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Effect of CO_2 and pH on drug-induced contractions of airway smooth muscle

Author
Duckles, Carolyn Sue

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EFFECT OF CO_2 AND pH ON DRUG-INDUCED CONTRACTIONS OF AIRWAY SMOOTH MUSCLE

by

Carolyn Sue Duckles
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DISSERTATION

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Effect of CO_2 and pH on drug-induced contractions of airway smooth muscle. C.S. Duckles.

Serotonin-induced contractions of isolated feline bronchial rings were relaxed rapidly and reversibly by increased CO_2 concentrations in the bath. However, acetylcholine-induced contractions were relatively insensitive to CO_2. Inhibition of acetylcholinesterase with physostigmine did not alter the selective effect of CO_2, eliminating the possibility that an effect of pH on acetylcholinesterase activity masked an effect of CO_2 on acetylcholine-induced contractions. Histological study of isolated bronchial rings indicated that the proportion of smooth muscle to total mass of the bronchial wall was less than 10%. Furthermore the presence of ganglia and nerves in this preparation was confirmed. Ten ganglia were found in each mm^3 of tissue with an average of 4.5 cells/ganglia. Changing pH by adding strong acid or base mimicked the effect of increasing or decreasing respectively the concentration of CO_2. Thus it can be concluded that the effect of short-term exposure to CO_2 depends on changes of extracellular pH. CO_2 does not act by altering the ionization of these drugs since the pKa of serotonin is 10.0, and the choline esters are quaternary ammonium compounds. The possibility that nervous elements mediate the effect of CO_2 was considered. However, the selective effect of CO_2 was not altered by blockade of the β-sympathomimetic receptor with propranolol or sotalol, by blockade of the cholinoreceptor with atropine, by blockade of nervous conduction with tetrodotoxin, or by ganglionic blockade with hexamethonium. In the presence of increased extracellular potassium, drugs still contracted bronchial smooth muscle. Furthermore, the effect of CO_2 persisted. Thus the membrane potential did not appear to play a role in drug-induced contractions

or the selective effect of CO_2. EGTA blocked drug-induced contractions; however, they were partially maintained by 0.12 mM calcium. Sensitivity of serotonin-induced contractions persisted in the presence of low calcium concentrations, eliminating the possibility that CO_2 alters the availability of calcium in the bathing medium. CO_2 does not act by influencing the activity of the contractile elements since contractions induced by potassium or acetylcholine were relatively insensitive to CO_2. However, sensitivity to CO_2 was not a unique property of the serotonin receptor system: contractions induced by several choline esters, carbachol, bethanechol and methacholine, were more sensitive to CO_2 than were contractions induced by acetylcholine itself. Differences in contraction rates among the choline esters could be attributed to differences in effective concentrations of these drugs. Distinctness of receptors for serotonin and these choline esters was verified by use of specific blocking agents, methysergide and atropine. It was also determined that at low pH, carbachol did not block acetylcholine-induced contractions. Tetramethylammonium-induced contractions were markedly sensitive to CO_2. Histamine, α-sympathomimetic receptor stimulants, prostaglandin $F_{2\alpha}$ and bradykinin did not contract this preparation. High concentrations of histamine relaxed carbachol-induced contractions. CO_2 could act by altering binding of drug to receptor or by influencing the specific transmission process from the receptor to the contractile elements. CO_2 had a reversible effect on tension during washout of serotonin- and carbachol-induced contractions which implies that CO_2 does not affect drug binding. However this depends on several assumptions, and direct measurement of drug binding would be necessary to reach a definite conclusion. Whether CO_2 affects drug binding or the transmission process from the receptor

to the contractile elements, it appears that there is more than one mechanism for contraction of airway smooth muscle by choline esters.

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INTRODUCTION

A. EFFECTS OF CO_2 ON BRONCHIAL TONE

CO_2 appears to influence airway smooth muscle tone by more than one mechanism. In animals with intact vagal innervation, ventilation with carbon dioxide increased airway resistance (Nadel and Widdicombe, 1962; Green and Widdicombe, 1966). Hypercapnia of the central nervous system had similar effects (Daly et al, 1953). The bronchoconstrictor effect of CO_2 was abolished when the vagus nerves were sectioned suggesting that reflex vagal pathways were involved in this response.

In contrast to the vagally mediated bronchoconstrictor effect of CO_2, bronchodilation appears to result from a direct effect of CO_2 on airway smooth muscle. However, CO_2 appears to act directly by more than one mechanism depending on the length of exposure to CO_2. Short-term exposure to CO_2 (on the order of several minutes) produces a selective relaxant effect: in vagotomized dogs bronchoconstriction produced by serotonin was markedly and rapidly relaxed by inhalation of CO_2, whereas bronchoconstriction produced by acetylcholine or vagal stimulation was affected only slightly (Green and Widdicombe, 1966; Sterling, et al, 1972). Similar selective effects have been demonstrated *in vivo* and *in vitro* in a variety of species (Gaddum and Stephenson, 1958; Atkinson et al, 1970; Pun et al, 1971; Sterling et al, 1972; Tang et al, 1972). On the other hand, prolonged exposure to CO_2 (more than 30 minutes) produced relaxant effects on contractions induced by acetylcholine or electrical stimulation (Stephens et al, 1968; Mitchell and Stephens, 1972).

There is evidence to suggest that the effect of short-term exposure to CO_2 depends on changes of extracellular pH whereas the effect of prolonged exposure to CO_2 is produced by change of intracellular pH. Thus, the short-term effect of CO_2 on serotonin-induced contractions was

mimicked by addition of acid to the bathing medium (Sterling et al, 1972), but contractions induced by electrical stimulation were not affected by changing extracellular pH when intracellular pH was presumably unaltered (Stephens et al, 1968).

Work by Nisell (1950) on isolated perfused lungs appears to contradict these findings. He found that short-term exposure to CO_2 relaxed constriction produced by either acetylcholine or vagal stimulation. However, the presence of other bronchoconstrictor substances (such as serotonin) released from the perfusing blood cannot be ruled out.

This review of the effects of carbon dioxide on airway smooth muscle shows that a number of questions remain unanswered. One aspect that appears to have particular pharmacological interest concerns the selective effect of CO_2. Short-term exposure to CO_2 inhibits markedly bronchoconstriction produced by serotonin but has much less effect on bronchoconstriction produced by either acetylcholine or vagal stimulation. Preliminary evidence also suggests that this effect of CO_2 depends on an alteration of extracellular pH. From these findings the hypothesis of this dissertation was formulated.

B. HYPOTHESIS

The finding that sensitivity to short-term exposure to CO_2 appears to be a unique property of serotonin-induced contractions coupled with the probable extracellular site of action of CO_2 leads to the hypothesis that CO_2 acts by altering the serotonin receptor system. Current ideas about the mechanism of drug-induced contractions of smooth muscle assume that the drug must bind to or interact with some specific component of the cell (the receptor). The receptor, however, remains hypothetical; only in a few special cases are drug receptors clearly identified. This

binding of the drug to its receptor initiates a series of steps that lead ultimately to a rise in the concentration of ionized calcium available to contractile proteins (Somlyo and Somlyo, 1968). The steps between binding of the drug to its receptor and a rise in free intracellular calcium also remain undefined. Processes such as membrane depolarization (due to increased membrane permeability or changes in electrogenic pump activity), increased membrane permeability to calcium, increased concentration of second messengers (cyclic AMP or GMP), and release of calcium from intracellular storage sites (sarcoplasmic reticulum, surface vesicles) are thought to comprise some of these steps in various types of smooth muscle. However, in no case is the full process understood. CO_2 could act by altering the binding of serotonin to its receptor, perhaps by changing the ionization of important chemical groupings. Alternatively, CO_2 could affect subsequent steps in the serotonin receptor mechanism. Although these steps are not clearly defined, by utilizing various pharmacological tools some aspects of these processes can be clarified, and the reasons for selectivity of the effect of CO_2 can be better understood. Therefore, the effect of short-term exposure to CO_2 was studied using an in vitro system, the feline isolated bronchial ring. The results and conclusions of this investigation will be presented in six sections as outlined below.

C. AIMS AND APPROACH

I. Histology of bronchial rings Study of the histology of isolated bronchial rings was carried out to determine the proportion of smooth muscle and the distribution of ganglia in the bronchial wall.

II. The selective effect of CO_2 on serotonin- and acetylcholine-induced contractions The effect of a range of CO_2 concentrations on

acetylcholine- and serotonin-induced contractions was investigated to determine the time course of the CO_2 effect and its reversibility. The dependence of the effect of CO_2 on drug concentration and the effect of CO_2 during washout after supramaximal drug-induced contractions were determined. The dependence of the effect of CO_2 on change of pH was studied to differentiate between extra- and intracellular sites of action.

III. Participation of nerves in the effect of CO_2 Various blocking agents were used to determine whether tissue nervous elements were involved in the bronchodilator effect of CO_2. The hypothesis that CO_2 causes release of β-sympathomimetic agonist from tissue stores was considered as well as the possibility that CO_2 acts on ganglia. The possibility that serotonin could cause acetylcholine release, a mechanism that might be sensitive to CO_2, was also investigated.

IV. The cholinoreceptor The insensitivity of acetylcholine-induced contractions to CO_2 was studied to determine if an effect of pH on acetylcholinesterase activity could mask an effect of CO_2 on acetylcholine-induced contractions. The sensitivity to CO_2 of contractions induced by other choline esters was also investigated.

V. Effects of calcium and potassium concentrations on sensitivity to CO_2 of drug-induced contractions The effect of CO_2 on drug-induced contractions in the presence of low extracellular calcium concentrations was studied to determine whether CO_2 acts by altering the availability of extracellular calcium. The effect of CO_2 was studied in the presence of increased extracellular potassium to investigate the importance of the membrane potential.

VI. Other stimulants The effect of CO_2 on other possible stimulants of this muscle, α-sympathomimetic agonists, increased extracellular po-

tassium, ganglionic stimulants, histamine, bradykinin and prostaglandins, was investigated.

METHODS

A. HISTOLOGICAL METHODS

Feline bronchial rings were excised and cleaned in the same manner as will be described for *in vitro* studies. For cross-sections of the bronchi, two intact rings were placed in Bouin's fixative. For serial sections one ring was cut open along the longitudinal axis of the tube to make a flat sheet which was placed in Bouin's solution for 15 min. After this initial fixation the opened bronchial tube was laid between two glass slides which were fastened together loosely with a rubber band so that the tissue would harden as a flat sheet. The intact rings for cross sections as well as the opened bronchial tube held flat between two glass slides were fixed in Bouin's solution for 5 hours.

After fixation the tissues were transferred to 70% ethyl alcohol and taken to the Histopathology Laboratory of the University of California Pathology Department, where the tissue was dehydrated with ascending alcohols, embedded in paraffin, and cut in sections of 5 microns thickness. For cross sections the plane of section was across the tube to produce circular sections. Serial sections were cut through the thickness of the flattened bronchial wall in the plane of the flat sheet. This produced a series of rectangular sections: the first was at the luminal surface, and the last was at the outer surface of the bronchial wall.

