number of likely possibilities. Both Berk *et al.* (1987) and Hatori *et al.* (1988) present evidence to suggest that these protons may enter via the plasma membrane calcium ATPase, which would be activated under conditions of high intracellular calcium concentration. Alternatively, protons may be released from intracellular binding sites also common to calcium. When intracellular calcium increases, these protons would be displaced into the cytosol. In support of this, Meech and Thomas have observed a reduction in intracellular pH upon microinjection of calcium into snail neurons (1977). In cardiac Purkinge fibres, Vaughan-Jones *et al.* (1987) have reported an intracellular acidification which is simultaneous with strophanthadin-induced increases in intracellular calcium, an effect which they also suggest results from competition of hydrogen and calcium ions for the same binding sites. It is possible that such a mechanism may account for our own observations.

In conclusion, with the development of this technique we have obtained data which supports the presence of the Na^+/H^+ exchanger in canine tracheal smooth muscle and demonstrates its importance in intracellular pH regulation. It has also allowed us to document the changes in intracellular proton concentration which occur in tandem with isometric tension development.

REFERENCES

Aikin, C.C. Direct measurement of intracellular pH and buffering power in smooth muscle cells of the guinea pig vas deferens. J. Physiol. (Lond.) **349**: 571-585, 1984.

Aronson, P.S., Nee, J., and Suhn, M.A. Modifier role of internal H^+ in activating the Na^+/H^+ exchanger in renal microvillus membrane vesicles. Nature **299**: 161-163, 1982.

Berk, B.C., Aronow, M.S., Brock, T.A., Cragoe, E. Jr., Gimbrone, M.A., Alexander, R.W. Angiotensin II-stimulated Na^+/H^+ exchange in cultured vascular smooth muscle cells. J. Biol. Chem. **262**: 5057-5064, 1987*a*.

Berk, B.C., Brock, T.A., Gimbrone, M.A. Jr., Alexander, R.W. Early agonist-mediated ionic events in cultured vascular smooth muscle cells. J. Biol.Chem. **262**: 5065-5072, 1987*b*.

Busa, W.B. and Nuccitelli, R. Metabolic regulation via intracellular pH. Am. J. Physiol. **246**: R409-R438, 1984.

Grinstein, S. and Rothstein, A. Mechanisms of regulation of the Na^+/H^+ exchanger. J. Mem. Biol. **90**: 1-12, 1986.

Hatori, N., Fine, B.P., Nakamur, A., Cragoe, E. Jr., and Aviv, A. Angiotensin II effect on cytosolic pH in cultured rat vascular smooth muscle cells. J. Biol Chem. **262**: 5073-5078, 1987.

Hellstrand, P. and Vogel, H.J. Phosphagens and intracellular pH in intact smooth muscle studied by ^{31}P-NMR. Am. J. Physiol. **248**: C320-C329, 1985.

Korbmacher, C., Helbig, H., Stahl, F., and Wiederholt, M. Evidence for Na/H exchange and Cl/HCO_3 transport in A10 vascular smooth muscle cells. Pfleugers Arch. **412**: 29-36, 1988.

Krampetz, I.K. and Bose, R. Relaxant effect of amiloride on canine tracheal smooth muscle. J. Pharmacol. Exp. Therap. **246**: 641-649, 1988.

Mahnensmith, R.L. and Aronson, P.S. The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. Circ. Res. **57**: 773-788, 1985.

Meech, R.W. and Thomas, P.C. The effect of calcium injection on the intracellular sodium and pH of snail neurons. J. Physiol. **265**: 867-879, 1977.

Rink, T.J., Tsien, R.Y. and Pozzan, T.. Cytoplasmic pH and free Mg^{++} in lymphocytes. J. Cell Biol. **95**: 1189-196, 1982.

Roos, A. and Boron, W.F. Intracellular pH. Physiol. Rev. **61**: 296-434, 1981.

Spurway, N.C., and Wray, S. A phosphorus nuclear magnetic resonance study of metabolites and intracellular pH in rabbit vascular smooth muscle. J. Physiol. (Lond.) **393**: 57-71. 1987.

Steinberg, S.F., Bilezikian, J.P., and Al-Awqati, Q. Fura-2 fluorescence is localized to mitochondria in endothelial cells. Am. J. Physiol. 253: C744-747, 1987.

Stephens, N.L., E.A. Kroeger, and Loh, W. Intracellular pH in hypoxic smooth muscle. Am. J. Physiol. **232**: E330-E335, 1977.

Suzuki, H., Morita, K. and Kuriyama, H. Innervations and properties of the smooth muscle of the dog trachea. Jap. J. Physiol. **26**: 303-320, 1976.

Vaughan-Jones, R.D., Lederer, W.J., and Eisner, D.A. Ca^{2+} ions can affect intracellular pH in mammalian cardiac cells. Nature **301**: 522-524, 1983.

Vermue, N.A. and Nicolay, K. Energetics of smooth muscle taenia caecum of guinea-pig: a ^{31}P-NMR study. FEBS Lett. **156**: 293-297, 1983.

Weissberg, P.L., Little, P.J., Cragoe, E.J. Jr., and Bobik, A. Na-H antiport in cultured rat aortic smooth muscle: its role in cytoplasmic pH regulation. Am. J. Physiol. **253**: C193-198, 1987.

Wray, S. Smooth muscle intracellular pH: measurement, regulation and function. Am. J. Physiol. **254**: C213-C225, 1988*a*.

Wray, S. Regulation of intracellular pH in rat uterine smooth muscle, studied by ^{31}P-NMR spectroscopy. Biochim. Biophys. Acta **972**: 299-301, 1988*b*.

PART III: THE MECHANISMS OF AMILORIDE-INDUCED RELAXATION OF CANINE TRACHEAL SMOOTH MUSCLE

ABSTRACT

Canine airway smooth muscle is relaxed by micromolar concentrations of the K^+-sparing diuretic amiloride. In this study, the mechanism of this effect was investigated. Amiloride does not appear to relax this tissue through stimulation of beta adrenoceptors or by promotion of the release of relaxant prostaglandins. Relaxation could not be correlated with an increase in tissue cyclic AMP levels. Chemically "skinned" muscle strips were only slightly relaxed with very high concentrations of amiloride (30% decrease in tension at 800 uM), and accumulation of the drug into intact tissue was negligible at effective concentrations of 100 uM. The ability of amiloride to block Na^+/H^+ exchange and the time course of this effect were assessed by measurement of intracellular pH in muscle strips using the fluorescent pH-sensitive intracellular dye BCECF and by simultaneous measurement of tension. Amiloride exposure produced intracellular acidification with a similar time course to the observed relaxation of contracted strips. Intracellular acidification of tracheal smooth muscle imposed by exposure to acetic acid also reduced tension development, apparently by reducing intracellular calcium concentration as measured with the calcium sensitive dye Fura-2. Amiloride itself significantly reduced the force of contraction elicited in calcium-free Krebs-Henseleit solution (containing 0.2 mM EGTA). When calcium (2.7 mM) was subsequently added to the bathing medium after contraction in calcium free solution, the initial rate of contraction was also significantly reduced by the drug. This suggested that amiloride altered both intracellular calcium release and calcium influx. Based on these data, it appears that amiloride relaxes tracheal smooth muscle by promoting intracellular acidification and reducing activator calcium.

INTRODUCTION

We have previously shown that micromolar concentrations of the K^+-sparing diuretic amiloride relax isometrically contracted tracheal smooth muscle strips. This relaxant effect was apparently independent of the original contractile stimulus since high potassium-, carbachol-, serotonin-, histamine-, and electrically-induced contractions were all relaxed by this compound (Krampetz and Bose, 1988). The universality with which the drug relaxed this tissue suggested that amiloride interfered with an essential step (or steps) in the contractile process common to all of these forms of contraction.

Probably the best documented property of amiloride in living systems is its ability to block passive sodium-dependent transport processes, specifically epithelial sodium channels, the Na^+/H^+ antiporter, and the Na^+/Ca^{2+} exchanger (Benos, 1982). Preliminary evidence indicated that in our preparation, the effect of amiloride was modified by changes in extracellular sodium and that the relaxant effect of exposure to acid could be augmented by this drug. Since the presence of the Na^+/H^+ antiporter has previously been described in canine *vascular* smooth muscle by Kahn *et al.* (1986), we consequently hypothesized that amiloride-induced relaxation was most likely mediated by inhibition of the Na^+/H^+ antiporter.

While inhibition of the plasmalemmal Na^+/H^+ antiporter appeared as the most probable explanation for the relaxant action of amiloride in our preparation, additional inhibitory effects of amiloride have been demonstrated and might adequately explain its relaxant properties. For example, there is evidence that amiloride can interact with adrenergic receptors (Palaty, 1986), including beta receptors (Howard *et al.*, 1987), and can inhibit prostaglandin production (Howard *et al.*, 1987). Amiloride may act intracellularly to inhibit kinases (Ozaki *et al.* 1987; Chatterjee *et al.*, 1988), and may alter release of calcium from intracellular stores (Deth *et al.* , 1985; Chatterjee *et al.*, 1988). In light of these data, it was the purpose of this study to test

several of the proposed hypotheses to explain the relaxant effect of amiloride in canine tracheal smooth muscle.

MATERIALS AND METHODS

Tissue

Tracheae were removed from pentobarbital-anaesthetized mongrel dogs of either sex immediately after lethal KCl injection to the heart. Tissue was quickly placed in cold ($4^{o}C$) Krebs-Henseleit solution (KHS). Smooth muscle was dissected free of epithelium, cartilage and connective tissue in cold KHS, and refrigerated (for up to 24 hours) if not immediately used.

Pharmacological studies

Muscle strips were cut to approximate 1-2 mm x 1mm x 10 mm dimensions and tied with 5.0 silk thread. These were suspended vertically by attachment to a surgical steel wire loop at one end and to a mechanical force transducer (Grass FT03) at the other in identical 10 ml capacity muscle baths containing oxygenated KHS (pH =7.41, $37^{o}C$, aerated with 95% O_2-5% CO_2, minimum PO_2= 400 Torr, PCO_2= 40$\pm$5 Torr). Signals were amplified and recorded with a Gould 260 6-channel chart recorder. Preload was initially adjusted to approximately 3 g, which resulted in a passive tension of approximately 1-2 g after the 1 hour equilibration period.

To determine whether relaxation was mediated by an interaction with beta adrenoceptors, amiloride was added to contracted tissue in the presence of the beta blocking drugs propranolol or sotalol (10uM). Similarly, to inhibit the release of prostaglandins, indomethacin (1 uM) was included in the bathing medium. Drugs were added to muscle strips (n=5) contracted with carbachol (1 uM) or KCl (80 mM added to KHS). Amiloride (100 uM) was introduced after isometric tension reached a plateau.

Cyclic AMP Assays

Smooth muscle tissue from each animal (n=4) was sectioned into 4 thin sheets of 150 to 250 mg (wet weight). These were randomly placed in individual 10 ml organ baths and suspended isometrically using a series of stainless steel hooks. (These ensured that the tissue remained suspended as a sheet). Tissue was incubated under conditions described above. Muscles were allowed to equilibrate for one half hour under a preload of approximately 1 g. One pair was stimulated with KCl (80 mM) and the other with carbachol (1uM). Amiloride (100 uM) was added to one of each pair when a stable tension had been achieved. Ten minutes later (after relaxation was observed), tension was fixed in each sheet and the tissue was rapidly blotted and immersed in liquid nitrogen. The samples were weighed, pulverized and then suspended in a small volume of 4 mM EDTA solution (pH 7.0). These suspensions were heated for 3 minutes in a boiling water bath, chilled in ice, and centrifuged. The supernatant was removed and placed on ice. The pellet was washed twice and centrifuged with cold EDTA solution. The supernatant and wash solutions from each sample were combined and lyophylized, then resuspended with 4 mM EDTA to a known volume. Duplicate samples were assayed for cAMP content using an Amersham cyclic AMP radioimmunoassay kit (Amersham, Arlington Heights, IL). Results were expressed per mg wet weight of tissue.

Skinned Muscle Studies

Thin muscle strips (10 x 1 x 0.5 mm) were skinned overnight with detergent (Triton X-100, Fisher Scientific, St. Louis, MO) using the method and solutions of Sparrow *et al.* (1984). Muscles were isometrically attached to fixed wire rods using Collodion adhesive (Fisher Scientific) and incubated in a circulating bath (3 ml volume) at 28°C. Tension was measured with a 2 g Shinkoh UL2240 transducer (Tokyo, Japan). Strips were placedin contracting solution. Once tension had reached a plateau, amiloride was added cumulatively to a total

concentration of 800 uM. (This concentration was near the solubility limit of amiloride in contracting solution.) Tension changes were measured as percent of plateau tension.

Amiloride Accumulation Study

In order to assess whether intracellular accumulation of this drug occurred over time, tissue strips were incubated in KHS containing carbachol (1 uM) and 100 uM amiloride for 1, 5, 10, 20 and 40 minutes. (Since the relaxant effect of amiloride was most prominent in carbachol-induced contractions, this agonist was included in the bathing solution.) After removal from the incubation medium, strips were blotted, weighed and dissolved over 48 hours using 1 ml of 50% methanol and 50% 250 ug/ml digitonin in Tris buffer (pH 7.0). Total volume was brought to 2.5 ml and amiloride concentration was assessed fluorimetrically using a Perkin-Elmer spectrophotofluorimeter. Excitation wavelength was set at 396 nm (maximum excitation wavelength) and emission wavelength at 430 nm (maximum emission wavelength). Dry weight subtracted from weight after blotting was designated as tissue water content. Concentrations (umoles/l tissue water) were measured from a standard curve.

Intracellular pH measurements

Tissue strips (prepared as described under Pharmacological studies) were vertically suspended (1 g preload) in a modified optical cuvette containing 3 ml of KHS aerated with 95% O_2 and 5% CO_2 and warmed to 37°C. The strip was held isometrically by a steel wire clamp at its lower edge and attached to a Grass FT03 force transducer with surigical thread. The muscle strip was positioned in the beam of a Perkin-Elmer LS-5 spectrophotofluorimeter (Part II, Figure 13) and was allowed to equilibrate for 30 minutes. Excitation wavelength was set at 503 nm and emission at 530 nm. Emission was measured periodically at an isobestic excitation wavelength (440 nm) throughout the experiment as an estimate of dye leakage. Before addition

of the fluoroprobe ester, autofluorescence changes during exposure to KCl (80 mM), carbachol (1 uM) and amiloride (100 uM) were monitored. The tissue was incubated in 2 uM of BCECF-AM (from freshly made 1 mM stock in DMSO) in KHS for 30 minutes. After another 30 minutes (to allow for continued hydrolysis of the compound), the muscle was contracted with KCl or carbachol for approximately 20 minutes. The agonist and incubating solution were then removed and replaced with fresh KHS. Following this, the strip was stimulated again and amiloride (100 uM) was added after approximately 10 minutes. At the termination of each experiment, intracellular pH was calibrated using 140 mM K^+ Hepes-buffered salt solution (see Drugs and Solutions section) titrated to approximately 6.7 and 7.4 pH units and containing 7 uM of the K^+/H^+ ionophore nigericin to equalize $[H^+]_{in}$ and $[H^+]_{out}$. This solution was aerated with 100% 0_2.

Calcium Influx and Release Studies

Since the direct effect of amiloride on intracellular calcium concentration could not be measured using fluorimetric techniques due to interference of amiloride with the fluorescence signal of Fura-2, an indirect method of calcium estimation was utilized. Muscle strips were prepared and equilibrated as previously described. Carbachol (1 uM) or KCl (80 mM) were used to repeatedly contract the strips isometrically with until a stable maximum tension was achieved. The strips were washed with KHS and were allowed to relax for 5 minutes, after which the bathing medium was replaced with calcium free KHS containing 0.2 mM EGTA. After 5 minutes in calcium-free medium, the agonist was added, and the muscle was allowed to contract for twenty minutes (KCl) or until tension had returned to baseline (carbachol). The maximum height of contraction in calcium-free medium was used as a measure of intracellular calcium release. After this period, $CaCl_2$ (2.7 mM) was introduced into the medium, which produced a contraction. The rate of this contraction during the first minute immediately after calcium addition was used as a relative measure of transmembrane calcium movement.

To assess the pharmacological effect of amiloride and calcium entry blockers on these two measured parameters, this experimental protocol was repeated in the same muscle strip in the presence of 100 uM amiloride, 10uM D-600 or 10uM nifedipine, respectively.

Intracellular calcium measurements

The effect of intracellular acidification on intracellular calcium concentration and contraction was investigated by decreasing intracellular pH through extracellular acidification with acetic acid. The preparation and apparatus were essentially the same as for intracellular pH measurements, however, dye loading was accomplished by incubating the tissue strips in KHS containing approximately 200 uM Fura-2·AM for 1 hour. The fluorescence ratio (excitation wavelengths 340 nm/ 380 nm) was measured at an emission wavelength of 500 nm using a Jasco CAF-100 Ca^{2+} analyser (Tokyo, Japan). In this experiment, the tissue was incubated in 10 ml of circulating, aerated KHS. This allowed for constant measurement of medium pH from a small reservoir remote from the tissue itself. The tracheal strip was contracted with carbachol (1 uM) or KCl (80 mM). When tension had reached a plateau, 10 ul of glacial acetic acid (per 10 ml KHS) was added. At the end of each experiment, digitonin (0.2 mM) was added to determine the maximum ratio, followed by addition of 0.5 mM EGTA (pH 7.0) to assess the minimum ratio value.

Experimental Solutions and Drugs

The composition of the Krebs-Henseleit solution was (in mM): NaCl 118, KH_2PO_4 1.4, $MgCl_2$ 1.2, Glucose 11, KCl 4.7, $NaHCO_3$ 25, $CaCl_2$ 2.5. To maintain a constant level of sodium ion between solutions, sodium concentration was not changed when tissue was stimulated using 80 mM KCl. (This solution was therefore hypertonic). Intracellular pH calibration solution contained (in mM): KCl 140, $CaCl_2$ 2.5, $MgSO_4$ 0.8, glucose 11, Hepes 10, nigericin 0.007.

Compounds were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Stock solutions were prepared from the following chemicals (concentrations in parentheses): amiloride hydrochloride (10 mM), atropine sulphate (0.1 mM), carbamyl choline hydrochloride (0.1 mM), histamine hydrochloride (20 mM), propranolol hydrochloride (1 mM), serotonin hydrochloride (1 mM), indomethacin (10 mM in ethanol, Merck, Sharp, & Dohme, Pointe Claire, Canada), sotalol hydrochloride (1 mM, Bristol-Meyers, Ottawa, Canada), and potassium chloride (4 M, Fisher Scientific, Winnipeg, Canada). In skinned muscle experiments, amiloride was dissolved in DMSO to 100 mM, and diluted as appropriate with contracting solution before addition. All solutions were prepared using distilled deionized water unless otherwise indicated. BCECF·AM and FURA-2·AM were obtained from Molecular Probes (Oregon, USA).

Statistical Procedures

Data were analyzed statistically using the Student's t-test or Duncan's New Multiple Range test, as indicated in the figure legends. Significant differences were considered at p values of less than or equal to 0.05. All results are expressed as mean $\pm$ SEM.

RESULTS

1. Effect of pharmacological agents on amiloride-induced relaxation.

The inclusion of indomethacin (1 uM) or the beta-blocking drugs, propranolol (1 uM) and sotalol (10 uM), in the bathing medium of the tracheal smooth muscle strips had no effect on amiloride-induced relaxation (n=5, Figure 21). This indicates that amiloride did not relax this tissue by a beta-agonist effect or through promotion or inhibition of prostaglandin release.

2. Effect of amiloride on cellular cyclic AMP levels.

Relaxation induced by amiloride was not coincident with an increase in cellular cyclic AMP levels. In carbachol contracted muscle, amiloride had no significant effect on the level of cAMP (73 ± 10 versus 55 ± 16 pmol/g wet wt., n=4), while in muscle contracted with KCl, the presence of amiloride significantly *decreased* cAMP levels from 160 ± 35 to 86 ± 21 pmol/g wet wt ($p < 0.05$, n=4; unpaired t-test).

3. Effect of amiloride on "skinned" muscle strips.

Contractions elicited in skinned tracheal smooth muscle were relaxed only at very high concentrations of amiloride, unlike the intact tissue. At 800 uM (near the solubility limit for amiloride in contracting solution), amiloride decreased tension by only 30% (Figure 22), in contrast with the effect of 100 uM amiloride in unskinned tissue (Figure 21). These data suggest that amiloride's action is either dependent on an intact membrane or that the drug may accumulate in the intact tissue.

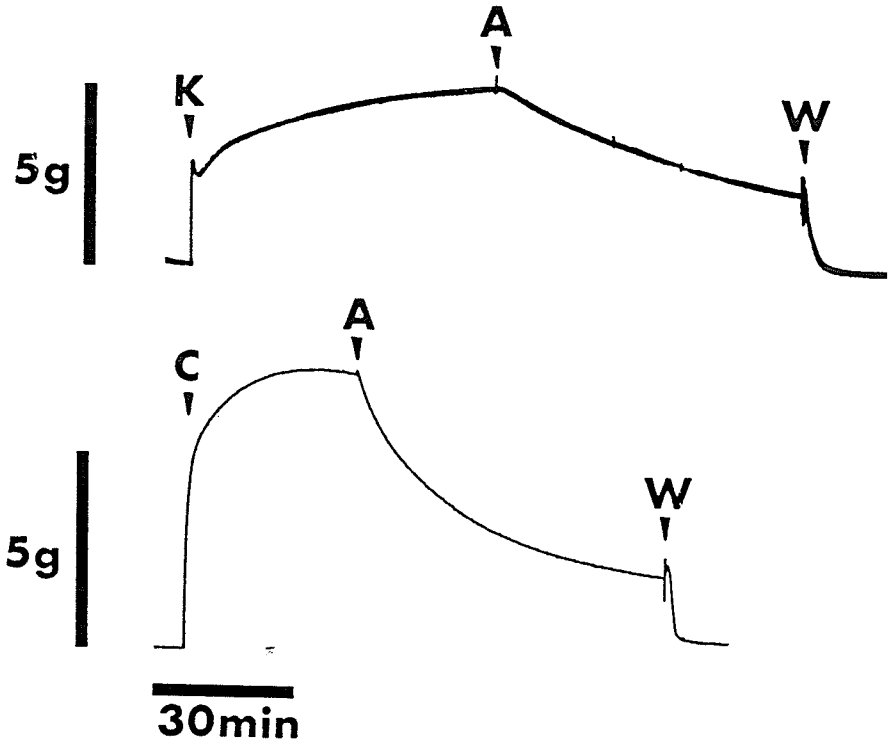

Figure 21: Amiloride-induced relaxation of canine tracheal smooth muscle. Strips (n=4) were contracted isometrically with 80 mM KCl (K) or 1 uM carbachol (C) in the presence of 100 uM indomethacin and 10 uM propranolol (shown) or sotalol. Amiloride (100 uM) induced relaxation was unchanged when compared to untreated controls.

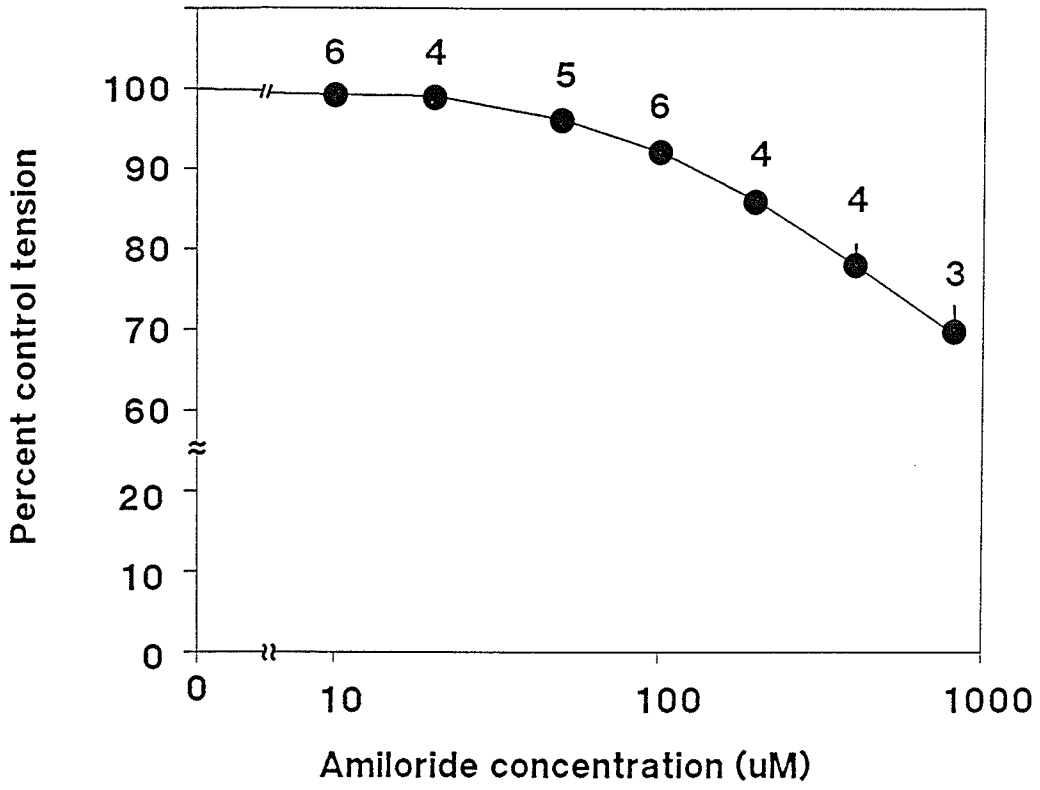

Figure 22: Effect of cumulative addition of amiloride on plateau tension in canine tracheal strips skinned using Triton X-100. Even at a concentration of 800 uM, tension was reduced by only 30%.

4. Accumulation of amiloride in tracheal smooth muscle.

In order for amiloride to relax this tissue through a direct effect on contractile elements (as suggested in the previous section), it must be shown to enter the cell interior in comparable concentrations. In the study of amiloride accumulation into this tissue, uptake of this drug was minimal during the time course of relaxation in the presence of carbachol. Even after a 40 minutes exposure to 100 uM of this compound, only 0.17 umoles of amiloride per litre of tissue water had accumulated (Figure 23).

5. Effect of amiloride on contraction in calcium-free medium and with calcium addition.

In canine tracheal strips, contractions could be elicited in calcium-free 0.2 mM EGTA KHS using either 1 uM carbachol (Figure 24) or 80 mM KCl (not shown). Although the maximum height of contraction was reduced in both cases, the characteristics of these contractions were somewhat different. Carbachol-induced contractions were phasic while KCl induced contractions were maintained. Both were only slightly reduced (Table 1) by the presence D-600 (10 uM) or nifedipine (10 uM), indicating that they were not dependent on extracellular calcium entry through voltage-sensitive channels.

The observation that a sustained KCl-induced contraction could be elicited in calcium-free medium, suggested the presence of a calcium source which is not accessible to chelation by extracellular EGTA. Even more surprising was the insensitivity of this contraction to the calcium channel blockers nifedipine and D-600, since Coburn (1977) has indicated that KCl-induced contractions are completely inhibited by these agents in this tissue. This may be related to drug concentration, since Weiss *et al.* (1985) have also observed contraction of canine trachealis muscle in the presence of similar amounts of D-600.