Sections were stained with iron hemotoxylin and aniline blue and examined in a Leitz Ortholux microscope. Photographs were taken with a Leitz Orthomat camera using Kodachrome II professional type A film.

B. PHYSIOLOGICAL METHODS

1. Isolated Muscle Technique 32 cats of either sex (weight, 2-4 kg) were anesthetized with 300 mg sodium pentobarbital administered intra-

peritoneally. The chest was opened, and the lungs were removed and placed in warm Kreb's bicarbonate solution. Intrapulmonary bronchi were dissected out and placed in warm (37°C), oxygenated Kreb's solution. When this gross dissection of bronchi was complete, each bronchus was cleaned of vascular and parenchymal tissue, side branches were cut off and bronchial rings (mean circumference, 6.5 $\pm$ 0.2 mm; mean length, 5.7 $\pm$ 0.1 mm) were cut. The rings were either used the same day or stored for 24 hours at 5°C. A total of 195 bronchial rings were studied.

Each ring was mounted by means of stainless steel wires in the muscle chamber pictured (Figure 1). Muscle chambers (volume, 23 ml) were manufactured by the University of California glassblower to our specifications and were mounted in a plexiglass tissue bath (Phipps and Bird). Temperature was maintained with a Phipps and Bird Controlled Heater Agitator Unit at 37°C unless otherwise specified.

Each bronchial ring was connected on one side to a stationary clamp and on the other side to a Grass FT03 force transducer to measure isometric tension. The output of the transducer was monitored on a Grass Model 5 polygraph using a Model 5P1 DC pre-amplifier. The usual paper speed was 0.25 mm/sec. The force transducer was calibrated before each experiment with known weights.

The force transducer was mounted via a Harvard Apparatus isometric tension clamp to a stationary metal framework attached to the tissue bath. By adjusting the isometric tension clamp the length of the bronchial ring could be altered to vary the resting tension. When the rings were mounted each was stretched to approximately 5 grams of tension 5 times in succession since stretching has been reported to accelerate the time course of recovery to a stable resting tension of smooth muscle

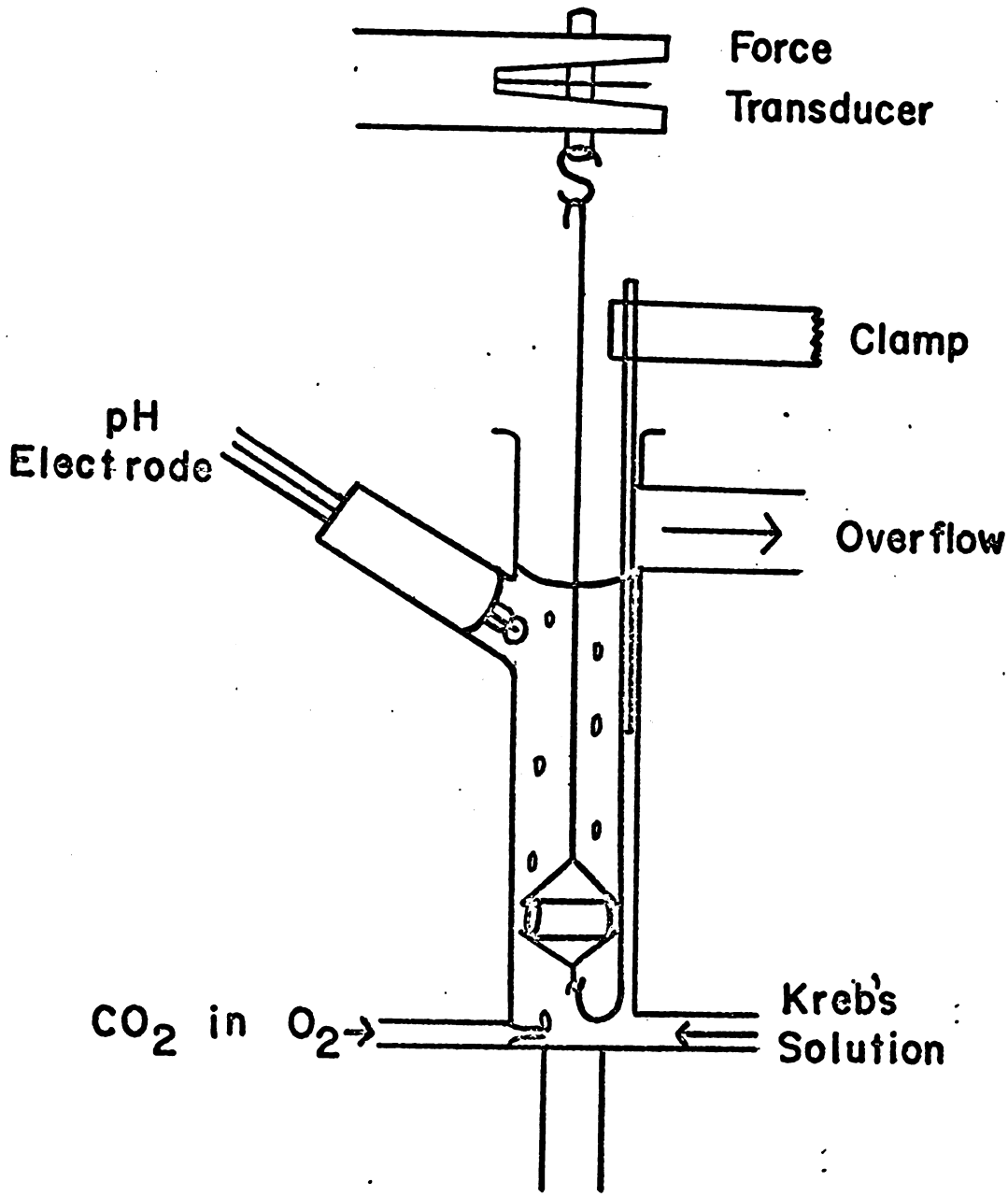


Figure 1: Drawing of bronchial ring mounted in muscle chamber. Inlets for Kreb's solution and gas are at the bottom of the chamber while outlet for overflow of solution and inlet for pH electrode are at the top. The bronchial ring is attached below to a clamped glass rod and above to the force transducer by means of stainless steel wires.

preparations (Wurzel et al, 1960). A resting tension of 1 gram was maintained throughout the experiment.

Testing of the muscle response began as soon as a steady resting tension was reached. No additional equilibration period was used because it was determined that muscles equilibrated for one hour did not respond consistently for a longer period of time than muscles that were not so equilibrated. Also, when tested shortly after mounting, muscles given repeated doses showed a decline in response with time that was similar to the decline of muscles that were not repeatedly stimulated (Figure 2).

Kreb's bicarbonate solution was of the following composition (mM): NaCl, 117; KCl, 4.75; $CaCl_2$, 2.82; KH_2PO_4, 1.19; $MgSO_4$, 1.19; $NaHCO_3$, 24.6; glucose, 5. Kreb's solution was made fresh each day by combination and dilution of three stock solutions. One stock solution contained $NaHCO_3$, another glucose, and a third the rest of the salts. These stock solutions were kept refrigerated for storage. Kreb's solution in the muscle chamber was bubbled with various concentrations of CO_2 in O_2 to vary pH; 5% CO_2 which gave a pH of 7.45 was used for control values. 2.5% CO_2 gave a pH of 7.63; 10%, 7.18; and 20%, 6.93. In some experiments pH was changed by addition of HCl or NaOH directly into the muscle chamber, while the CO_2 concentration was maintained at 5%. Kreb's solutions with high potassium concentrations were made by substituting equimolar amounts of KCl for NaCl. This method has been shown to produce membrane depolarization without increasing water content or intracellular potassium concentration of guinea pig taenia coli (Casteels and Kuriyama, 1966). Calcium was omitted from the Kreb's solution and EGTA added to study the effects of calcium-free solutions, and in some

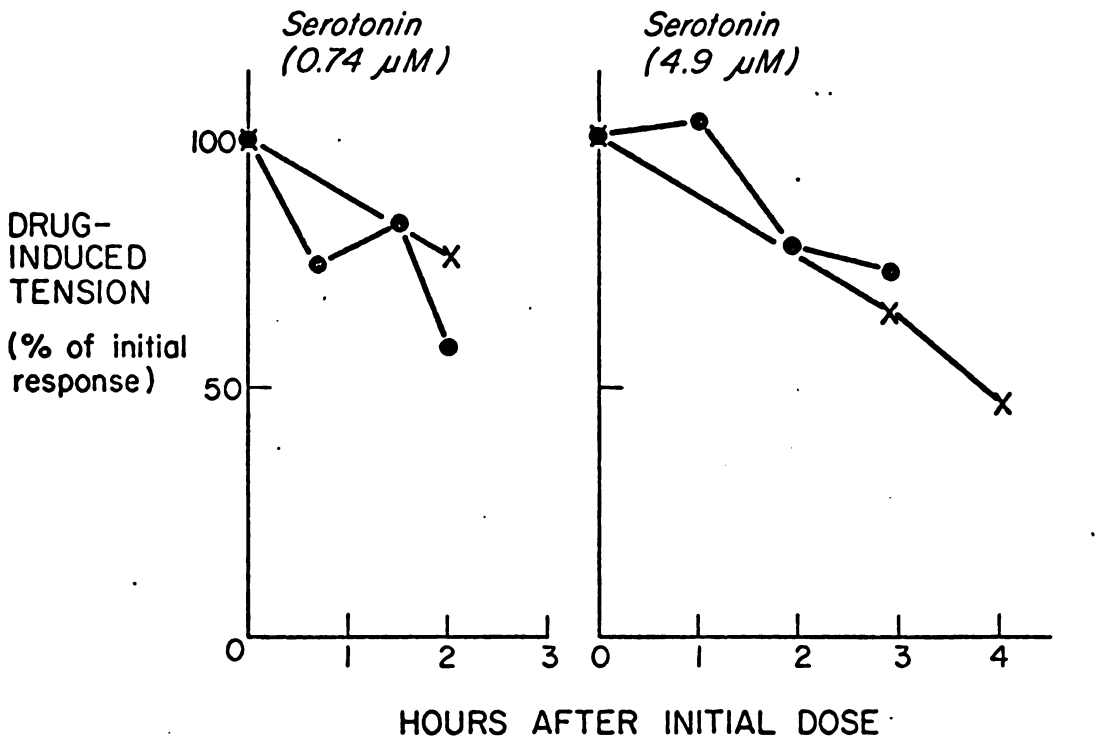


Figure 2: Effect of equilibration time of muscle in bath on contractile response to serotonin with 5% CO_2. Each section compares responses of one bronchial ring given repeated doses of serotonin (X—X) to those of another ring that was allowed to equilibrate for several hours (●—●).

experiments the calcium concentration was reduced to 0.12 mM.

Acetylcholine, bethanechol and norepinephrine were made up in Kreb's solution each day of use. Stock solutions of serotonin, carbachol and physostigmine were made up in weakly acidic (pH 5.4) phosphate buffer. Prostaglandin $F_{2\alpha}$ stock solution was made with 95% ethanol; control ethanol solutions had no effect on bronchial ring tension. The tetrodotoxin preparation consisted of freeze-dried tetrodotoxin and citrate buffer which had a pH of less than 5 when made up with distilled water. Other drug stock solutions were made up in Kreb's solution. All stock solutions were kept refrigerated for up to two weeks except tetrodotoxin and prostaglandin $F_{2\alpha}$ stock solutions which were kept frozen. For use in each experiment, stock solutions or fresh drugs were diluted with Kreb's solution and injected into the muscle chamber in a volume of 1 ml via a polyethylene catheter. For convenience propranolol, sotalol, atropine and physostigmine were made up in large volumes of the Kreb's solution which was used to wash the muscle. After each drug injection, air was injected through the catheter to flush out remaining solution. The catheter was flushed with Kreb's solution during drug washout. All drug concentrations are reported as final molar concentration in the bath.

Tension was measured 10 min after addition of a stimulating drug by which time a steady tension had been achieved. Changes in P_{CO_2} and pH were superimposed on these maintained contractions. Drug washout was accomplished by allowing fresh solution to flow from suspended bottles into the bottom of the muscle chamber and out the top by overflow. Temperature equilibration of fresh solution was achieved by allowing the solution to run through a length of glass tubing in the 37°C water

bath before entering the muscle chamber.

pH was monitored with an A.H. Thomas combination pH electrode #4858-L15 mounted directly in the muscle chamber through a rubber stopper in the side arm. Continuous monitoring was achieved with an amplifier designed by U.C. Research and Development and a Fairchild Digital Multimeter Model 7000 A allowing for a continuous digital readout of millivolt values. The amplified pH signal was also connected to one channel of the Grass polygraph by means of the DC 1 meg input. pH was calibrated in the following manner:

1. Short out leads to amplifier by cross-connection and adjust reading to 0 mV with adjustable control.
2. Connect amplifier to pH electrode in Beckman pH 7.00 buffer equilibrated to a temperature of 37°C. (At 37°C this buffer will be at a pH of 6.98.)
3. Obtain mV reading and calculate pH value for 0 mV using the following formula (Dr. Leslie Benet, personal communication):

$$pH(x) = pH\ (s) + \frac{(Ex-Es)\ F}{RT\ \ln\ 10}$$

x = unknown
s = standard

$$\text{at } 37^{\circ}C\ \frac{F}{RT\ \ln\ 10} = \frac{1}{.0615}$$

4. Short-out connection to amplifier to obtain 0 mV reading again. Adjust Grass write-out to the calculated corresponding pH value using base-line control of Grass driver amplifier.
5. Connect amplifier to pH electrode in pH 6.98 buffer. Adjust Grass write-out to 6.98 using sensitivity control of pre-amplifier.
6. Alternate steps 4 and 5 until no further adjustment is necessary.
7. Mount pH electrode in muscle chamber.

2. Experimental Design and Statistics To determine the length of time muscles could be studied with reproducible results, 14 bronchial rings were given repeated doses of serotonin for up to 6 hours (Figure 3). Since reproducible results were obtained from most muscles for up to 3 hours, all experiments were designed to be complete in approximately 3 hours.

For comparison of the effects of CO_2, pH and antagonist drugs on contractions induced by various agonists, concentrations of agonists that gave half-maximal contractions were used. Concentrations that gave 50% of a maximal contractile response were determined from control (pH 7.45) concentration-effect curves. Concentration-effect curves for acetylcholine and serotonin were constructed in the following manner. The concentrations of the agonist that gave a maximal contractile response in 5% CO_2 and that gave no response were determined. A series of concentrations between these two points was then chosen for construction of the concentration-effect curve. Each muscle was tested for response to a maximal concentration, and then three other concentrations in random order were tested, varying CO_2 concentration each time. At the end of the experiment, the maximum drug concentration was administered. Results were discarded from muscles in which the response to the maximum concentration given at the end of the experiment was less than 75% of the initial response. Contractile response to a maximum concentration in 5% CO_2 was designated as 100% and used as a reference from which the percent responses at other drug and CO_2 concentrations were calculated.

Standard errors were calculated using a program for the Hewlett Packard 9100B calculator. Student's t-test for paired or unpaired

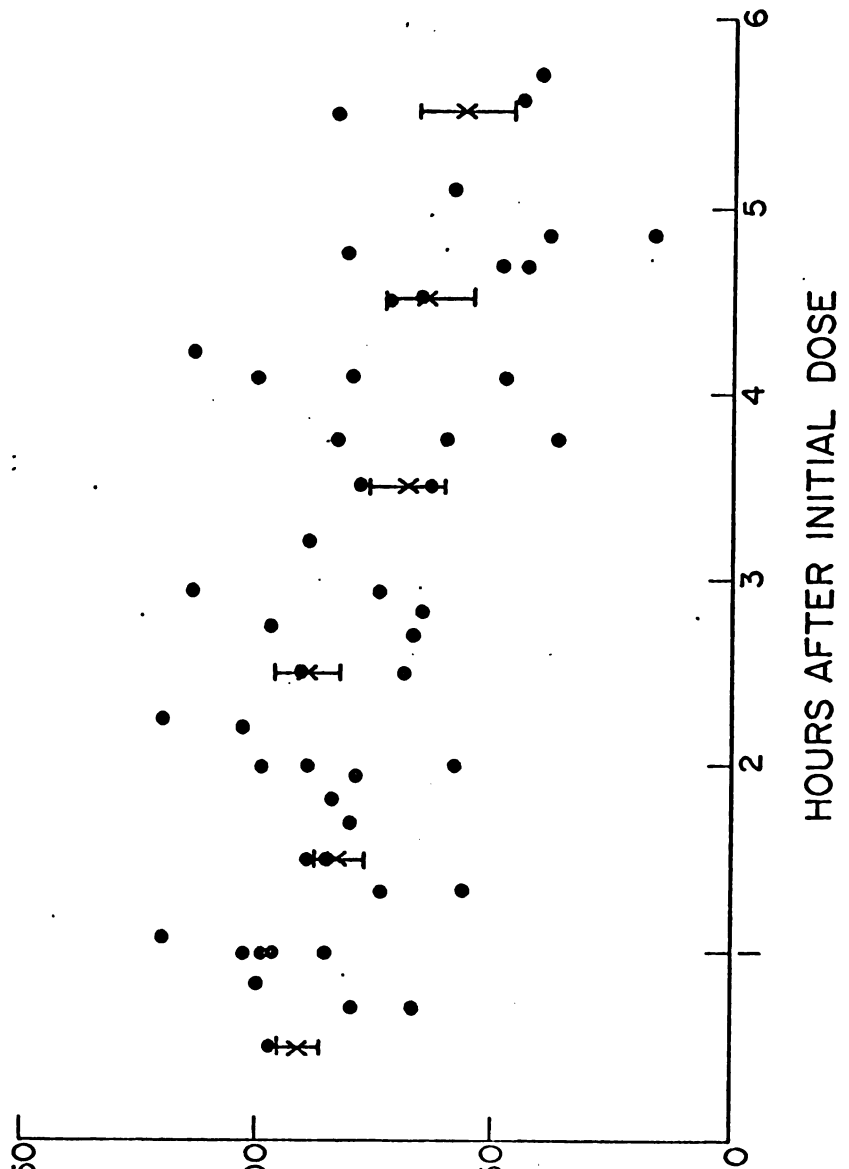


Figure 3: Effect of time on responsiveness to serotonin of fourteen bronchial rings with 5% CO_2 in the bath. ●, individual data points; X, grouped data for each hour ± S.E.

samples was used as appropriate for determination of p values. Calculations of regression lines and comparison of their slopes were carried out by analysis of covariance (Snedecor and Cochran, 1967).

3. Drugs and Chemicals The drugs and chemicals utilized in this dissertation have been listed here by source:

Allied Chemical, reagent grade
Calcium Chloride, dihydrate

Ayerst
Propranolol HCl, powder

Brewer and Company
Nicotine Bitartrate

Burroughs Wellcome and Co.
Methoxamine HCl (Vasoxyl), ampoules

California Biochemicals
Histamine Dihydrochloride, powder

K and K Laboratories
Tetramethylammonium Chloride
Hexamethonium Chloride

Mallinckrodt, analytical reagents
Sodium Chloride, crystals
Potassium Phosphate Monobasic, crystals
D-glucose Anhydrous, granular
Sodium Bicarbonate, powder
Potassium Chloride, granular
Magnesium Sulfate, crystals

Mead Johnson
Sotalol (MJ 1999), courtesy Dr. McKinney

Merck, Sharp and Dohme
Bethanechol Chloride (Urecholine), ampoules
Carbachol Chloride (Carcholin), powder
Methacholine Chloride (Mecholyl), ampoules

Nutritional Biochemicals
Serotonin Creatinine Sulfate (5-hydroxytryptamine, 5HT), powder

Sandoz
Bradykinin, synthetic, ampoules
Methysergide (UML-491), ampoules

Sankyo
Tetrodotoxin, Crystalline 3X (TTX)

Sigma
Acetylcholine Chloride (Ach), powder
EGTA [ethyleneglycol-bis (β-amino ethyl ether) N,N'-Tetra-Acetic Acid]

UC Pharmacy
Physostigmine (eserine salicylate), powder
Atropine sulfate, powder

Upjohn Company, courtesy of Dr. J. E. Pike
Prostaglandin $F_{2\alpha}$ Tromethamine salt

Winthrop
Norepinephrine Bitartrate (NE, Levophed), ampoules

RESULTS AND CONCLUSIONS

I. HISTOLOGY OF BRONCHIAL RING

A. RESULTS

1. Smooth Muscle Eight cross sections of one bronchial ring utilized for in vitro studies showed that smooth muscle comprised only a small proportion of the total mass of tissue (Figure 4). Cartilage, mucous glands and connective tissue accounted for the remainder. It could be estimated by eye from cross sections that smooth muscle made up less than 10% of the tissue.

Smooth muscle fibers appeared to have a generally circular orientation. However, sections in the plane of these fibers showed the branching character of the smooth muscle net. Groups of fibers were often seen crossing each other.

2. Ganglia and Nerves Serial sections of one bronchial ring were made to determine whether ganglion cells were actually present in isolated rings. Because of their large size, large distinct nuclei and brown color under the staining conditions used, ganglion cells could be identified even at low magnification (Figure 5). Ganglion cells were always found in close association with nerves, either forming discrete groups of cells in nerves or as chains of cells distributed along the nerve itself. An estimate of the number of ganglia gave a value of 10 ganglia/mm^3. Each ganglion contained on the average 4.5 cells. Ganglia were found both outside the cartilage (extrachondral) and in the connective tissue between cartilage and muscle (subchondral).