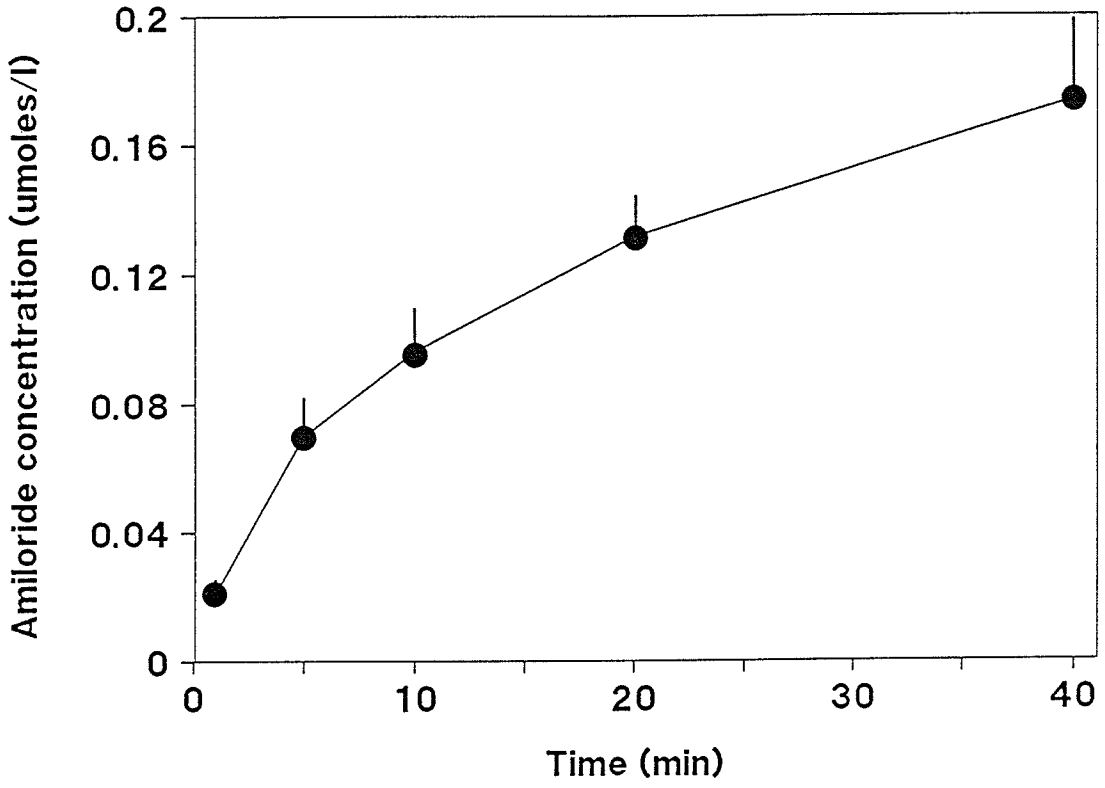

Figure 23: Amiloride accumulation in stimulated tracheal strips incubated in 100 uM of this compound in KHS. Standard error bars are omitted where this value was less than the span of the marking symbol.

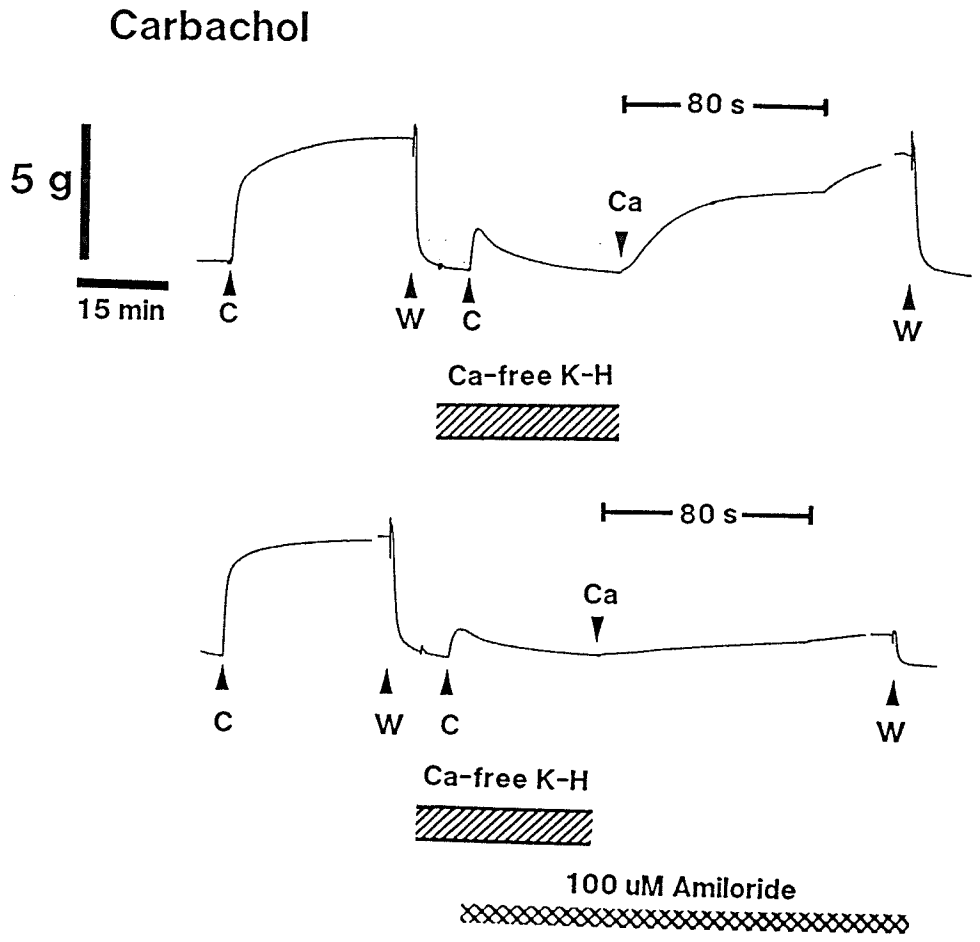

Figure 24: Effect of amiloride on contraction in calcium free medium and on contraction upon calcium addition. Note the reduction in height of contraction in calcium free medium and the reduced rate of contraction in the presence of 100 uM amiloride (bottom trace) as compared to the control (top trace).

Maximum tension developed in calcium-free KHS (proportion of maximum tension developed in normal KHS)

Stimulus	n	Control	Amiloride (100 uM)	D-600 (10 uM)	Nifedipine (10 uM)
carbachol	6	0.39±0.05	0.22±0.05* (44%)	-	-
(1 uM)	4	0.40±0.04	-	0.22±0.02(45%)	-
	3	0.33±0.04	-	-	0.20±0.01(39%)
KCl	7	0.40±0.07	0.33±0.07(17%)	-	-
(80 mM)	5	0.47±0.08	-	0.30±0.06* (36%)	-
	3	0.47±0.03	-	-	0.34±0.02* (28%)

Rate of contraction upon calcium addition to calcium-free KHS (proportion of maximum control tension attained during first minute in 2.7 mM $CaCl_2$ KHS)

Stimulus	n	Control	Amiloride (100 uM)	D-600 (10 uM)	Nifedipine (10 uM)
carbachol	6	0.41±0.05	0.13±0.04* (68%)	-	-
(1 uM)	3	0.43±0.90	-	0.20±0.05* (53%)	-
	3	0.43±0.90	-	-	0.33±0.11(23%)
KCl	5	0.33±0.60	0.13±0.04* (61%)	-	-
(80 mM)	4	0.36±0.06	-	0(100%)	-
	4	0.36±0.06	-	-	0(100%)

Table 1: Effect of amiloride on tension development in calcium-free KHS and on the initial rate of contraction upon calcium addition to the medium. Effect of calcium channel antagonists D-600 and nifedipine are also shown for comparis on. Maximum tension developed in calcium-free medium is expressed as a proportion of maximum tension achieved in KHS. Initial rate of contraction is expressed as the proportion of maximum control tension developed during the first minute after calcium addition to the medium. Asterisks denote a significance level of $p \leq 0.05$, as determined using the paired Student t-test. Percentage decrease from control values is shown in parentheses.

The presence of amiloride (100 uM) significantly reduced carbachol elicited contraction in calcium-free medium from 39±5% (of control tension) to 22±5% (n=6 Duncan's NMR test), suggesting inhibition of intracellular calcium release. However, KCl-elicited contraction was not decreased by amiloride (control 40±7% versus amiloride treated 33±7%) (Table 1).

In contrast, contraction resulting from calcium addition to the medium was completely blocked by these drugs in KCl-induced contraction, while the initial rate of contraction in carbachol stimulated strips was reduced by only a small proportion by the presence of these compounds. This would suggest that transmembrane flux was reduced by 100 uM amiloride in both KCl and carbachol stimulated tissues. These results are summarized in Table 1. Since amiloride does not directly inhibit post-receptor events, this suggests that amiloride is directly inhibiting calcium entry or acting indirectly through acidification.

7. Effect of amiloride on intracellular pH

Using the pH-sensitive intracellular dye, BCECF, we have determined that relaxation of canine tracheal smooth muscle by amiloride (100 uM) is coincident with intracellular acidification (Figure 25). Intracellular pH was reduced by 0.17±0.03 units in KCl-stimulated tissue (n=8) and 0.26±0.13 units in carbachol-stimulated tissue (n=6). In both cases, the time course of tension decrease was closely paralleled by acidification, yet carbachol-induced contraction were more sensitive to a similar change in intracellular pH.

A greater sensitivity to intracellular acidification is also seen in tissue where intracellular pH was changed by exposure to acetic acid (Figure 26, lower trace). In spite of the fact that the induced pH changes were essentially identical (Figure 27), carbachol contractions were more markedly reduced.

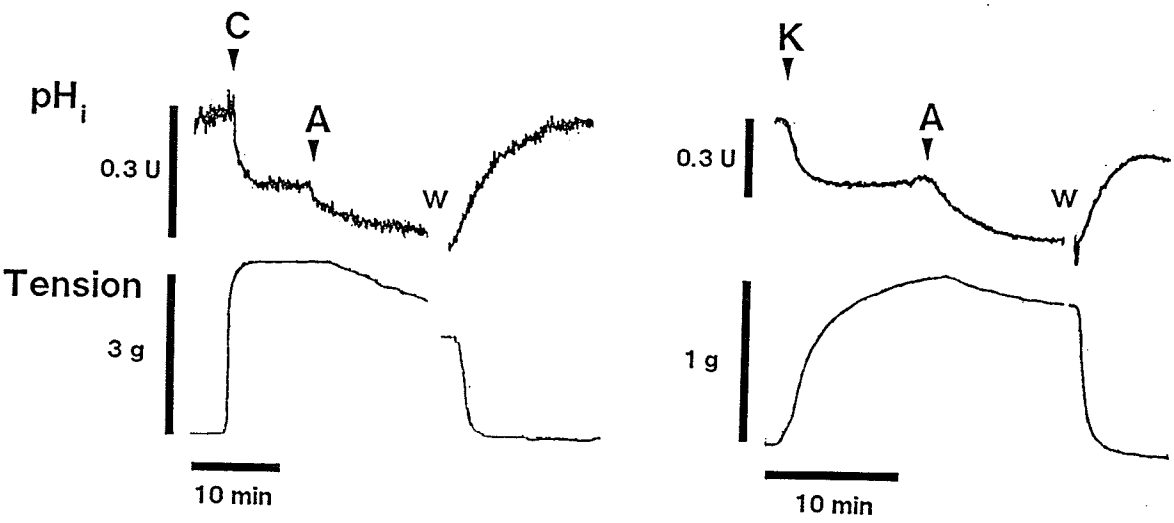

Figure 25: Intracellular pH measurement and simultaneous tension recordings from intact tracheal strips. Contraction with carbachol (C) and KCl (K) typically resulted in intracellular acidification. Subsequent addition of 100 uM amiloride (A) further acidified the cell interior, an effect which coincided with tissue relaxation. Note the greater sensitivity of carbachol-induced contractions to the similar degree of acidification.

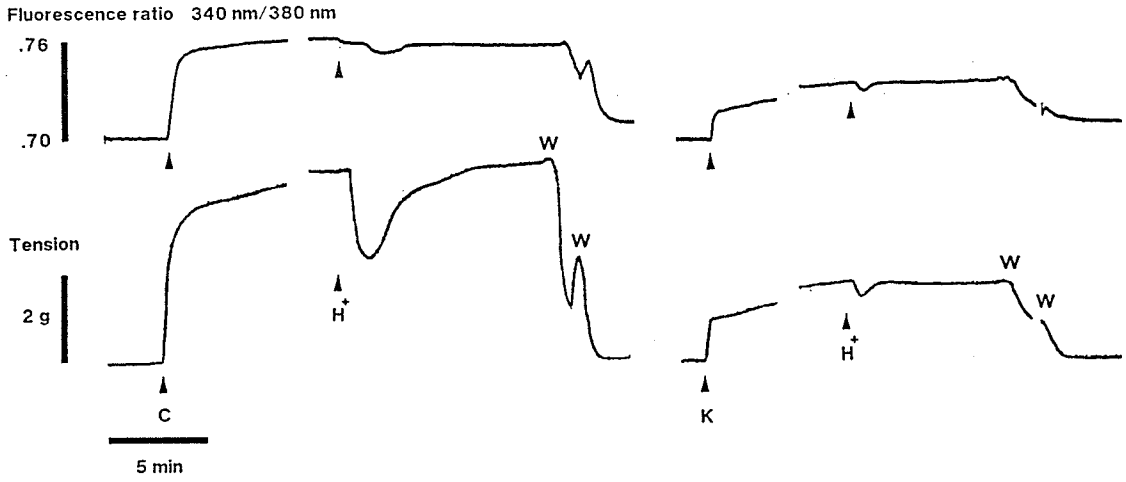

Figure 26: Simultaneous changes in intracellular calcium concentration and tension in carbachol(C)- and KCl(K)- induced contractions upon exposure to glacial acetic acid (H^+) in the same muscle strip. In spite of the similar change in intracellular pH with this treatment (Figure 27), carbachol contractions showed a greater and more prolonged decrease in intracellular calcium compared to KCl contraction. This was also reflected in the tension traces (representative of 4 experiments).

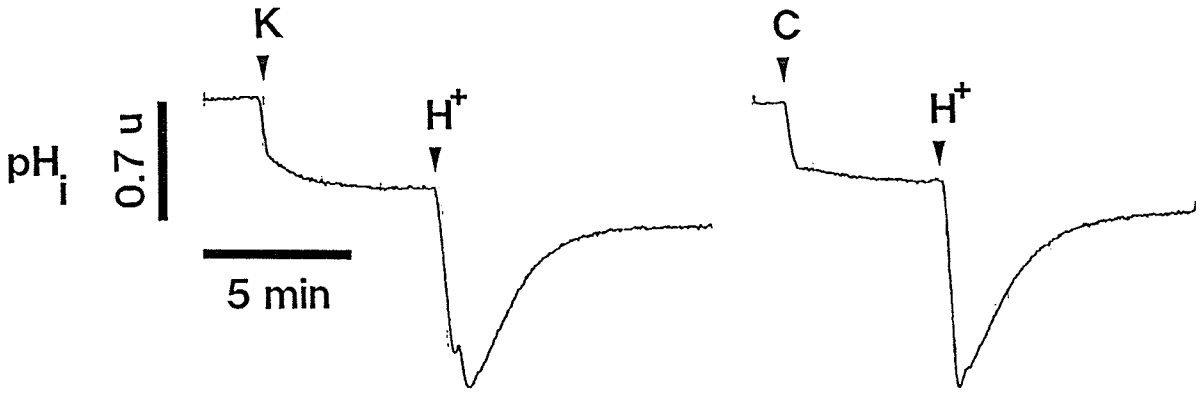

Figure 27: Intracellular pH changes imposed with the addition of glacial acetic acid (10 ul/ 10 ml KHS) during KCL (K) and carbachol-induced contractions in the same strip (C). The traces are essentially identical (representative of 6 experiments).