B. CONCLUSIONS

According to Macklin (1929) the bronchial musculature can be described as a branched, tubular net. Thus, when the lungs are in the inflated state, the bronchial muscle coat is not closed and continuous

Figure 4: Cross section of bronchial ring showing small proportion of smooth muscle. C = cartilage, M = mucous glands, S = smooth muscle, E = ciliated epithelium.

I 10 microns

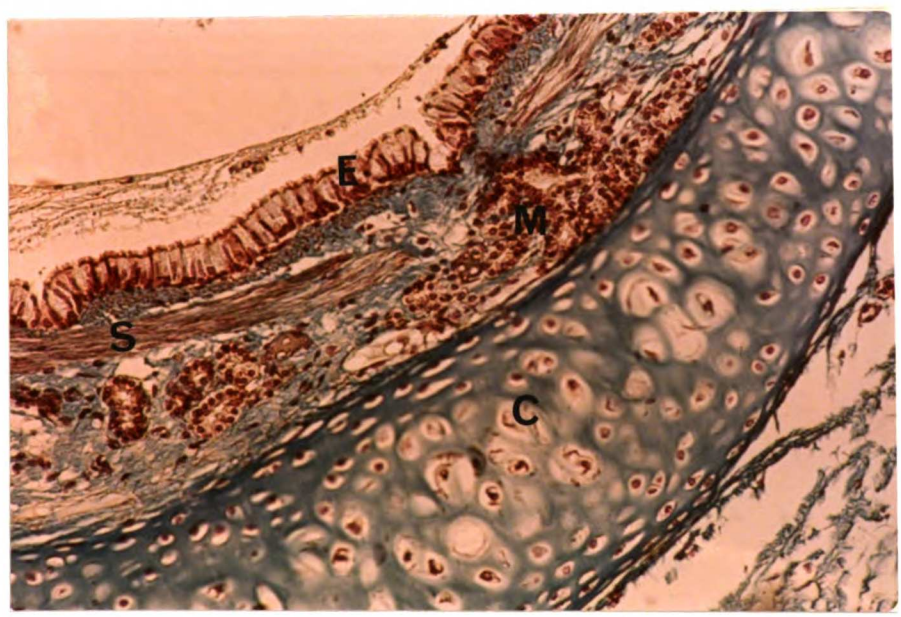
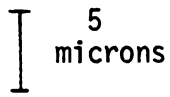
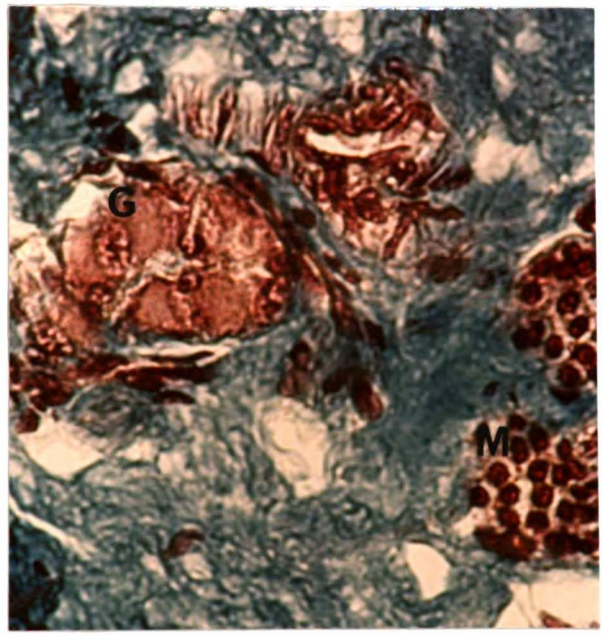

E
M
S
C

Figure 5: Section of feline bronchial ring showing brown staining ganglion cells which were located extrachondrally. G = ganglion cell, M = mucous glands.




G
M

but open. Elastic fibers are in close relationship with the smooth muscle. The small proportion of smooth muscle in the bronchial wall makes certain types of studies difficult. For example, attempts to measure the effects of pH on binding of a particular drug to smooth muscle might be unsuccessful, because the large amount of non-specific binding of the drug to cartilage and other components of the bronchial wall would probably obscure any changes in specific drug binding.

Smooth muscle of the bronchi is innervated by both parasympathetic and sympathetic fibers (Dahlstrom et al, 1966; Hirsch and Kaiser, 1969; Mann, 1971; Nagaishi, 1972). According to Dahlstrom et al (1966), bronchial muscle of the cat has a general adrenergic innervation of high density, an innervation that extends as far as the respiratory bronchioles. Peribronchial ganglia were found to lack specific catecholamine fluorescence, nor were adrenergic nerve terminals observed in ganglia. In a study of the bronchial tree in 5 mammalian species, it was determined that acetylcholinesterase-containing fibers greatly outnumbered those containing catecholamines (Mann, 1971). Extrachondral ganglia were found to contain groups of intensely fluorescing cells. Ganglion cells are located in both the extra- and subchondral nerve plexuses (Nagaishi, 1972).

Numerous questions remain to be answered about the innervation of smooth muscle of the lung. As pointed out by Nagaishi (1972), the function of ganglion cells is still in question. It is not definitely established whether they belong to the parasympathetic or sympathetic nervous system. The functions of extra- and subchondral ganglia also need clarification. Is there a functional difference between ganglia in these two locations? A third question that remains open to speculation concerns

the possible existence of cross connections between sympathetic and parasympathetic systems.

II. THE SELECTIVE EFFECT OF CO_2 ON SEROTONIN- AND ACETYLCHOLINE-INDUCED CONTRACTIONS

A. RESULTS

1. Effect of CO_2 on Serotonin-Induced Contractions Increasing CO_2 concentration from 5% to 10% relaxed serotonin-induced contractions; the onset of this effect occurred in less than 1 min, and it was maximum in approximately 4 min (Figure 6). Upon return to the control (5%) CO_2 concentration, the effect of 10% CO_2 was reversed completely. This effect could not be accounted for by changes in resting tension since CO_2 did not alter bronchial ring tension in the absence of a stimulating drug. Decreasing CO_2 concentration from 5% to 2.5% produced an increase in serotonin-induced tension; CO_2 concentrations from 2.5% to 20% were found to alter markedly the magnitude of serotonin-induced contractions (Figure 7). The relaxant effect of CO_2 occurred over a wide range of serotonin concentrations (Figure 8): contractions produced by lower concentrations of serotonin were relaxed completely by 10% and 20% CO_2, and the maximum serotonin response was altered significantly by changing CO_2 concentration.

During washout of serotonin from the bathing medium, the muscle relaxed slowly. Full relaxation after supramaximal serotonin-induced contractions took from 40 to 60 min, making it possible to observe the effect of CO_2 when the muscle was still contracted, but when serotonin concentration in the bath was low. When CO_2 concentration was increased to 10% during the washout phase muscle tension decreased. This relaxant effect of CO_2 was also reversible: when the CO_2 concentration was alternated from 5% to 10% the time course of relaxation was unchanged

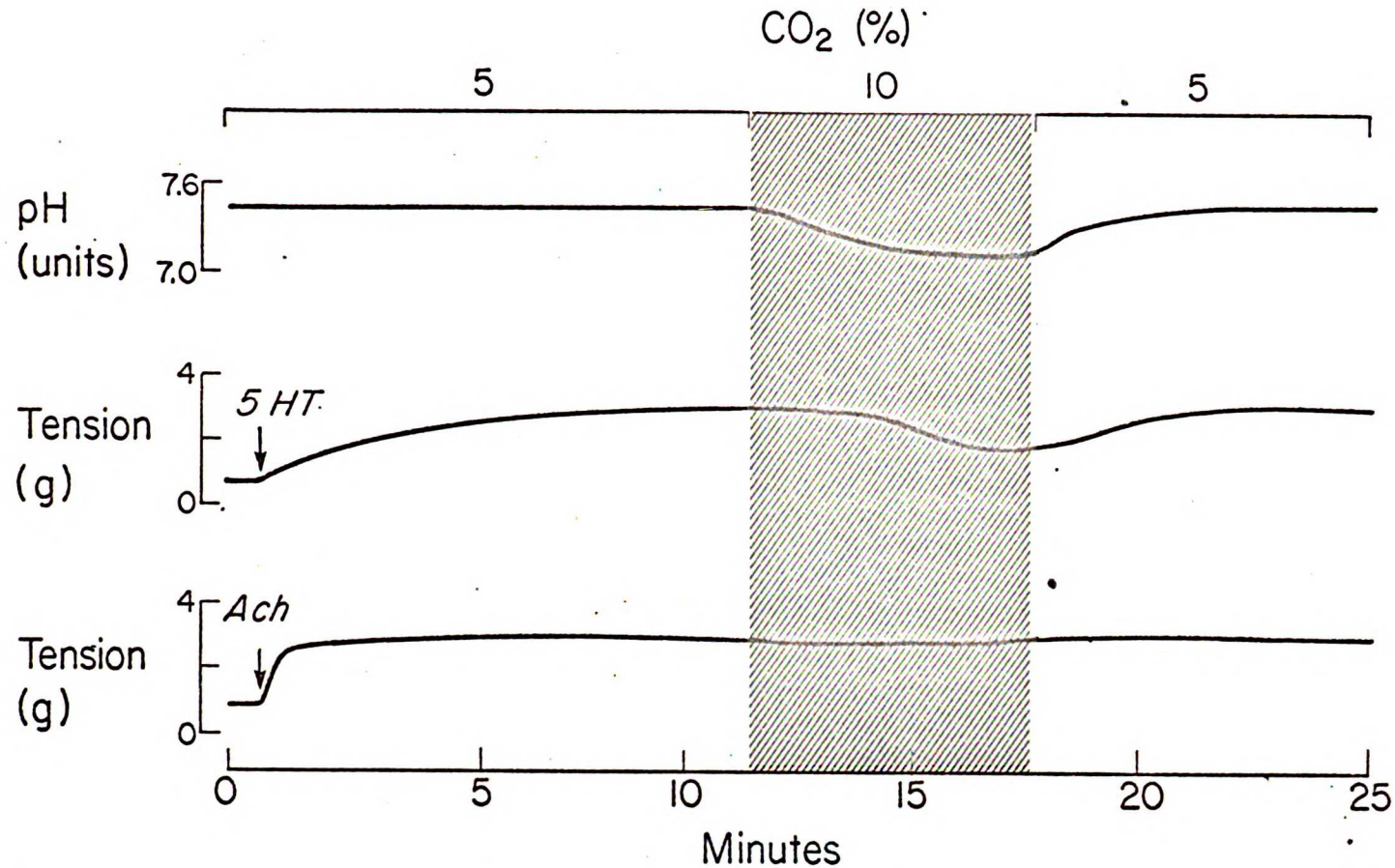


Figure 6: Effect of changing CO_2 concentration in the bath on contractions induced by serotonin (5HT; 0.54 μM) and acetylcholine (Ach; 22 μM). Drug concentrations were chosen to produce half-maximal contractions with 5% CO_2. Upper tracing shows simultaneous recording of pH in the bath. Shaded area indicates exposure to 10% CO_2.