8. Effect of intracellular acidification on cytosolic calcium concentration.

Direct measurements of intracellular calcium concentration showed that acidification of the cell interior through exposure to external acid also resulted in a transient decrease in intracellular calcium concentration (Figure 26). Carbachol-induced contractions were more sensitive to acid exposure, both in terms of tension and reduction in cytosolic calcium concentration. Regardless of stimulus, calcium levels recovered along with tension and intracellular pH in spite of reduced external pH. This indicated that the observed change in cytosolic calcium was not primarily mediated by a change in external proton concentration.

DISCUSSION

In the current study, six possible mechanisms through which amiloride could induce relaxation in canine tracheal smooth muscle were investigated. These were: (1) a beta-adrenoceptor mediated relaxation; (2) a release of relaxant prostaglandins; (3) a stimulation of adenylate cyclase; (4) a direct effect on the smooth muscle contractile elements; (5) the inhibition of calcium influx and/or intracellular release of calcium; and (6) intracellular acidification resulting from Na^+/H^+ antiporter blockade. The results suggest that amiloride inhibits contraction by promoting intracellular acidification and also altering calcium mobilization processes.

In our preparation, the minor effect of exposure of "skinned" tracheal smooth muscle fibres to amiloride, even at concentrations exceeding those seen by the intact tissue, and the lack of significant uptake indicated that this compound could have no direct action on the contractile elements. Yet, others have suggested that a direct effect may explain the relaxant action of amiloride in other smooth muscles. Ozaki *et al.* (1987) have shown that amiloride can relax saponin-skinned guinea pig teania coli, albeit at concentrations 4.5 times higher than that required to inhibit contraction in intact tissue. Chatterjee *et al.* (1988) have similarly observed a decrease in contraction of saponin-skinned rabbit aorta at 100 and 300 uM amiloride, although a 45 minute incubation period was required before contraction. Only the former authors have examined drug accumulation. Their results would indicate an active accumulation of amiloride in the taenia - after 40 minutes in 1 mM amiloride, the tissue to medium ratio of the drug was approximately 1.25. This characteristic was not found in the canine trachea, since a 40 minute incubation in 100 uM amiloride resulted in a ratio of approximately 0.002. In this respect, canine trachealis would appear to be similar to human red blood cells, which under comparable conditions also show a low rate of uptake (Benos *et al.*, 1983). In the absence of significant

uptake, the contribution of a direct effect on the contractile elements in tracheal tissue remains trivial.

The prominent point of this study is that amiloride produces an intracellular acidification which is coincident with relaxation. Acidification upon exposure to amiloride and amiloride analogs has been previously described in vascular smooth muscle preparations by Korbmacher *et al.* (1988), Hatori *et al.* (1987), Berk *et al.* (1987) and by Weissberg *et al.* (1987). However, since these studies were performed with isolated or cultured vascular smooth muscle cells, none measured tension simultaneously.

While the similar time course of acidification and relaxation does not necessarily imply a cause-and-effect relationship, the induction of intracellular acidosis by alternative means (exposure to acid medium) also resulted in a decrease in tension in our preparation. Intracellular acidification has also been demonstrated to decrease contraction of other muscle types. In sheep purkinje fibres, a reduction in intracellular pH coincided with a fall in force (Vaughan-Jones *et al.*, 1987). This may be linked to a decrease in the sensitivity of calcium-dependent processes. Fabiato and Fabiato (1978) have shown decreased calcium sensitivity of skinned cardiac and skeletal muscle myofilaments with increased proton concentration. This is also the case in vascular smooth muscle as demonstrated by Mrwa *et al.* (1974) in glycerinated hog carotid artery. Another likely explanation is that a decrease in pH_i could directly affect $[Ca]_i$ regulating systems. In guinea-pig and rabbit cardiac ventricular muscle, for example, intracellular acidosis is able to depress calcium entry through the slow inward current (Fry and Poole-Wilson, 1981). Philipson *et al.* (1982) have demonstrated that in cardiac sarcolemmal vesicles isolated from dog ventricle, Na^+-dependent Ca^{2+}-uptake was reduced by 50% at pH 6.7. Thus, another conductive pathway for calcium, the Na^+/Ca^{2+} exchanger, is also affected by proton concentration.

The significant decreases in both the carbachol-induced contraction in calcium-free medium and the rates of KCl- and carbachol-induced contraction (with the subsequent addition of calcium to the medium) observed in the presence of amiloride could be explained by the above effects. The observation that intracellular acidification imposed by acetic acid exposure also reduced intracellular calcium concentration, would appear to support this.

The decreased amplitude of carbachol-elicited contraction in calcium-free medium could be explained by impaired release of intracellular calcium. In support of this, Chatterjee *et al.* (1988) have demonstrated that amiloride reduces calcium efflux from intact norepinephrine-stimulated rabbit aorta. The authors attribute this to a decrease in calcium release from intracellular sites, although they propose no mechanism for this effect. This may also be an effect of decreased intracellular pH, since Grover *et al.* (1986) have demonstrated that acidification (7.6 to 6.8) increases the oxalate stimulated(active) uptake of calcium from ER-enriched membrane vessels of porcine coronary arterial smooth muscle.

While amiloride has previously been shown to relax intact tracheal muscle irrespective of stimulus, differences in the characteristics of amiloride-induced relaxation were observed particularly between the carbachol and KCl stimulated strips, as stated in the introduction (Krampetz and Bose, 1988). In particular, it was observed that during potassium-induced contraction (80 mM KCl) amiloride addition produced a slow monophasic dose dependent relaxation (IC_{50} = 12.3 uM). In carbachol contracted strips, concentrations up to 10 uM amiloride induced a slow monophasic relaxation. Yet, with the addition of 35 to 250 uM amiloride, an initial rapid phase of relaxation (IC_{50} = 75.5 uM) was superimposed onto this slow phase (IC_{50} = 23.5 uM), resulting in a biexponential relaxation. We hypothesized that the slow phase of relaxation in both carbachol and KCl contracted muscle represented a common mechanism of relaxation, and that the additional phase observed in carbachol-induced contraction was suggestive of a second site of action for this compound. One of the major

differences between these two forms of contraction in tracheal smooth muscle appears to be a reliance on different calcium pools. Muscarinic agonists such as carbachol allow the entry of this ion through receptor-operated membrane channels, but they also stimulate release of calcium from intracellular sources (Kirkpatrick, 1975; Coburn, 1977; Farley and Miles, 1978; Weiss *et al.*, 1985). It may be that the additional phase of relaxation observed represents inhibition of calcium release from intracellular sites.

A final possibility remains that amiloride may directly inhibit calcium movement in this tissue, rather than indirectly through acidification. Amiloride has been demonstrated to selectively block low threshold T-type calcium channels in mammalian neural and atrial tissue at relatively low concentrations (K_D = 30uM) using the patch clamp technique (Tang *et al.*, 1988). Recently, the presence of a T-type channel has been documented in canine airway smooth muscle cells with characteristics described as "very similar to the T current or fast calcium current reported in pituitary, neuronal, atrial and smooth muscle cells" (Kotlikoff, 1988). Whether amiloride may directly block extracellular calcium entry through such a channel remains unknown.

REFERENCES

Benos, D.J.: Amiloride: A molecular probe of sodium transport in tissues and cells. Am. J. Physiol. **242**: C131-C145, 1982.

Benos, D.J., Reyes, J., and Shoemaker, D.G.: Amiloride fluxes across erythrocyte membranes. Biochim. Biophys. Acta. **734**: 99-104, 1983.

Berk, B.C., Brock, T.A., Gimbrone, M.A., and Alexander, R.W.: Early agonist-mediated ionic events in cultured vascular smooth muscle cells. J. Biol. Chem. **262**:5065-5072, 1987.

Chatterjee, M., Chiu, P.J.S., Doll, R.A., and Sybertz, E.J.: Effect of amiloride on regulatory mechanisms of vascular smooth muscle contraction. Biochem. Pharmacol. **37**: 813-818, 1988.

Coburn, R.F.: The airway smooth muscle cell. Fed. Proc. **36**: 2692- 2697, 1977.

Deth, R.C., Poirier, J.S., and Proulx, J.R.: Amiloride-induced relaxation of arterial smooth muscle. Fed. Proc. **44**:1105, 1985.

Fabiato, A. and Fabiato, F.: Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. J. Physiol. (Lond.) **276**:233-255, 1978.

Farley, J.M. and Miles, P.R.: The sources of calcium for acetylcholine-induced contractions of dog tracheal smooth muscle. J. Pharmacol. Exp. Ther. **207**: 340-346, 1978.

Fry, C.H., and Poole-Wilson, P.A.: Effects of acid-base changes on excitation-contraction coupling in guinea-pig and rabbit ventricular muscle. J. Physiol. (Lond.) **313**: 141-160, 1981.

Grover, A.K. and Samson, S.E.: Pig coronary artery smooth muscle: Substrate and pH dependence of the two calcium pumps. Am. J. Physiol. **251**: C529-C534, 1986.

Hatori, N., Fine, B.P., Nakamura, A., Cragoe, E. Jr., and Aviv, A.: Angiotensin II effect on cytosolic pH in cultured rat vascular smooth muscle. J. Biol. Chem. **262**: 5073-5078, 1987.

Howard, M.J., Mullen, M.D., and Insel, P.A.: Amiloride interacts with renal α- and β-adrenergic receptors. Am.J. Physiol. **253**:F21-F25, 1987.

Kahn, A.M., Shelat, H. and Allen, J.C.: Na^+/H^+ exchange is present in the sarcolemmal vesicles from dog superior mesenteric artey. Am.J. Physiol. **250**:H313-H319, 1986.

Kirkpatrick, C.T. Excitation and contraction in bovine tracheal smooth muscle. J. Physiol. (Lond.) **244**: 263-281, 1975

Korbmacher, C., Helbig, H., Stahl F., and Wiederholt, M.: Evidence for Na/H exchange and Cl/HCO_3 exchange in A10 vascular smooth muscle cells. Pflugers Arch./ Eur. J. Physiol.**412**: 29-36, 1988.

Kotlikoff, M.I.: Calcium currents in isolated canine airway smooth muscle cells. Am.J. Physiol. **254**:C793-C801, 1988.

Krampetz, I.K. and Bose, R.: Relaxant effect of amiloride on canine tracheal smooth muscle. J. Pharmacol. Exp. Ther. **246**:641-648, 1988.

Mrwa, U., Achtig, I. and Ruegg, J.C.: Influences of calcium concentration and pH on tension development and ATPase activity of the arterial actomyosin contractile system. Blood Vessels **11**:277-286, 1974.

Ozaki, H., Kojima, T., Moryama, T., Karaki, H., Urakawa, N., Kohama, K., and Nonomura, Y.: Inhibition by amiloride of contractile elements in smooth muscle of guinea pig taenia cecum and chicken gizzard. J. Pharmacol. Exp. Ther. **243**: 370-377, 1987.

Palaty, V.: Amiloride acts as an alpha-adrenergic antagonist in the isolated rat tail artery. Can. J. Physiol. Pharmacol. **64**: 931-933, 1986.

Philipson, K.D., Bersohn, M.M., and Nishimoto, A.Y.: Effects of pH on Na^{+}-Ca^{2+} exchange in canine sarcolemmal vesicles. Circ. Research **50**: 287-293, 1982.

Sparrow, M.P., Pfitzer, G., Gagelmann, M, and Ruegg, J.C.: Effect of calmodulin, Ca^{2+}, and cAMP protein kinase on skinned tracheal smooth muscle. Am. J. Physiol. **246**:C308-C314, 1984.

Tang, C.-M., Presser, F., and Morad, M.: Amiloride selectively blocks the low threshold (T) calcium channel. Science **240**: 213-215, 1988.

Vaughan-Jones, R.D., Eisner, D.A., and Lederer, W.J.: Effects of changes of intracellular pH on contraction in sheep cardiac purkinje fibers. J. Gen. Physiol. **89**: 1015-1032, 1987.

Weiss, G.B., Pang, I.-H., and Goodman, F.R. Relationship between ^{45}Ca movements, different calcium components and response to acetylcholine and potassium in tracheal smooth muscle. J. Pharm. Exp. Therap. **233**: 389-394, 1985.

Weissberg, P.L., Little, P.L., Cragoe, E. J., Jr., and Bobik, A.: Na-H antiport in cultured rat aortic smooth muscle: its role in cytoplasmic pH regulation. Am J.Physiol. **253**:C193-C198, 1987.

PART IV: THE EFFECT OF AMILORIDE ON CARBACHOL-INDUCED BRONCHOCONSTRICTION IN ANAESTHETIZED DOGS

ABSTRACT

Previous studies have shown that amiloride relaxed canine tracheal smooth muscle strips *in vitro* and attenuated contraction when present before stimulation. In this study, these actions were assessed *in vivo*. Sodium pentobarbital-anesthetized, open-chested dogs (n=6) were placed in a pressure-compensated plethysmograph and exposed to aerosolized carbachol to induce bronchoconstriction. Airway resistance (R_{aw}) was measured with the lung oscillation technique. Treatment with aerosolized amiloride (10 mM solution) significantly reduced the peak bronchoconstrictive response to carbachol challenge to 2.53 $\pm$ 0.35 cm H_2O/l/s from a control value of 7.78 $\pm$ 2.07 cm H_2O/l/s. Mean plateau R_{aw} after carbachol challenge was 2.27 $\pm$ 0.40 cm H_2O/l/s. Subsequent treatment with aerosolized amiloride decreased this plateau R_{aw} to a level not significantly different from control (1.24 $\pm$ 0.18 versus 0.62 $\pm$ 0.07 cm H_2O/l/s). This study indicates that amiloride reduces the bronchoconstrictive effect of carbachol and suggests a possible therapeutic and prophylactic use for this drug in the treatment of asthma and other bronchospastic disease.