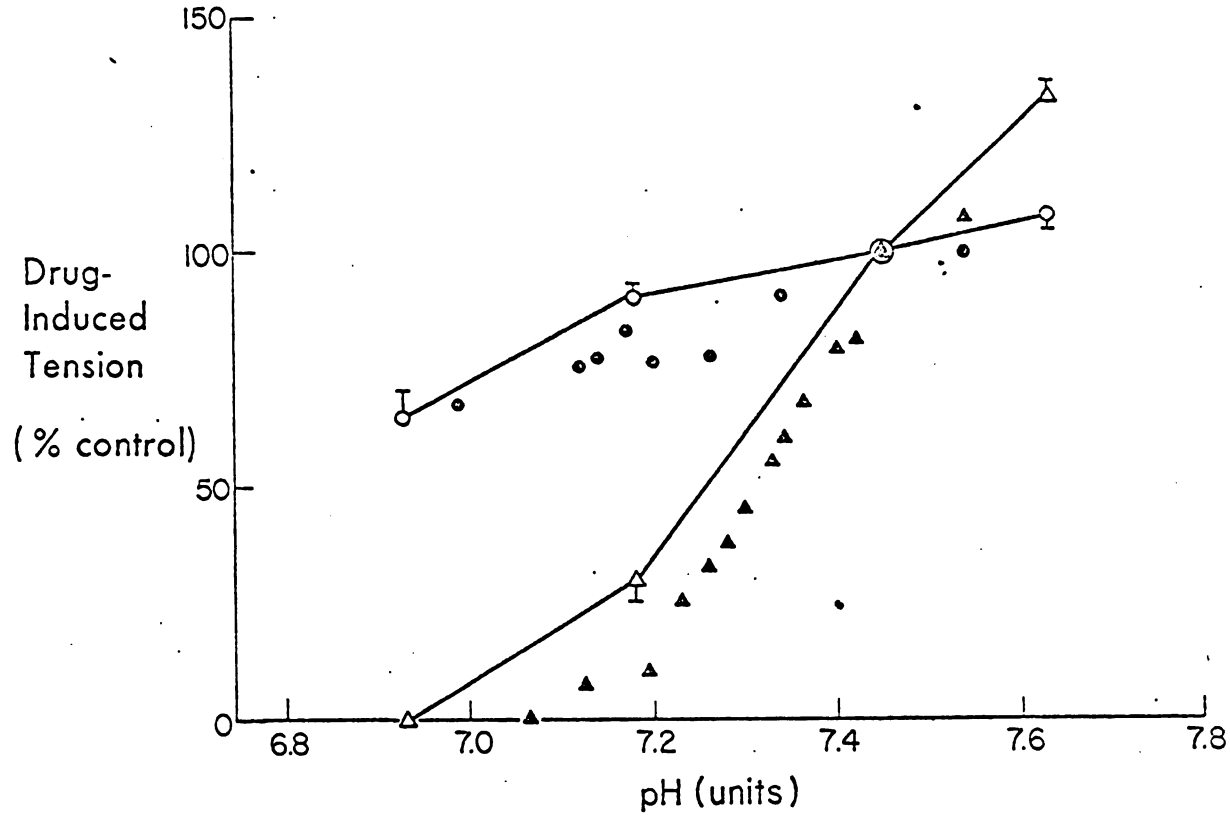


Figure 7: Comparison of effect of changing pH by altering CO_2 concentration in the bath (open symbols connected by solid lines) and by adding HCl or NaOH with CO_2 maintained at 5% (closed symbols). Drug concentrations were chosen to produce half-maximal contractions with 5% CO_2: circles, acetylcholine (22 μM); triangles, serotonin (0.54 μM). Drug-induced tension (ordinate) is expressed as percent of control contractile response (pH 7.45) and plotted as a function of pH (abscissa). The open symbols are means ± S.E. (n=4); the closed symbols are single observations.

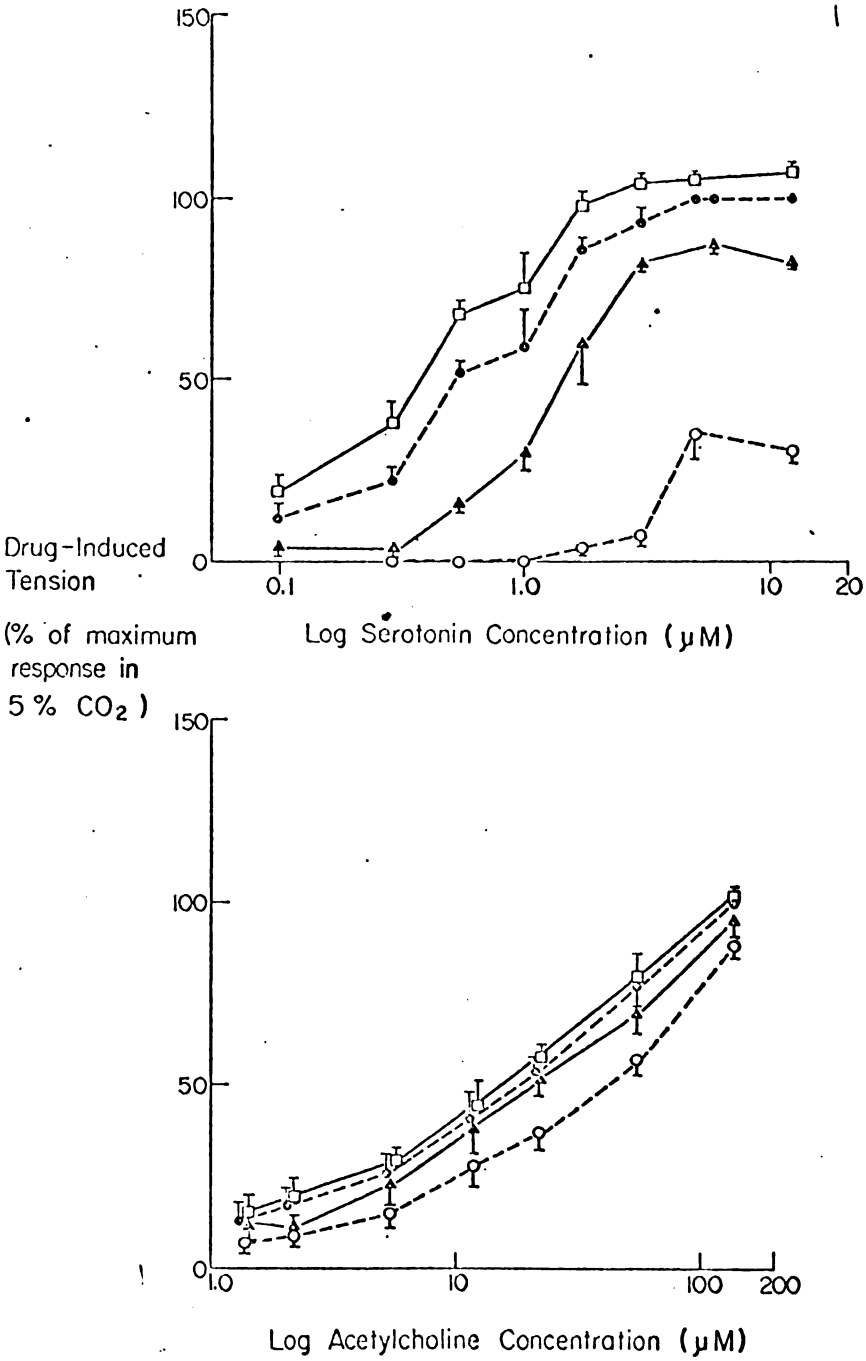


Figure 8: Concentration-effect curves for serotonin (upper) and acetylcholine (lower) with 4 different concentrations of CO_2 in the bath. Drug-induced tension (ordinate) is expressed as percent of maximal contraction with 5% CO_2 in the bath. Values are means ± S.E. (n=4). The four lines represent results with different concentrations of CO_2: □—□, 2.5% CO_2; ●---●, 5%; ▲—▲, 10%; and O---O, 20%.

(Figure 9).

The effect of changing CO_2 concentration at the plateau of serotonin-induced contractions (as illustrated in Figure 6) was compared to the effect of changing CO_2 concentration before adding serotonin (Figure 10). In two bronchial rings with a serotonin concentration of 0.98×10^{-6} M, changing CO_2 at the plateau produced inhibition of $50.2 \pm 3.9\%$, whereas changing CO_2 before adding the drug gave an inhibition of $56.6 \pm 13.6\%$. These two effects of CO_2 were not significantly different ($p > 0.5$).

2. Effect of CO_2 on Acetylcholine-Induced Contractions Sensitivity to CO_2 of acetylcholine-induced contractions presented a marked contrast to the sensitivity of serotonin-induced contractions: 10% CO_2 had only a slight effect on half-maximal acetylcholine-induced contractions (Figure 6): indeed, over a wide range of CO_2 concentrations the magnitude of acetylcholine-induced contractions was altered only slightly (Figure 7). Calculation of the linear regression lines from Figure 7 and comparison of their slopes indicated that the effects of CO_2 on contractions produced by acetylcholine were significantly different from the effects of CO_2 on serotonin-induced contractions (Table 1) ($p < 0.005$). Concentration-effect curves for acetylcholine indicated that CO_2 had relatively little effect over a wide range of acetylcholine concentrations (Figure 8). Acetylcholine-induced contractions were never relaxed completely by concentrations of CO_2 that completely relaxed serotonin-induced contractions. Relaxation after supramaximal acetylcholine-induced contractions was much faster than after serotonin-induced contractions (less than 5 min and 40-60 min respectively).