INTRODUCTION

Krampetz and Bose have previously shown that micromolar concentrations of amiloride relax airway smooth muscle *in vitro* (1988). This action appears to be attributable to inhibition of the Na^+/H^+ antiporter on the airway smooth muscle membrane. A number of interesting effects of this drug were demonstrated using the isolated tracheal smooth muscle preparation. Amiloride relaxed contractions elicited with different stimulants, including KCl, serotonin, histamine, and carbachol - even in the presence of beta-blockers and indomethacin. This indicated that the relaxant effect of amiloride was not specific to a particular form of stimulation, nor was it mediated by beta-agonists or by relaxant prostaglandins. However, carbachol-induced contractions were most effectively relaxed. In addition to the pharmacological characteristics, it was observed that the effect of amiloride was easily reproducible in the same muscle strip over several hours (no tachyphylaxis), and had no long term detrimental effects on the ability of the tissue to develop isometric tension. These specific properties of amiloride - the ability to relax respiratory muscle regardless of contractile stimulus without tachyphylaxis or toxic effect - are also essential properties of an effective bronchorelaxant. This suggested that amiloride might serve as a relaxant of airway smooth muscle *in vivo*.

The present study was performed to investigate the bronchodilatory actions of amiloride in the dog. Because amiloride was found to be most effective in relaxing and blocking carbachol-induced contractions *in vitro*, this compound was chosen to induce bronchoconstriction *in vivo*. Based on the previously described studies, two fundamental questions were addressed. Could amiloride reduce bronchoconstriction induced by aerosolized carbachol, and could it limit the extent of bronchoconstriction if given prior to challenge? The results of this study suggest that when introduced as an aerosol *in vivo*, amiloride retains airway relaxant properties .

METHODS

Animals

Dogs of either sex weighing approximately 20 kg each were used in the experiment. The animals were anesthetized with nembutal, i.v. (30mg/kg). A catheter was inserted into the femoral artery in order to monitor arterial blood pressure. The chest was opened by a sternum splitting procedure and the trachea canulated with a steel tracheal tube. (An open-chest preparation was utilized to eliminate artifactual changes in pleural pressure measurements which could result from changes in esophageal tone when the esophageal balloon method is used.) Animals were ventilated with room air at approximately 20 breaths per min at a tidal volume of approximately 20 ml/kg, and were placed in a pressure compensated volume displacement plethysmograph (Figure 28). Lung volume was measured by a Krogh spirometer placed at the rear of the plethysmograph. A pneumotachygraph located beneath the spirometer was used to measure flow. The frequency response of the flow and volume signal has been previously described by Mink *et al.* (1979).

A large bore catheter was attached to a port in the tracheal tube to measure airway opening pressure (P_{ao}). Pressure at the lung surface was (P_{pl}) was measured by a catheter lying free in the plethysmograph (PE 205; length = 80 cm; diameter = 1.57 mm). Transpulmonary pressure ($P_{ao} - P_{pl}$) was measured by a differential pressure transducer (Validyne MP-45).

Airway resistance measurement techniques.

Airway resistance (R_{aw}) measurements were obtained by forced oscillation in a manner similar to that described by Wood and co-workers (1976). After a standard volume history to total lung capacity (30 cm H_2O, transpulmonary pressure) by a positive pressure source, the

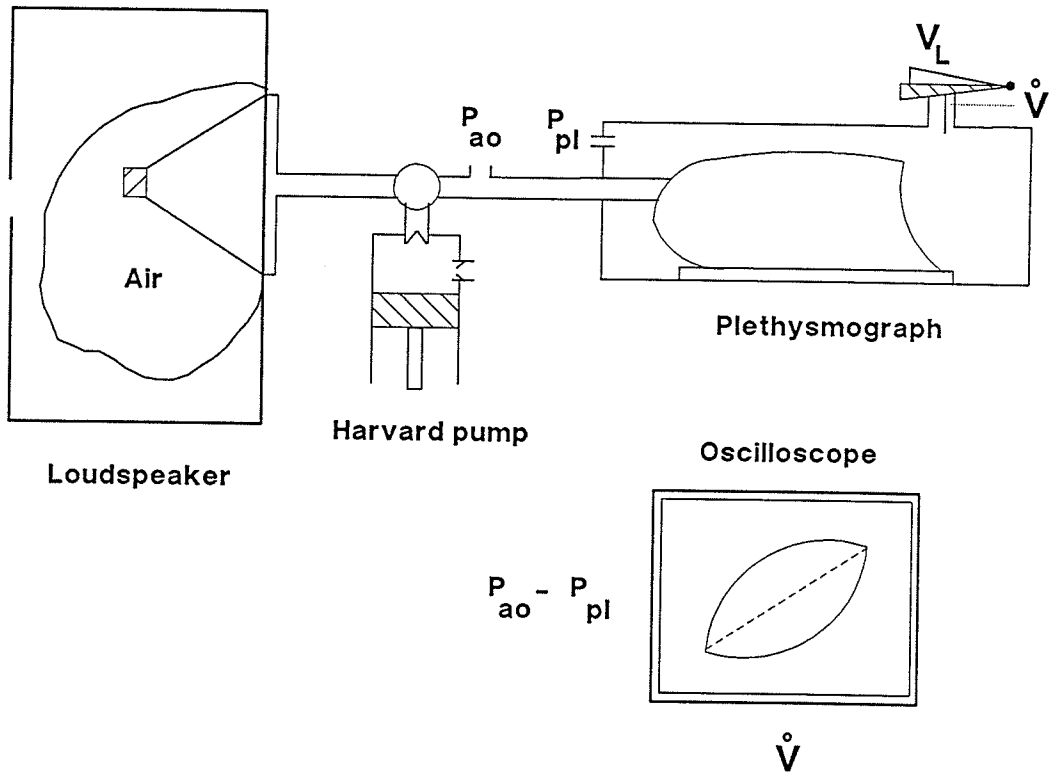

Figure 28: Schematic diagram of experimental apparatus used for the measurement of airway resistance. Anaesthetized open-chested dogs were placed in a body plethysmograph and ventilated with room air by a Harvard pump or oscillated by a loudspeaker. During the oscillation procedure, the differential pressures the airways and pleural space were displayed against flow, and the resulting pressure-flow loop was closed electronically (Modified from Wood et al., 1976).

lungs were slightly deflated and maintained at 5 cm H_2O transpulmonary pressure. The lungs were oscillated at 4 Hz at approximately 1 litre per second peak to peak by a loudspeaker powered by a sine wave generator. $P_{ao} - P_{pl}$ was plotted against flow on an oscilloscope. The resulting pressure-flow loop was closed by electrically subtracting the elastic and accelerative components of pressure. With the loop closed, pressure was in phase with flow and the slope of the resulting loop represented airway resistance. Flow and pressure signals, as well as lung volumes and blood pressure were displayed on a recorder (Hewlett Packard 7758P). Airway resistance was calculated by dividing the measured amplitude of the pressure wave forms by the amplitude of the flow oscillations.

Aerosols

Aerosol solutions were prepared from the following chemicals obtained from the Sigma Chemical Co. (St. Louis, MO): 10 mM carbamyl choline hydrochloride (in saline) and 10 mM amiloride hydrochloride. Carbachol was employed as stimulus because it is not hydrolyzed by acetylcholinesterase, and for aforementioned reasons (see introduction). Amiloride was dissolved in distilled deionized water due to its limited solubility in saline solution. The aerosol route of administration was chosen to ensure high local concentrations near the airway smooth muscle and to avoid large systemic doses. Aerosols were generated with a Bennet respiration unit (Model PR-2) nebulizer and delivered via the endotracheal tube. The particle size generated by this model has been estimated by Ryan et al. (1981) to be 3.6 (mass median diameter) $\pm$ 3.47 um (geometric standard deviation) at a flow rate of 7 l/min. Since we employed a flow rate of approximately twice this value (15 l/min), the nebulized particles produced would be somewhat smaller. This would allow the aerosol to be deposited diffusely throughout the lung. During aerosol delivery, tidal volume was increased to 500 - 750 ml. The aerosol reservoir contained 5 ml of the test solution. Under these conditions, a 4 minute aerosolization delivered approximately 2 ml of amiloride solution (as measured by volume

change in the reservoir), which corresponded to a 5.3 mg dose or 20 umoles of the drug. This amount was chosen for two reasons. Firstly, previously obtained *in vitro* evidence indicated that micromolar concentrations are required for relaxation of isolated respiratory smooth muscle. Secondly, this amount also closely corresponds to the lowest daily therapeutic dose prescribed in humans (5 mg). To avoid the documented antihypertensive actions and reduce the possibility of undesired systemic effects, higher doses were not employed.

Experimental Design

Each experiment (n=6) was conducted within 5 hours on the same day according to the following protocol. After a one minute application of aerosolized normal saline, four measurements of airway resistance (R_{aw}) were made about three minutes apart to ensure that the preparation was stable and the measurements reproducible in each of the animals. (This saline treatment was followed by a 4 minute exposure to aerosolized distilled water to establish the effect of the carrier alone, without prior carbachol exposure). Bronchoconstriction was then induced by exposing each dog to aerosolized carbachol in saline for one minute. In order to obtain a sustained and measurable increase in airway resistance, the exposure time was then adjusted (shortened or prolonged) for each individual animal so that peak response was at least 5 times baseline R_{aw} (*i.e.* > 2.5 cm H_2O/l/s). Since the ability of carbachol to induce sufficient bronchoconstriction varied between dogs, the actual exposure times to the aerosol ranged from 45 to 180 seconds. Once determined, this exposure time remained constant for each subsequent challenge within the same animal. To ensure sufficient time for recovery, a minimum of 30 minutes was allowed to elapse between challenges.

In every animal, exposure to aerosolized carbachol resulted in an immediate and dramatic rise in airway resistance. Although the peak height of this value did vary widely between animals, it remained relatively constant in the same individual. Thus, each animal

would serve as its own control. This peak response was followed by a gradual reduction in airway resistance. A new steady state of R_{aw} values generally occurred within 20 minutes subsequent to carbachol treatment (Fig30). At this point three "steady state" R_{aw} readings were obtained. The mean of these readings was designated as mean R_{aw} post carbachol.

Exposure to aerosolized carbachol produced a high initial peak resistance, followed by an increased steady state resistance compared with baseline. The characteristic time course of this carbachol-induced bronchoconstriction made it obvious that single point measurements of R_{aw} were inadequate for our purposes and might have been misleading. To reduce this source of error, repeated assessments of were made, and mean values used in analysis. Multiple measurements of airway resistance were made for two reasons. It was felt that a more accurate measurement of changes in this parameter would be obtained (mean value), and also that it allowed for a comparison of the time course of treatment effects to be made.

Once a stable level of bronchoconstriction had been attained, 5 of the 6 animals were treated with carrier solution (distilled water) following carbachol-induced bronchoconstriction. This control treatment preceded exposure to amiloride in order to eliminate the possibility of a carry-over effect. The protocol was identical to that of amiloride treatment (see below): after a stable elevated R_{aw} was achieved, approximately two milliliters of distilled water were delivered via aerosol over four minutes. Measurements of airway resistance were made every two minutes for approximately twenty minutes or until a new plateau was obtained. In this manner, we could establish the effect of the carrier solution *and* time on carbachol-induced bronchoconstriction independent of amiloride.

After carrier treatment, the dogs were rechallenged with carbachol and were allowed to reach a steady state bronchoconstriction. Aerosolized amiloride solution was then applied for 4

minutes. Airway resistance was measured at 2 minute intervals for approximately 20 minutes or until a new plateau was attained.

An assessment of amiloride's prophylactic activity was made in six dogs according to the following procedure: animals were treated with a 4 minute exposure to aerosolized amiloride followed immediately by another carbachol challenge of identical duration to the initial titrated challenge. Peak R_{aw} was measured within 2 minutes of challenge. In this instance, peak R_{aw} values before and after amiloride treatment were compared in the statistical analysis.

Statistical Analysis

Data were analyzed statistically using Duncan's New Multiple Range (NMR) test, where indicated in the figure legends. Student's t-test for unpaired data was employed for comparison of rate of decrease in R_{aw} after amiloride (n=6) and after sham treatment (n=5). Student's t-test for paired data was used for comparison of peak carbachol response before and after amiloride treatment. Significant differences were considered at p values of less than or equal to 0.05. All results are expressed as mean $\pm$ SEM.

RESULTS

With the described oscillation procedure, *in phase* sinusoidal waveforms of pressure and flow were obtained, the height of which varied with the different treatments (Figure 29). Airway resistance was calculated by dividing the mean amplitude of the pressure waveforms by that of the flow. These values were then plotted as a function of time for each dog .

Typical changes in airway resistance resulting from each of the described treatments in one animal are shown in Figure 30. After exposure to aerosolized saline, mean baseline airway resistance for each animal was calculated from at least 4 readings, resulting in a grand mean of 0.62 ± 0.07 cm H_2O/l/s from the 6 dogs. In two animals, including that shown in figure 30, treatment with the carrier solution itself (before carbachol treatment) produced a transient increase in airway resistance. However, in both cases, this elevation was short-lived, and R_{aw} returned to its pretreatment level. The dog used in the depicted experiment (and *only* this individual) also demonstrated a transient increase in R_{aw} with carrier exposure *after* carbachol treatment. This suggested that this animal may have been sensitive to the reduced osmolarity of the carrier aerosol, however, when this animal was later exposed to aerosolized saline (0.9% NaCl) after carbachol challenge (data not shown) a similar increase in R_{aw} was observed (with a peak airway resistance of 1.16 cm H_2O/l/s). As with the distilled water exposure, airway resistance subsequently returned to the pre-saline treatment level.

Following this carrier treatment, the dog was re-challenged with carbachol, resulting in a similar magnitude of peak bronchoconstriction as measured in the previous challenge. After four identical measurements of R_{aw} (defined as "steady state" R_{aw}), the dog was exposed to aerosolized 10 mM amiloride. This treatment resulted in an approximate 50 % decrease in airway resistance from the plateau

Dog 29/01/87

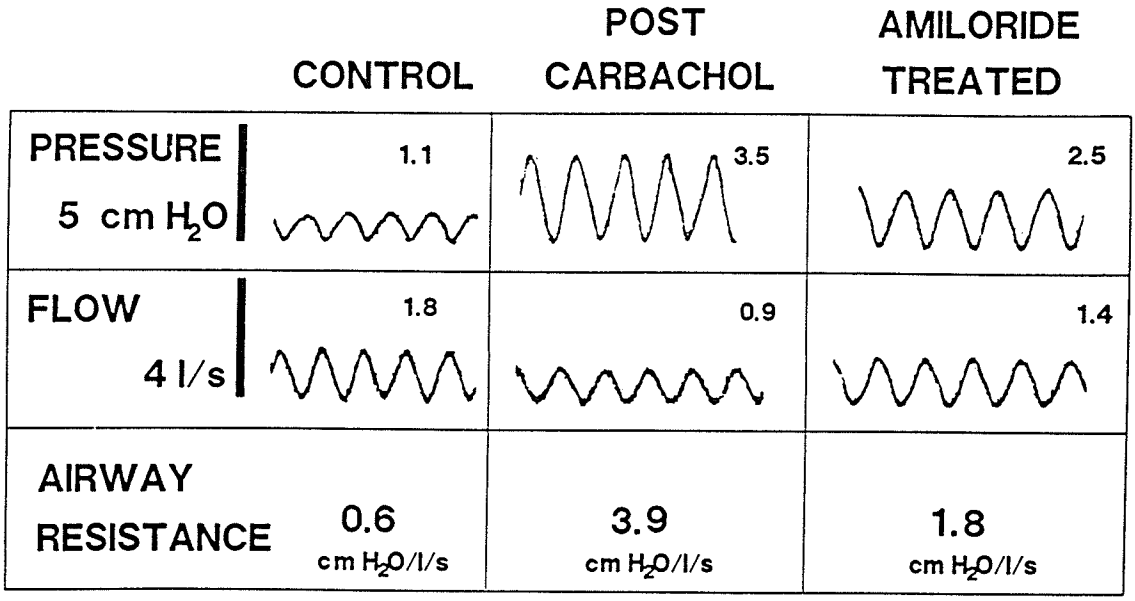

Figure 29: Oscillatory traces (4 Hz) of pressure and flow obtained throughout the experiment. Airway resistances were calculated via the division of amplitude of pressure tracings by amplitude of flow oscillations. Shown are typical control traces, post carbachol and amiloride treatment traces.

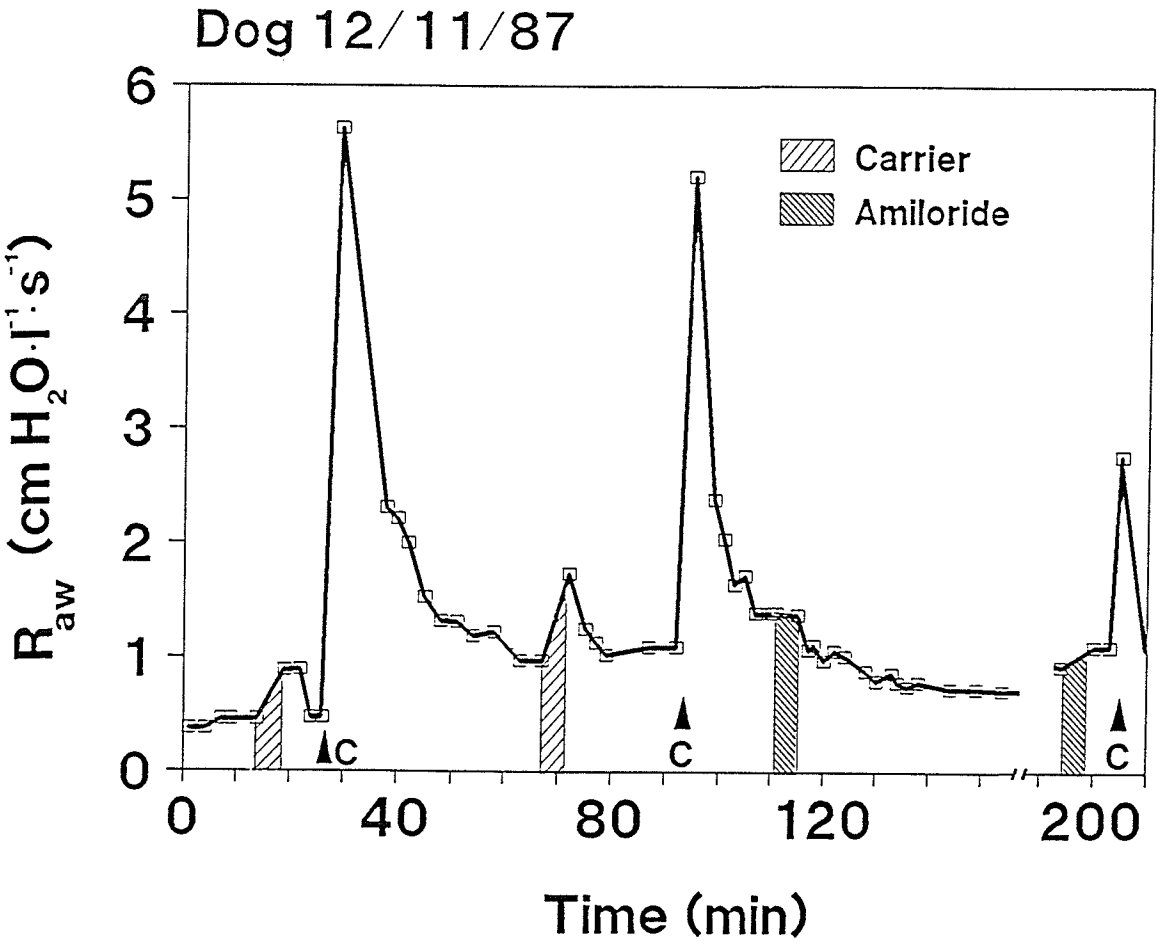

Figure 30: Plot of airway resistance changes observed in one animal during a typical experiment. Arrows (C) represent a one minute exposure to aerosolized carbachol. Shaded areas represent a 4 minute exposure to aerosolized carrier (distilled water) or 10 mM amiloride. Treatment with aerosolized carrier solution resulted in a transient increase in R_{aw}, which rapidly returned to pretreatment levels. Carbachol exposure produced a dramatic rise in airway resistance, followed by a decay and an elevated steady state R_{aw}. Note the contrast in the steady state R_{aw} maintained subsequent to carbachol exposure with amiloride and the carrier alone. Note also the relative decrease in peak height of bronchoconstriction after amiloride exposure.

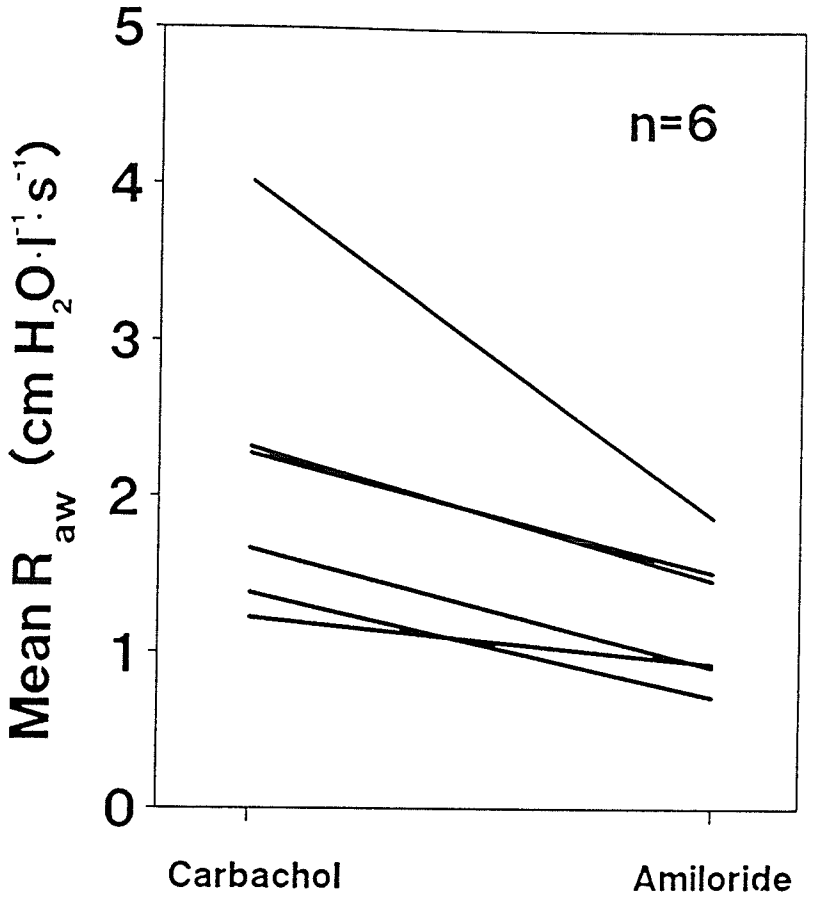

Figure 31: Individual mean steady state R_{aw} values obtained after bronchoconstriction with carbachol and with subsequent amiloride treatment (n=6). While post carbachol values are significantly elevated ($p<0.01$, Duncan's NMR test), subsequent amiloride treatment reduces R_{aw} to levels not different from controls ($p<0.05$, Duncan's NMR test).

bronchoconstriction. This reduction was not due to the carrier solution since the carrier alone actually produced a *bronchoconstriction*, which amiloride was also apparently able to inhibit. The effect of amiloride was not a time effect since the reduction was greater than that observed over a similar period in controls (see below). The animal was challenged again with carbachol (data not shown), to establish the reproducibility of the bronchoconstriction. Subsequent to treatment with amiloride aerosol, the peak response to carbachol was markedly decreased when compared to those achieved previously.

A summary of the individual data representing steady state airway resistance before and after amiloride treatment are presented in figure 31. The mean steady state R_{aw} value after carbachol treatment was significantly increased from baseline values (2.27 $\pm$ 0.41 cm H_2O/l/s versus 0.62$\pm$0.07 cm H_2O/l/s). After amiloride treatment, the mean airway resistance was reduced to 1.24 $\pm$ 0.18 cm H_2O/l/s, a value which was not statistically different from the original baseline R_{aw}.

Evidence indicates that these effects were primarily independent of a decay in bronchoconstriction caused by the carrier and time. This was examined by a comparison of the rates of decrease in airway resistance after amiloride treatment (n=6) and with the sham treatment (n=5). Results indicated that rate of decrease with amiloride treatment was six-fold greater than the carrier - 0.06$\pm$0.02 versus 0.01$\pm$0.01 cm H_2O/l/s per minute.

Aerosolized amiloride pretreatment also markedly decreased peak carbachol-induced bronchoconstriction. As shown in figure 30(final challenge), exposure to amiloride produced an obvious reduction in peak airway resistance. The effect was most marked when amiloride was applied immediately prior to carbachol challenge. Individual results from this experimental series are shown in figure 31. When a paired comparison was made of these data using a one-sided unpaired Student t-test,

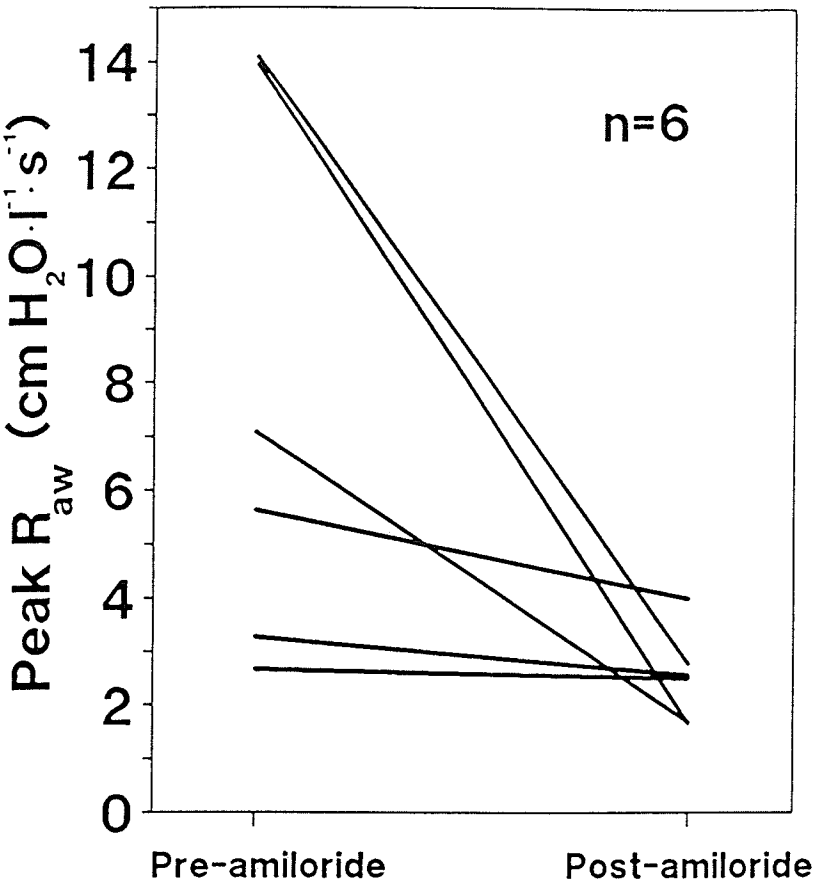

Figure 32: Peak airway resistance values before and after exposure to aerosolized amiloride. Analysis of data using a paired t-test (1-sided), indicated a significant decrease in R_{aw} (p=0.031).

the mean peak airway resistance after amiloride exposure was found to be significantly decreased ($p=0.03$) to 2.53 ± 0.35 cm H_2O/l/s from 7.78 ± 2.07 cm H_2O/l/s. This indicates that amiloride indeed exhibits a prophylactic action *in vivo*.

DISCUSSION

Our results show that aerosolized amiloride lessens bronchoconstriction induced by carbachol to a level which is not significantly different from that before challenge. In addition to this, an assessment of the prophylactic actions of amiloride indicated that pretreatment with this compound also markedly reduced peak bronchoconstriction. These results are not attributable to an effect of the carrier solution or of time, indicating that amiloride itself possesses bronchodilatory actions. This is in agreement with the previously described *in vitro* relaxant properties of this compound in isolated canine tracheal smooth muscle (Krampetz and Bose, 1988).