3. Effect of pH vs CO_2 on Drug-Induced Contractions The effect of CO_2 could be due to a change in the concentration of CO_2 or to a change

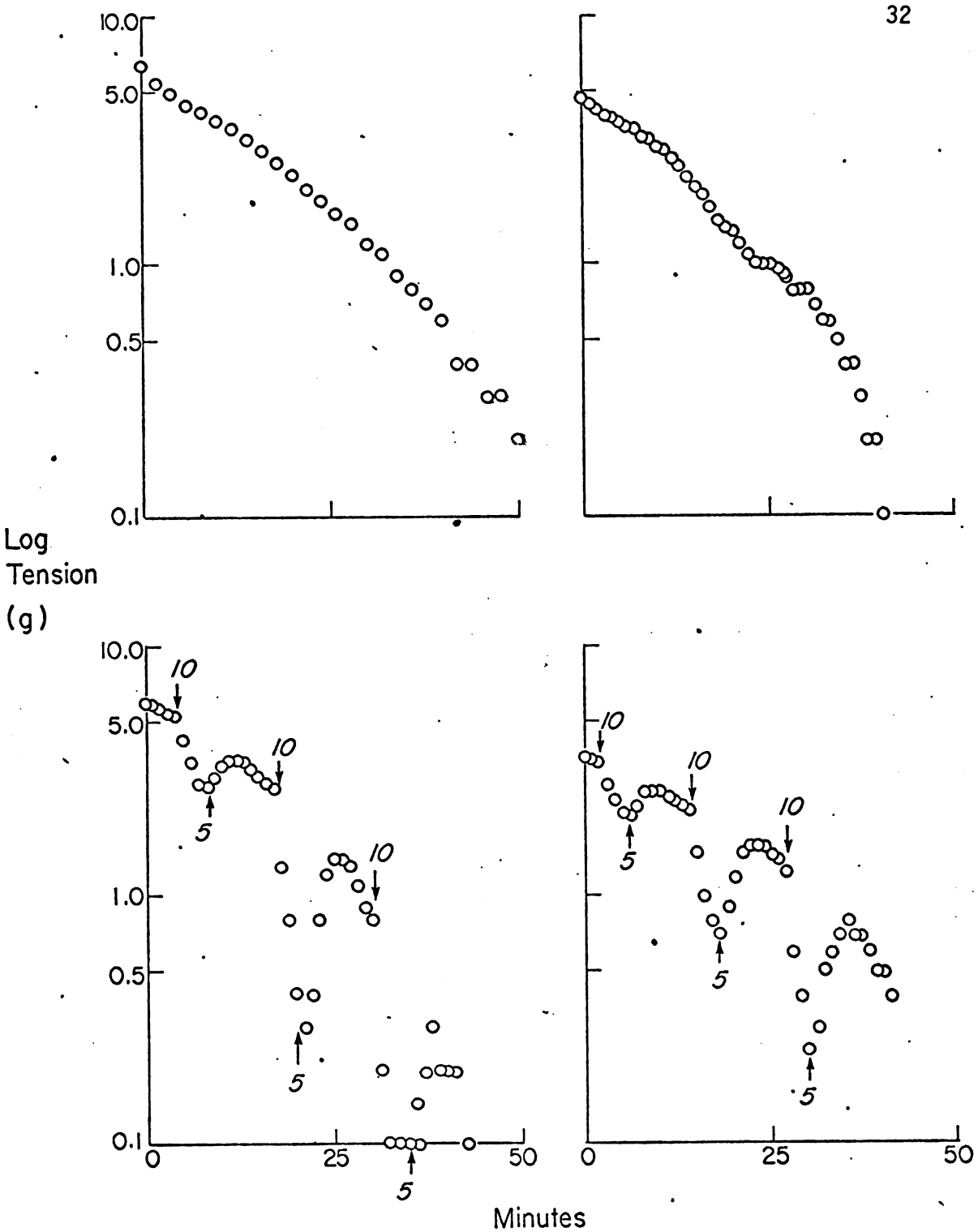


Figure 9: Rate of decrease in muscle tension during washout of serotonin (5 μM) following supramaximal contraction. Log of muscle tension (ordinate) is plotted as a function of time (abscissa). The CO_2 concentration in the bath was maintained at 5% for two bronchial rings (upper), while for two others the CO_2 concentration was alternated from 5% to 10% (lower). All four washouts began with 5% CO_2 in the bath. 5↑=change to 5% CO_2; 10↓=change to 10% CO_2.

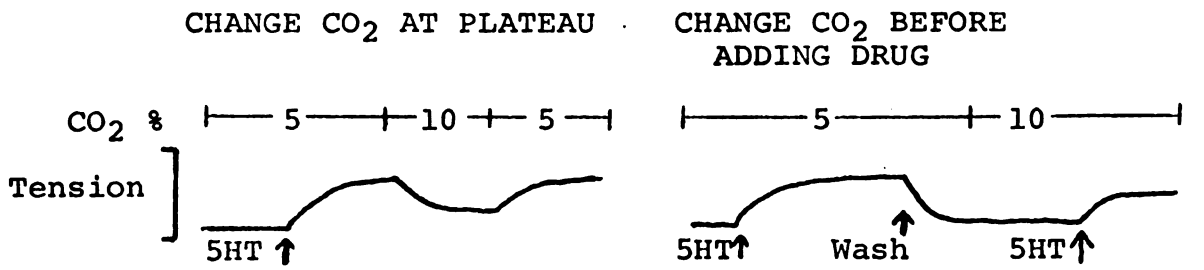


Figure 10: Schematic diagram to illustrate two different times for changing CO_2 concentration. Addition of serotonin (5HT) and washes are indicated. CO_2 concentrations are indicated above the tension tracings.

TABLE 1

Comparison of sensitivities to pH of acetylcholine- and serotonin-induced contractions. For each drug slopes of regression lines for the relationship of tension vs. pH were calculated (for data see Figure 7). Drug concentrations were chosen to produce half-maximal contractions with 5% CO_2 in the bath.

Drug	Slope of Regression Line
22 μM Acetylcholine	59.4
0.54 μM Serotonin	229.2

in pH. Therefore, we investigated the effect of adding HCl or NaOH while holding CO_2 concentration constant at 5% in 7 bronchial rings. Changing pH by adding HCl or NaOH had substantially the same magnitude of effect as changing pH by increasing or decreasing respectively the CO_2 concentration (Figure 7). The effect of adding HCl or NaOH was as rapid as the effect of changing CO_2 concentration.

B. CONCLUSIONS

CO_2 had a profound relaxant effect on serotonin-induced contractions. This effect was rapid, reversible, and occurred over a wide range of both CO_2 and serotonin concentrations. However, acetylcholine-induced contractions were relatively insensitive to similar concentrations of CO_2. Maximum serotonin-induced contractions were altered by changing CO_2 concentration implying that CO_2 and serotonin are not in competition for a single receptor site.

In a wide variety of tissues intracellular pH has been shown to be sensitive to changes in extracellular CO_2 concentration but virtually

unaffected by addition of HCl or $NaHCO_3$ to the bathing medium (Waddell & Bates, 1969; Paillard, 1972). Thus, in our experiments, since HCl was as effective and as rapidly acting as CO_2 in relaxing serotonin-induced contractions we have concluded that CO_2 has no short-term effects on contractility except those mediated by change of extracellular pH. This conclusion assumes that airway smooth muscle is similar to other tissues in respect to intracellular pH.

These findings add further support to the concept that CO_2 can directly affect airway smooth muscle by at least two mechanisms: in the present study short-term exposure to CO_2 produced a selective effect that was dependent on extracellular acidosis. On the other hand, relaxation can also be caused by intracellular acidosis after prolonged exposure to CO_2 (Stephens et al, 1968). The magnitude of the intracellular and extracellular effects of CO_2 are also markedly different: long term exposure to CO_2 (pH 7.0) reduced contractions of bronchial rings produced by acetylcholine or supramaximal electrical stimulation by only 30% and 20% respectively (Mitchell and Stephens, 1972) while in our experiments half-maximal serotonin-induced contractions were relaxed by more than 60% by a pH of 7.18.

The finding that CO_2 concentration influences tension reversibly during washout of serotonin-induced contractions but does not change the time course of relaxation suggests that pH does not influence serotonin binding if we make the following assumptions: 1. The relaxation phase is due to slow release of serotonin. 2. If serotonin is released from receptor binding the rate of serotonin release from the muscle will be accelerated. The first assumption seems to be justified: since relaxation from acetylcholine-induced contractions was much faster than from

serotonin-induced contractions, it is unlikely that relaxation from serotonin-induced contractions was limited by the viscous properties of the muscle or the maximum rate of calcium pumping. The second assumption requires that serotonin bound to the receptor should be a significant fraction of the total serotonin remaining in the muscle. If decreased pH acted by releasing serotonin from its receptor and the above assumptions are correct, total serotonin efflux would be accelerated when pH was decreased. When the pH was subsequently increased, drug that was released from the receptor could not be replenished, and the effects of CO_2 on the relaxation phase would not be reversible. However, actual measurements of specific drug binding at different levels of pH would be necessary to determine conclusively whether or not pH alters serotonin binding.

To summarize: CO_2 acted selectively on drug-induced contractions of airway smooth muscle; this effect was rapid and reversible. The effect of CO_2 was mimicked by addition of HCl or NaOH to the bathing medium which suggests that the effect of CO_2 depends on change of extracellular pH. CO_2 influenced tension reversibly during washout of serotonin-induced contractions suggesting that CO_2 does not alter binding of serotonin to its receptor. Furthermore, since maximum serotonin-induced contractions were altered by CO_2, serotonin and CO_2 do not appear to act competitively.

III. PARTICIPATION OF NERVES IN THE EFFECT OF CO_2

A. RESULTS

1. Blockade of the β-sympathomimetic Receptor Since bronchial smooth muscle is innervated by sympathetic nerves (Dahlstrom et al, 1966) and β-sympathomimetic agents relax this preparation, the hypothesis that the CO_2 effect involves release of catecholamines from tissue stores was tested. Two β-blocking agents were used, propranolol and sotalol. To determine the effective concentration of these blocking agents various concentrations were tested for effectiveness against the relaxant effect of 5.9×10^{-6} M norepinephrine. A concentration of 5×10^{-5} M of propranolol and 3×10^{-5} M of sotalol effectively blocked norepinephrine-induced relaxation. The protocol for experiments reported in this section is illustrated in Figure 11.

Propranolol had no effect on acetylcholine-induced contractions in 5% CO_2 ($p > 0.5$) and did not alter the effect of 10% CO_2. On the other hand, propranolol reduced serotonin-induced contractions in 5% CO_2 ($p < 0.02$) but did not alter sensitivity to CO_2 (Figure 12).

Sotalol reduced carbachol-induced contractions in 5% CO_2 ($p < 0.05$) and tended to reduce acetylcholine-induced contractions in 5% CO_2 although this was not statistically significant ($p > 0.1$). Sotalol had no effect on serotonin-induced contractions in 5% CO_2 ($p > 0.7$). The effect of 10% CO_2 on contractions produced by these three agents was unchanged in the presence of sotalol (Figure 13).

2. Blockade of Acetylcholine Receptor In some tissues it has been maintained that serotonin acts by stimulating ganglia to release acetylcholine (Brownlee and Johnson, 1963). It is possible that such a mechanism in airway smooth muscle could be sensitive to CO_2, accounting for

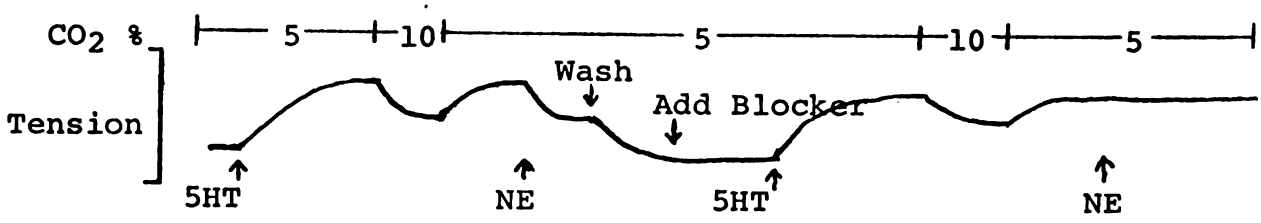


Figure 11: Schematic diagram of protocol to investigate effects of β-sympathomimetic receptor blocking agents. 5HT=serotonin; NE= norepinephrine.

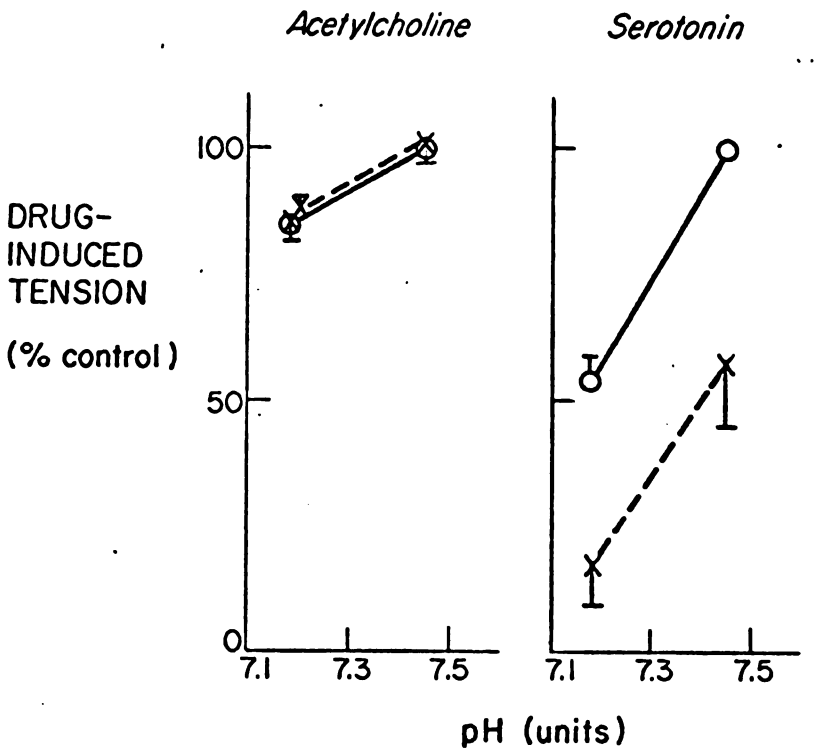


Figure 12: Effect of pH on contractions induced by acetylcholine (22 μM) and serotonin (0.54 μM) with and without propranolol (5 x 10^{-5} M). Acetylcholine and serotonin concentrations were chosen to produce half-maximal contractions with 5% CO_2 in the bath. Drug-induced tension (ordinate) is expressed as percent of control contractile response (pH 7.45; without propranolol) and plotted as a function of pH (abscissa). pH was varied by changing CO_2 concentration. Values are means ± S.E. (n=6 for serotonin, n=5 for acetylcholine). O—O, without propranolol; X---X, with propranolol.

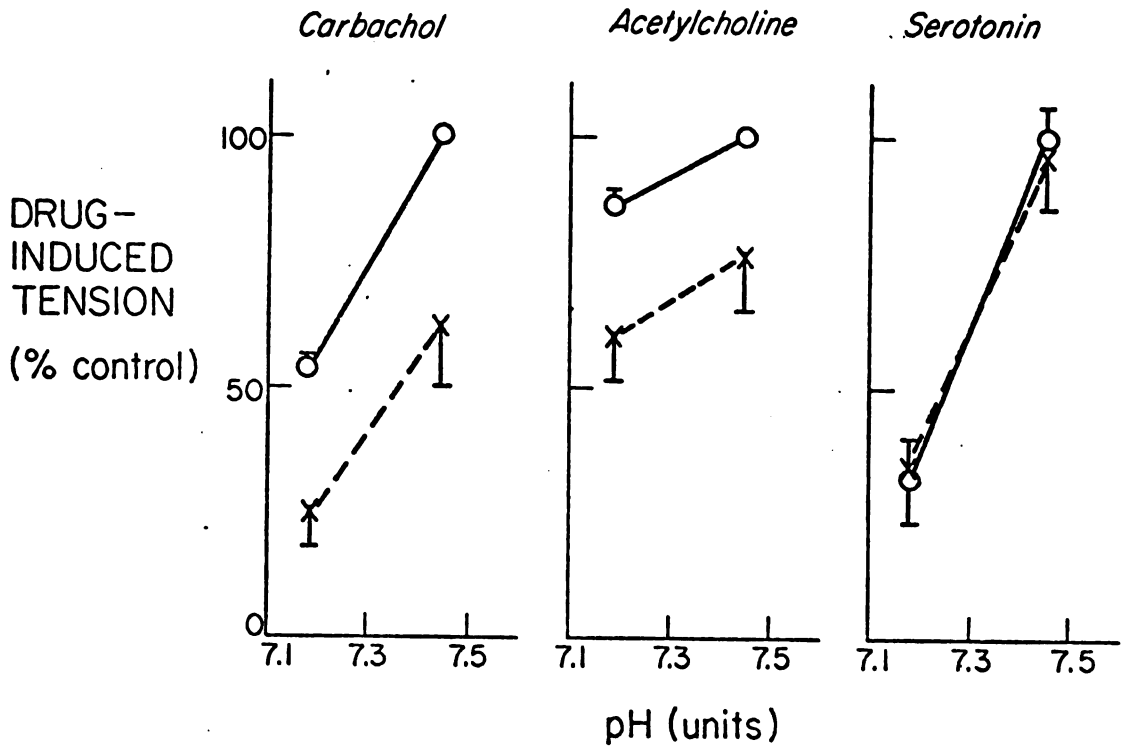


Figure 13: Effect of pH on contractions induced by carbachol (0.19 μM), acetylcholine (22 μM) and serotonin (0.54 μM) with and without sotalol (3×10^{-5} M). Acetylcholine, serotonin and carbachol concentrations were chosen to produce half-maximal contractions with 5% CO_2 in the bath. Drug-induced tension (ordinate) is expressed as percent of control contractile response (pH 7.45; without sotalol) and plotted as a function of pH (abscissa). pH was varied by changing CO_2 concentration. Values are means ± S.E. (n=4). O——O, without sotalol; X---X, with sotalol.

the effect of CO_2 on serotonin-induced contractions (Figure 14). However, in 7 bronchial rings a concentration of atropine (2×10^{-9} M) that completely blocked acetylcholine-induced contractions reduced serotonin-induced contractions in 5% CO_2 by an average of only 6%. In addition serotonin-induced contractions were just as sensitive to CO_2 in the presence of atropine (Figure 15).

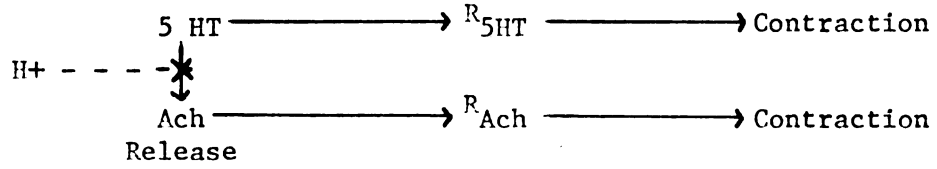


Figure 14: Schematic diagram to illustrate hypothesis that serotonin (5HT) acts by stimulating acetylcholine (Ach) release. - - - - X indicates inhibition. R=drug receptor.

3. Blockade of Nervous Conduction Although tetrodotoxin blocks conduction in nerves (Narahashi et al, 1964) it does not affect smooth muscle electrical or contractile activity that is independent of nervous stimulation (Kuriyama et al, 1966). Tetrodotoxin (3×10^{-7} M) did not alter the selective effect of 10% CO_2, although both acetylcholine- and serotonin-induced contractions with 5% CO_2 in the bath were reduced by tetrodotoxin ($p<0.01$) (Figure 16).

4. Ganglionic Blockade To determine whether part of the action of acetylcholine was due to ganglionic stimulation, the effect of hexamethonium, a ganglionic blocking agent was tested. Tetramethylammonium was chosen as a ganglionic stimulant, and concentrations of $0.09\text{-}0.36 \times 10^{-3}$ M produced maintained contractions. The lowest concentration of hexamethonium that blocked the effect of tetramethylammonium was then selected, and this concentration ranged from 0.37 to 3.7×10^{-3} M in different bronchial rings. The effect of CO_2 on acetylcholine-induced contractions

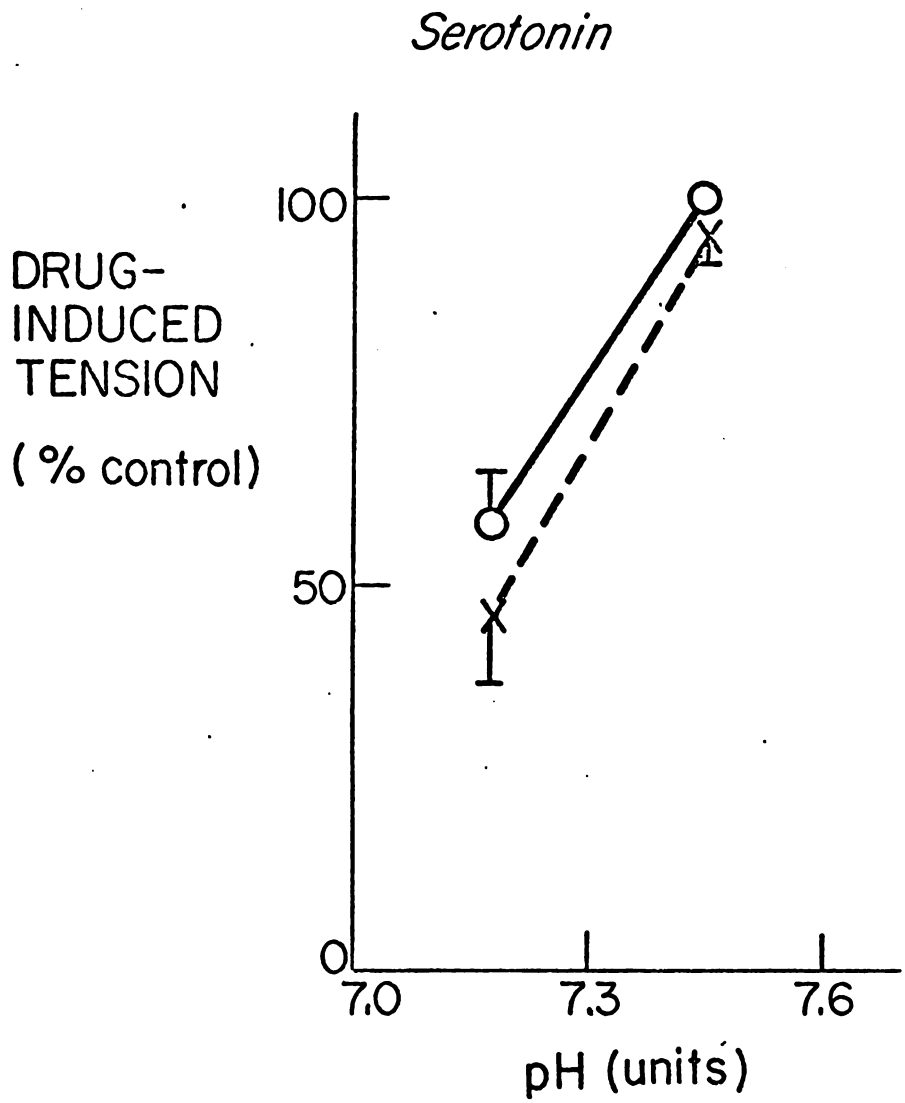


Figure 15: Effect of pH on contractions induced by serotonin (0.54 μM) with and without atropine (2×10^{-9} M). The serotonin concentration was chosen to produce half-maximal contractions with 5% CO_2 in the bath. Drug-induced tension (ordinate) is expressed as percent of control contractile response (pH 7.45; without atropine) and plotted as a function of pH (abscissa). pH was varied by changing CO_2 concentration. Values are means ± S.E. (n=7). O—O, without atropine; X---X, with atropine.