Amiloride - which is currently prescribed as a K^+-sparing diuretic - appears to exert is primary pharmacological actions via blockade of passive sodium transport across the cell membrane (Benos, 1982). This has been demonstrated in many diverse tissues using micromolar concentrations of the drug (Benos, 1982; Mahnensmith and Aronson, 1985). It is hypothesized that this is the origin of its current therapeutic effects, both diuresis and natriuresis (Palmer, 1986) and vascular smooth muscle relaxation (Haddy *et al.*,1985; Pinon and Fabre, 1985). In addition to the ability to relax vascular smooth muscle, amiloride has also been demonstrated to relax respiratory smooth muscle (Krampetz and Bose, 1988). Evidence indicates that this action may be based on the ability of amiloride to block the Na^+/H^+ antiporter in airway smooth muscle cell membranes.

While it is clear that amiloride can relax isolated respiratory smooth muscle, this ability is not immediate proof of its mechanism of action in the whole animal. For example, it may be suggested that the contrast in effect of the carrier solution versus the drug solution which we observed merely resulted from the difference in osmolarity of the distilled water versus 10 mM amiloride hydrochloride solution. Distilled water-induced bronchoconstriction has been

demonstrated in some asthmatics and has been attributed to hypo-osmolarity (Bascom and Bleeker, 1986; Echerbacher *et al.*, 1984). In the data shown in figure 30,distilled water did increase airway resistance, however, in this dog exposure to a 4 minute aerosol of 0.9% saline solution *also* provoked bronchoconstriction subsequent to carbachol challenge. In light of these observations, it seems unlikely that the slightly higher osmolarity of the amiloride solution could have been an important factor in the observed bronchorelaxation.

A critical point for the proposed mechanism of relaxation is whether amiloride was able to cross the epithelium to reach this proposed target site, and if so, was it present in sufficient quantities. In fact, Mentz *et al.* have presented evidence that amiloride is actively translocated in the lumenal to submucosal direction in epithelium from sheep trachea and bronchi (1986). These investigators, have also shown that the disappearance of amiloride from the airway surface liquid could be described by two exponential rates - an initial rapid decrease (half-time, 10.5 min) followed a slower rate of disappearance (half-time, 254 min). The initial reduction was dramatic - a decrease in concentration from 700 to 66 uM in 30 minutes. This indicates that amiloride could reach and even accumulate near the underlying respiratory smooth muscle during the time of the observed reduction of airway resistance. The somewhat slow time course of the bronchorelaxant effect of this drug may reflect the involvement of an accumulatory process. Tissue levels of this drug wre not assessed in our study. However, by employing an aerosol solution of high amiloride concentration (10 mM for 4 minutes, delivering approximately 20 umoles or 5.3 g of the drug) it was presumed that this tissue would then be exposed to the micromolar concentrations required to produce relaxation, without risking the systemic effects that greater quantities would have incurred.

The effect of amiloride on the respiratory epithelium itself must also be considered. Mentz *et al.*, have also demonstrated that amiloride alters the function of this tissue. Local application of amiloride on the trachea of sheep resulted in an increase in airway surface liquid

volume (1986). In addition to this, there is evidence that nebulized amiloride increases mucocilliary and cough clearance in humans, specifically those afflicted with cystic fibrosis (Kohler *et al.*,1986). Based on these data, amiloride does appear to affect the respiratory epithelium. We have not yet evaluated what contribution this might have towards the observed reduction in airway resistance, yet these effects may be beneficial in the treatment of asthma, where the production of thick, tenacious mucus can exacerbate airway closure.

Amiloride can also alter the function of inflammatory cells. Wright *et al.* (1986) have determined that superoxide production and lysozyme release in neutrophils in response to opsonized zymosan or concanavalin A is an amiloride-sensitive process. Lymphocyte activation and subsequent DNA synthesis is also inhibited by this compound (Heikkila *et al.*, 1983). However, of greatest relevance to airway function is the observation by Linnebjerg *et al.* (1988) that histamine and arachidonic acid release from rat mast cells is supressed by amiloride. While these antiinflamatory properties are probably of minor importance in our model of bronchoconstriction, they nonetheless suggest an additional beneficial effect from the use of this compound in allergic bron chospasm.

Our results indicate that amiloride is an effective bronchorelaxant in the dog and may exert its actions via a direct relaxant effect on respiratory smooth muscle and/or epithelium. Since amiloride is currently safely prescribed for humans in an oral form (as a diuretic), it is conceivable that both the acute actions and prophylactic properties which we observed, along with the mucus clearing effects observed by others will prompt further investigation into the use of this drug as an aerosol in the pharmacotherapy of human bronchospastic disease.

REFERENCES

Bascom, R., and Bleeker, E.R. Bronchoconstriction induced by distilled water. Am. Rev. Resp. Dis. **134**:248-253, 1986.

Benos, D.J. Amiloride: a molecular probe of sodium transport in tissues and cells. Am. J. Physiol. **242**: C131-C145, 1982.

Dolovich, M.B., Sanchis, J., Rossman, C., and Newhouse, M.T. Aerosol penetrance: a sensitive index of periferal airways obstruction. J. Appl. Physiol. **40**:468-471, 1976.

Eschenbacher, W.L., Boushey, H.A., and Sheppard, D. Alteration in osmolarity of inhaled aerosols cause bronchoconstriction and cough but absence of permeant anion causes cough alone. Am. Rev. Resp. Dis. **129**:211-215, 1984.

Haddy, F.J., Pamnani, M.B., Swindall, B.T., Johnson, J., and Cragoe, E.J. Jr. Sodium channel blockers are vasodilators as well as natriuretic and diuretic agents. Hypertension **7**(suppl): I121-I126, 1985.

Kohler, D., App, E., Schmitz-Schumann, M., Wurtemberger, G., and Matthys, H. Inhalation of amiloride improves the mucociliary and cough clearance in patients with cystic fibrosis. Eur. J. Respir. Dis. **69**(suppl): 319-326, 1986.

Krampetz I.K., and Bose, R.: Relaxant effect of amiloride on canine tracheal smooth muscle. J. Pharm. Exp. Therap. **246**:641-648 1988.

Linnebjerg, H., Hansen, H.S., and Jensen, B. Inhibition by amiloride and by Na^+-depletion of A23187-stimulated arachidonic acid and histamine release from rat mast cells. FEBS. Lett. **240**:167-170, 1988.

Mahnensmith, R.L., and Aronson, P.S. The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. Circ. Res. **57**:773-788 1985.

Mentz, W.M., Brown, J.B., Friedman, M., Stutts, M.J., Gatzy, J.T., Boucher, R.C. Deposition, clearance and effects of aerosolized amiloride in sheep airways. Am. Rev. Respir. Dis. **134**: 938-943, 1986.

Mink, S., Ziesmann, M., and Wood, L.D.H. Mechanisms of increased maximum expiratory flow during HeO_2 breathing in dogs. J. Appl. Physiol.: Respirat. Environ. Exercise Physiol. **47**:490-502, 1979.

Palmer, L.G. The epithelial sodium channel. *In* New Horizons in Therapeutics, ed. by G. Poste and S.T. Crooke, pp. 327-344, Plenum press, New York, 1986.

Pinon, J.F., and Fabre, J. Inhibition of potassium-induced contracture of the isolated rat aorta by amiloride. Arzneim. Forsch./Drug Res. **35**: 421-423, 1985.

Ryan, G., Dolovich, M.B., Ominkski, G., Cockcroft, D.W., Juniper, E., Hargreave, F.E., and Newhouse, M.T. Standardization of inhalation provocation tests: Influence of nebulizer output, particle size, and method of inhalation. J. Allergy. Clin. Immunol. **67**: 156-161, 1981.

Smith, A.J., and Smith, R.N. Kinetics and bioavailability of two formulations of amiloride in man. Br. J. Pharm. 48:646-649, 1973.

Wood, L.D.H., Engel, L.A., Griffin, P., Despas, P., and Macklem, P.T. Effect of gas physical properties and flow on lower pulmonary resistance. J. Appl. Physiol. **41**: 234-244, 1976.

GENERAL DISCUSSION

The studies outlined in this thesis grew from the initial observation that the K^+-sparing diuretic amiloride relaxed canine tracheal smooth muscle in vitro. After this effect had been clearly established, the characteristics of amiloride-induced relaxation were examined, including concentration-response characteristics. Preliminary evidence suggested that amiloride might have been interfering with the sodium-hydrogen antiporter, therefore, the presence of this transport process in tracheal smooth muscle was confirmed. Once this had been established, the possibility that antiporter inhibition or other likely mechanisms were responsible for amiloride-induced relaxation of canine tracheal smooth muscle was investigated. Finally, a study was made to determine whether amiloride's airway smooth muscle relaxing effects were operative *in vivo*.

The characteristics of amiloride-induced relaxation in isolated tracheal smooth muscle were related to the original stimulus, with "high affinity" sites present during both potassium and carbachol induced contractions (IC_{50} = 12.3 and 23.5uM, respectively) and the "low affinity" amiloride-sensitive site appearing only with addition of high amiloride concentrations to carbachol-contracted muscle strips (IC_{50} = 75.5uM). In order to elucidate the origin of each of these relaxation phases, their characteristics were examined. The attenuation of amiloride-induced relaxation in both KCl- and carbachol-contracted strips observed under conditions of increased external sodium concentration indicated that both of the aforementioned sites were sensitive to external sodium concentration. This supports the contention that amiloride was interacting with one or more sodium dependent processes.

As outlined in the introduction of the thesis, amiloride does inhibit several sodium-dependent membrane transport processes. Because of this ability and the apparent sodium sensitivity of amiloride-induced relaxation of canine trachealis, it was of primary concern to

clarify whether inhibition of these transport processes could be responsible for amiloride-induced relaxation of canine tracheal smooth muscle. To address this, evidence was required to support that (a) the process was present in this tissue, and (b) that the concentration of amiloride present was within the known range for inhibition of that process. In addition, it was necessary to examine a number of other alternate mechanisms by which amiloride might relax this smooth muscle.

Based on current evidence, it is not likely that epithelial type sodium channels are present in mammalian smooth muscle. Inhibition of these channels occurs at a low concentration of amiloride (IC_{50} values less than 1 uM). Since little or no relaxation of canine trachea smooth muscle was observed at this concentration of amiloride, this either suggest that these channels are not present in tracheal smooth muscle or that epithelial type channels are not important the relaxant effect of this drug.

For similar reasons, it is unlikely that Na^+/Ca^{2+} exchange inhibition is a major contributor to the observed relaxation phenomenon. To date, this process has not been demonstrated to occur in canine tracheal smooth muscle, and its presence and importance in the contraction of airway smooth muscle from other species is apparently unclear (Kirkpatrick and McDaniel, 1976; Bullock *et al.* 1981). Amiloride is not a specific or potent inhibitor of Na^+/Ca^{2+} exchange (see Introduction), since IC_{50}'s for this process are in the millimolar range. At this concentration, a number of key enzymes are also affected, including the myosin light chain kinase, Na^+/K^+ ATPase, and protein kinase C, all of which appear to have important roles in smooth muscle function.

In fact, only one of the sodium gradient-dependent, amiloride-sensitive transport processes previously described has been clearly shown in a canine smooth muscle preparation. Kahn *et al.* (1986) have demonstrated an amiloride sensitive, pH gradient-stimulated, ^{22}Na

uptake in sarcolemmal vesicles from dog superior mesenteric artery, indicating the presence of a Na^+/H^+ exchanger in canine vascular smooth muscle. Part II of this thesis demonstrated the presence and importance of the Na+/H+ exchanger in intracellular pH regulation in canine tracheal smooth muscle. Although direct measurements of ionic movement were not made, it was apparent from these data that intracellular pH regulation was markedly affected by reversal of the sodium gradient or by the presence of amiloride in the incubating solution. These data are consistent with those of other investigators who have examined this process in vascular smooth muscle preparations, and indicate an important role for this process in the regulation of intracellular proton concentration in canine airway smooth muscle.

The presence of a Na^+-dependent mechanism for the regulation of intracellular pH, and demonstration of its inhibition by amiloride is not, however, conclusive evidence that relaxation of canine tracheal smooth muscle was caused by inhibition of this process. Part III of the thesis examined this and a number of alternative mechanisms for amiloride-induced relaxation of canine airway smooth muscle. In these studies, it was determined that relaxation was not mediated through an interaction of amiloride with beta adrenoceptors, since the presence of propranolol or sotalol had no effect on amiloride-induced relaxation. [Both beta-1 and beta-2 receptors mediate relaxation in canine tissue (Barnes et al., 1983).] Relaxation was also not mediated by the release of cyclooxygenase products from the tissue, since indomethacin did not alter the effect of amiloride. The possibility that amiloride relaxed tracheal smooth muscle by elevating intracellular levels of cyclic AMP (either through stimulation of adenlylate cyclase or through inhibition of phosphodiesterase) was also tested, and revealed that no increase in concentration of tissue cAMP occured. Other airway smooth muscle relaxants, forskolin and theophylline, apparently relax airway smooth muscle through these mechanisms (Burka, 1983). Another alternative - that amiloride relaxed tracheal strips through increasing intracellular cyclic GMP concentrations - was not tested, although it has recently been shown

that elevated levels of this compound in isolated tracheal smooth muscle are associated with relaxation (Ishii and Murad, 1989).

While other investigators (Ozaki *et al.* 1987; Chatterjee *et al.*, 1988) have suggested that a direct effect of amiloride on the contractile elements may explain the relaxant action of amiloride in other smooth muscles, in our preparation amiloride had only a minor effect on contraction of "skinned" tracheal smooth muscle fibres, even when exposed to concentrations exceeding those seen by muscle strips with an intact membrane. In addition, no significant uptake of this compound was observed in our preparation. This indicated that amiloride could have no direct action on the contractile elements.. Because amiloride relaxed skinned guinea pig taenia coli, Ozaki *et al.* (1987) examined the effect of amiloride on activity of chicken gizzard myosin light chain kinase (MLCK) and presented evidence that amiloride can inhibit this enzyme *in vitro*. The concentrations required for significant inhibition were near the millimolar range, and therefore would again be dependent on active intracellular accumulation of the drug - a phenomenon absent in canine tracheal smooth muscle.

With the development of a technique which allowed for the simultaneous measurement of intracellular pH and tension (Part II), it was observed that amiloride produced an intracellular acidification in both KCl- and carbachol-stimulated muscle which was coincident with relaxation. Acidification upon exposure to amiloride and amiloride analogs has been previously described in vascular smooth muscle preparations by Korbmacher *et al.* (1988), Hatori *et al.* (1987), Berk *et al.* (1987) and by Weissberg *et al.* (1987). However, since these studies were performed with isolated or cultured vascular smooth muscle cells, the effect of this treatment on tension was not measured.

While the similar time course of acidification and relaxation does not necessarily imply a cause-and-effect relationship, the induction of intracellular acidiosis by alternate means

(exposure to acid medium) also resulted in a decrease in tension in our preparation. Intracellular acidification has been demonstrated to decrease contraction of other muscle types. In sheep purkinje fibres, a reduction in intracellular pH coincided with a fall in force (Vaughan-Jones *et al.*, 1987). This may be linked to a decrease in the sensitivity of calcium-dependent processes. Fabiato and Fabiato (1978) have shown decreased calcium sensitivity of skinned cardiac and skeletal muscle myofilaments with increased proton concentration. This is also the case in vascular smooth muscle as demonstrated by Mrwa *et al.* (1974) in glycerinated hog carotid artery. In this study, it was shown that a decrease in one pH unit (from 7.0 to 6.0) resulted in a hundredfold decrease in calcium sensitivity. A similar result was obtained while measuring Mg^{2+}-activated ATPase activity of actomyosin. While this may be a rather large and unphysiological shift in hydrogen ion concentration, a smaller decrease in pH (such as the 0.2 U decrease observed with exposure to 100 uM amiloride) would nonetheless tend to decrease the tension producing ability of the contractile apparatus under conditions of constant calcium concentration.

Another likely explanation is that a decrease in pH_i could directly affect $[Ca]_i$ regulating and transport systems. In guinea-pig and rabbit cardiac ventricular muscle, for example, intracellular acidosis is able to depress calcium entry through the slow inward current (Fry and Poole-Wilson, 1981). Philipson *et al.* (1982) have demonstrated that in cardiac sarcolemmal vesicles isolated from dog ventricle, Na^+-dependent Ca^{2+}-uptake was reduced by 50% at pH 6.7. Thus, another conductive pathway for calcium, the Na^+/Ca^{2+} exchanger, can also be affected by proton concentration.

The data presented in Part III suggest that amiloride may effect calcium movement in canine tracheal smooth muscle, specifically that intracellular calcium release and calcium influx can be altered by this drug. This may help to explain the distinct effects of amiloride in carbachol- and potassium-induced contractions. One of the major differences between these

two forms of contraction in tracheal smooth muscle appears to be a reliance on different calcium pools (Coburn and Yamaguchi, 1977; Farley and Miles, 1977). Muscarinic agonists such as carbachol increase entry of this ion through receptor-operated membrane channels, but they also stimulate release of calcium from intracellular sources (Farley and Miles, 1978), probably via phosphoinositide production. Potassium induces contraction in the trachealis through membrane depolarization and subsequent activation of voltage dependent channels, which allows the influx of calcium from extracellular sites and thus permits contraction. It has been purported that these contractions are solely dependent on extracellular calcium sources (Coburn, 1977), however, Kannan *et al.* (1987) have shown that a considerable proportion of contraction can be elicited in calcium free medium (containing 0.1 mM EGTA) even in the presence of 0.1 uM nifedipine. Data within this thesis also support this, and suggest that another source of calcium may be available during potassium-induced contraction.

Amiloride reduced carbachol-induced contraction in calcium free medium, but had no significant effect on KCl-elicited contraction under the same conditions. Contraction which occured upon calcium addition to the medium was reduced under both forms of stimulation. It may be that the additional effect of amiloride observed during carbachol stimulation represents inhibition of calcium release from intracellular sites. Bund *et al.* (1987) have also suggested that the differential effect of amiloride and ethylisopropyl amiloride on contractions of human resistance arterioles elicited with KCl or norepinephrine may be due to interference with phosphoinositide mediated contraction. It is possible that activation of the inositide pathway during muscarinic stimulation may present another amiloride sensitive site in our preparation, and may explain the greater sensitivity of carbachol contractions. Chatterjee *et al.* (1988) have demonstrated that amiloride reduces calcium efflux from intact norepinephrine-stimulated rabbit aorta. The authors attribute this to a decrease in calcium release from intracellular sites, although they propose no mechanism for this effect. It is possible that the "low" affinity site for amiloride observed exclusively in carbachol contraction could represent a direct or indirect

interaction with these processes. Whether this additional effect is mediated by a direct action of amiloride or through intracellular acidification is not clear. However, a greater sensitivity of carbachol-induced contraction to intracellular acidification is clearly demonstrated by the marked effect of acid addition on carbachol contracted strips as compared to the relatively small perturbation in tension seen during a potassium-induced contraction.

A final possibility remains that amiloride may directly inhibit calcium movement in this tissue, rather than indirectly through acidification. Amiloride has been demonstrated to selectively block low threshold T-type calcium channels in mammalian neural and atrial tissue at relatively low concentrations (K_D = 30uM) using the patch clamp technique (Tang *et al.*, 1988). Recently, the presence of a T-type channel has been documented in canine airway smooth muscle cells with characteristics described as "very similar to the T current or fast calcium current reported in pituitary, neuronal, atrial and smooth muscle cells" (Kotlikoff, 1988). Whether amiloride may directly block extracellular calcium entry through such a channel and whether this would have physiologically relivant effects remains speculative.

The results presented in Part IV indicate that aerosolized amiloride can lessen carbachol-induced bronchoconstriction. When applied prior to challenge amiloride reduced the peak bronchoconstriction developed during subsequent carbachol challenge. The mechanism of this bronchorelaxant effect *in vivo* was not investigated, although the *in vitro* evidence would suggest a direct action on the smooth muscle of the airway. In the anaesthetized dog, other effects of amiloride may be important, particularly its effect on the respiratory epithelium. There is increasing evidence that this tissue may play a role in the modulation of airway calibre. It is known that ion transport across the airway epithelium is altered by amiloride (Mentz *et al.*, 1986), but the significance of this toward our own observations is unknown.

The most significant information obtained in this study may be summarized into two important points. Through the use of amiloride, it has been shown that intracellular pH regulaton by the Na+/H+ antiporter is important in excitation-contraction coupling of canine airway smooth muscle (Figure 33). In addition, these data - especially the *in vivo* studies - suggest that amiloride's effects on airway smooth muscle contraction may be utilized in the treatment of bronchospastic disease. Since amiloride is currently prescribed for humans in an oral form (as a diuretic), it is conceivable that this may prompt further investigation into the use of this drug as an aerosol in the pharmacotherapy of human bronchospastic disease.

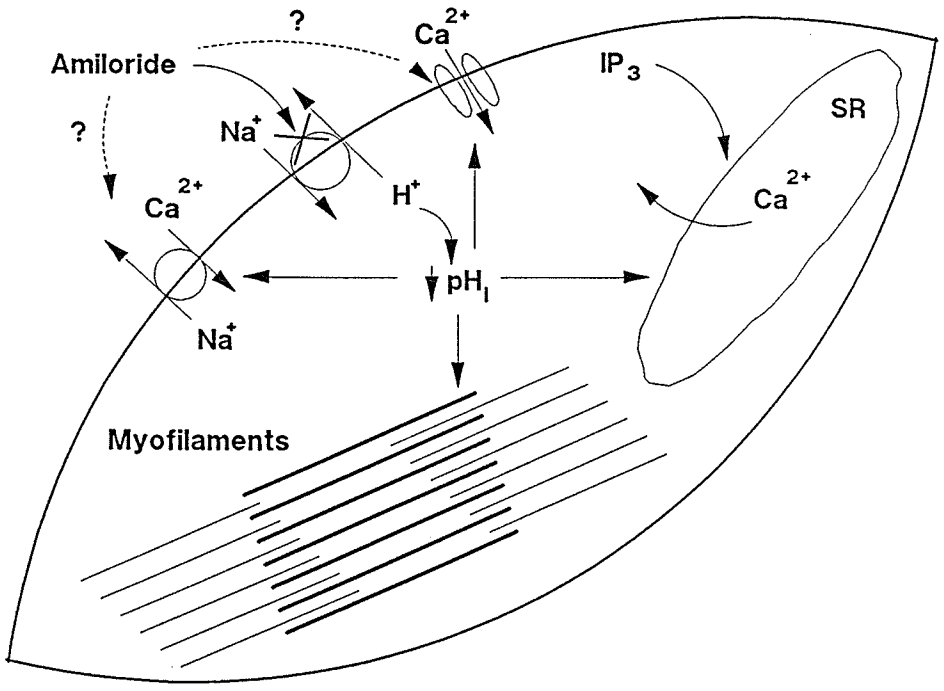

Figure 33: Schematic diagram of an airway smooth muscle cell and the proposed mechanism for amiloride-induced relaxation.

REFERENCES

Barnes, P.J., Nadel, J.A., Skoogh, B.-E., Roberts, J.M. Characterization of *beta* adrenoceptor subtypes in canine airway smooth muscle by radioligand binding and physiological responses. J. Pharm. Exp. Therap. **225**: 456-461, 1983.

Berk, B.C., Aronow, M.S., Brock, T.A., Cragoe, E. Jr., Gimbrone, M.A., Alexander, R.W. Angiotensin II-stimulated Na^+/H^+ exchange in cultured vascular smooth muscle cells. J. Biol. Chem. **262**: 5057-5064, 1987.

Bullock, C.G., Fettes, J.J.F. and Kirkpatrick, C.T. Tracheal smooth muscle - second thoughts on sodium-calcium exchange. J. Physiol. (Lond.) **318**: 46P, 1981.

Bund, S.J., Heagerty, A.M., Issard, A.S., and Swales, J.D. Amiloride-induced relaxation of human resistance arterioles: evidence for a central role of Na+/H+ exchange in sustained smooth muscle contraction. J. Physiol. (Lond.) **391**:25P, 1987.

Burka, J.F. Effects of selected bronchodilators on antigen- and A23187-induced contraction of guinea pig trachea. J. Pharm. Exp. Therap. 225: 427-435, 1983.

Chatterjee, M., Chiu, P.J.S., Doll, R.A., and Sybertz, E.J.: Effect of amiloride on regulatory mechanisms of vascular smooth muscle contraction. Biochem. Pharmacol. **37**: 813-818, 1988.

Coburn, R.F.: The airway smooth muscle cell. Fed. Proc. **36**: 2692- 2697, 1977.

Coburn, R.F. and Yamaguchi, T. Membrane potential-dependent and -independent tension in the canine tracheal muscle. J. Pharm. Exp. Therap. **210**: 276, 1975.

Deth, R.C., Poirier, J.S., and Proulx, J.R.: Amiloride-induced relaxation of arterial smooth muscle. Fed. Proc. **44**:1105, 1985.

Fabiato, A. and Fabiato, F.: Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. J. Physiol. (Lond.) **276**:233-255, 1978.

Farley, J.M. and Miles, P.R. Role of depolarization in acetylcholine-induced contractions of dog trachealis muscle. J. Pharmacol. Exp. Ther. **201**:199-205, 1977.

Farley, J.M. and Miles, P.R.: The sources of calcium for acetylcholine-induced contractions of dog tracheal smooth muscle. J. Pharmacol. Exp. Ther. **207**: 340-346, 1978.

Fry, C.H., and Poole-Wilson, P.A.: Effects of acid-base changes on excitation-contraction coupling in guinea-pig and rabbit ventricular muscle. J. Physiol. (Lond.) **313**: 141-160, 1981.

Hatori, N., Fine, B.P., Nakamura, A., Cragoe, E. Jr., and Aviv, A.: Angiotensin II effect on cytosolic pH in cultured rat vascular smooth muscle. J. Biol. Chem. **262**: 5073-5078, 1987.

Ishii, and Murad, F. ANP relaxes bovine tracheal smooth muscle and increases cyclic GMP. Am. J. Physiol. **256**: C495-C500, 1989.

Kahn, A.M., Shelat, H. and Allen, J.C.: Na^+/H^+ exchange is present in the sarcolemmal vesicles from dog superior mesenteric artey. Am.J. Physiol. **250**:H313-H319, 1986.

Kirkpatrick, C.T. and McDaniel, D.G. Tracheal smooth muscle - failure to demonstrate Na-Ca exchange. Pflugers Arch. 361: 301-301, 1976.

Korbmacher, C., Helbig, H., Stahl F., and Wiederholt, M.: Evidence for Na/H exchange and Cl/HCO_3 exchange in A10 vascular smooth muscle cells. Pflugers Arch./ Eur. J. Physiol.**412**: 29-36, 1988.

Kotlikoff, M.I.: Calcium currents in isolated canine airway smooth muscle cells. Am.J. Physiol. **254**:C793-C801, 1988.

Mentz, W.M., Brown, J.B., Friedman, M., Stutts, M.J., Gatzy, J.T., Boucher, R.C. Deposition, clearance and effects of aerosolized amiloride in sheep airways. Am. Rev. Respir. Dis. **134**: 938-943, 1986.

Mrwa, U., Achtig, I. and Ruegg, J.C.: Influences of calcium concentration and pH on tension development and ATPase activity of the arterial actomyosin contractile system. Blood Vessels **11**:277-286, 1974.

Ozaki, H., Kojima, T., Moryama, T., Karaki, H., Urakawa, N., Kohama, K., and Nonomura, Y.: Inhibition by amiloride of contractile elements in smooth muscle of guinea pig taenia cecum and chicken gizzard. J. Pharmacol. Exp. Ther. **243**: 370-377, 1987.

Philipson, K.D., Bersohn, M.M., and Nishimoto, A.Y.: Effects of pH on Na^+-Ca^{2+} exchange in canine sarcolemmal vesicles. Circ. Research **50**: 287-293, 1982.

Tang, C.-M., Presser, F., and Morad, M.: Amiloride selectively blocks the low threshold (T) calcium channel. Science **240**: 213-215, 1988.

Vaughan-Jones, R.D., Eisner, D.A., and Lederer, W.J.: Effects of changes of intracellular pH on contraction in sheep cardiac purkinje fibers. J. Gen. Physiol. **89**: 1015-1032, 1987.

Weissberg, P.L., Little, P.J., Cragoe, E.J. Jr., and Bobik, A. Na-H antiport in cultured rat aortic smooth muscle: its role in cytoplasmic pH regulation. Am. J. Physiol. **253**: C193-198, 1987.