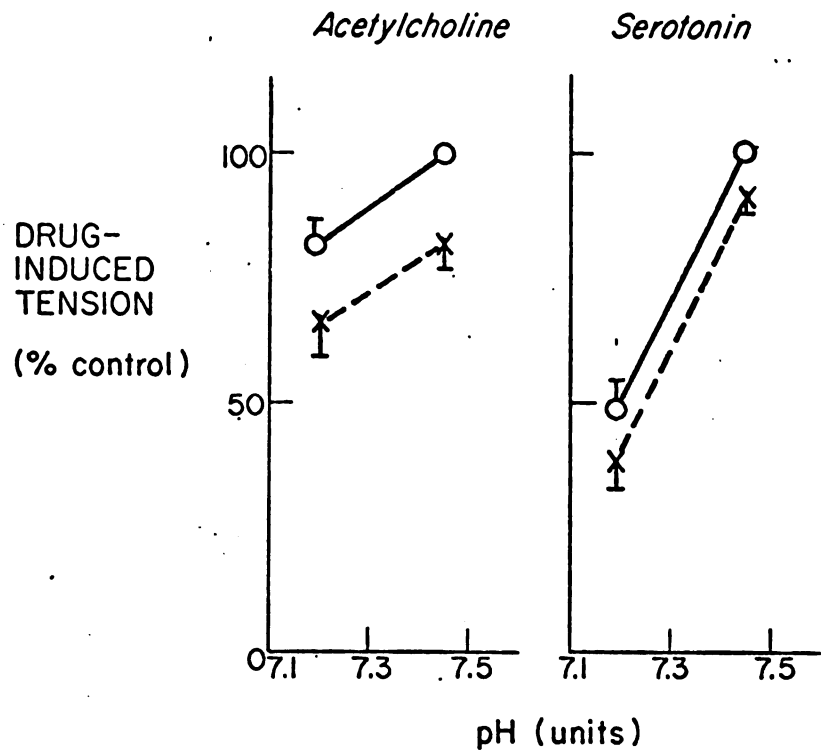


Figure 16: Effect of pH on contractions induced by acetylcholine (22 μM) and serotonin (0.54 μM) with and without tetrodotoxin (3 x 10^{-7} M). Acetylcholine and serotonin concentrations were chosen to produce half-maximal contractions with 5% CO_2. Drug-induced tension (ordinate) is expressed as percent of control contractile response (pH 7.45; without tetrodotoxin) and plotted as a function of pH (abscissa). pH was varied by changing CO_2 concentration. Values are means ± S.E. (n=9 for acetylcholine; n=6 for serotonin). O—O, without tetrodotoxin; X---X, with tetrodotoxin.

was not increased in the presence of hexamethonium, although with 5% CO_2 in the bath the effect of some concentrations of acetylcholine but not of others was increased (Figure 17).

B. CONCLUSIONS

Although propranolol blocked serotonin-induced contractions and sotalol blocked acetylcholine- and carbachol-induced contractions with 5% CO_2 in the bath, neither of these blocking agents altered the selective effect of CO_2. The effectiveness of these concentrations of propranolol and sotalol in blocking norepinephrine-induced relaxation was tested in each muscle, but both these drugs appeared to have additional effects. However, since these other effects were not the same for sotalol and propranolol, these cannot be attributed to blockade of the β-sympathomimetic receptor. Propranolol has a direct depressant action on cell membranes (Nickerson, 1970) which may account for the depression of serotonin-induced contractions. The retention of the selective effect of CO_2 in the presence of these two blocking drugs rules out the hypothesis that the effect of CO_2 involves release of catecholamines from tissue stores.

Since atropine blocked serotonin-induced contractions in 5% CO_2 only slightly, most of the action of serotonin is not due to acetylcholine release. The effect of CO_2 on serotonin-induced contractions was retained in the presence of atropine.

Blockade of nervous conduction with tetrodotoxin did not alter the selective effect of CO_2. The effective concentration of tetrodotoxin was taken from other work (Gershon, 1967), and tests of its effectiveness in blocking nervous conduction in bronchial rings were not performed in the present study. This concentration of tetrodotoxin had a small but

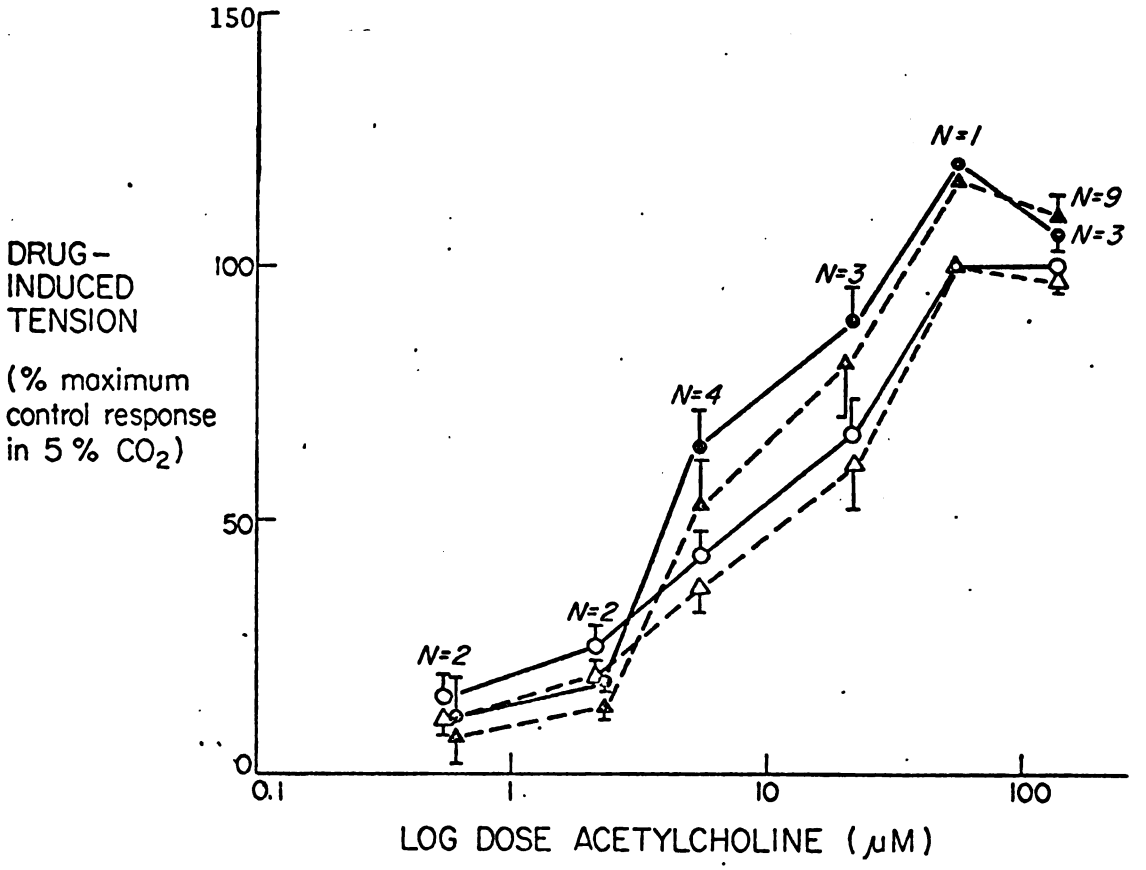


Figure 17: Concentration-effect curves for acetylcholine with and without hexamethonium ($0.37 - 3.7 \times 10^{-3}$ M) with 2 different concentrations of CO_2 in the bath. Drug-induced tension (ordinate) is expressed as percent of maximal contractions with 5% CO_2 and without hexamethonium. Number of experiments and standard errors are indicated. Without hexamethonium: O—O, 5% CO_2; Δ---Δ, 10% CO_2. With hexamethonium: ●—●, 5% CO_2; ▲---▲, 10% CO_2.

significant effect on both acetylcholine- and serotonin-induced contractions. Whether this indicates a small neural component to contractions of each of these agents or a direct action on smooth muscle requires further study.

The choice of the concentration of hexamethonium used in this study depended on its effectiveness in blocking contractions induced by tetramethylammonium. However, according to Hawkins and Paton (1958) contractions of guinea pig bronchial rings induced by tetramethylammonium were not antagonized by hexamethonium except in high concentrations although they were abolished by atropine. Concentrations of hexamethonium (3×10^{-6} M) reported by these workers to block effects of nicotine were, indeed, much lower than the concentrations used here. Thus the concentration of hexamethonium used in this study was probably greatly in excess of that required for blockade of ganglia. For that reason it is difficult to determine why acetylcholine-induced contractions were potentiated.

This could be attributed to blockade of acetylcholinesterase by these high concentrations of hexamethonium. However, contractions produced by low acetylcholine concentrations were not potentiated. Nevertheless, the effect of CO_2 on acetylcholine-induced contractions was not increased by hexamethonium.

Although the results from each individual blocking drug were not clear-cut because of the lack of complete specificity of pharmacological tools, when all the results are examined together, it becomes apparent that blockade of nervous elements did not alter the selective effect of CO_2. The effect of CO_2 on serotonin-induced contractions was not decreased by any of these blocking agents, nor was the effect of CO_2 on acetylcholine-induced contractions increased.

IV. THE CHOLINORECEPTOR

A. RESULTS

1. Inhibition of Acetylcholinesterase The hypothesis that the effect of CO_2 on airway smooth muscle involves an action on the serotonin receptor system depends on the finding that contractions induced by agonists acting on other receptors are unaffected by CO_2. Indeed, acetylcholine-induced contractions were relatively insensitive to CO_2 (Figure 8). However, as illustrated in Figure 18, an effect of CO_2 on acetylcholinesterase activity could mask an effect of CO_2 on acetylcholine-induced contractions.

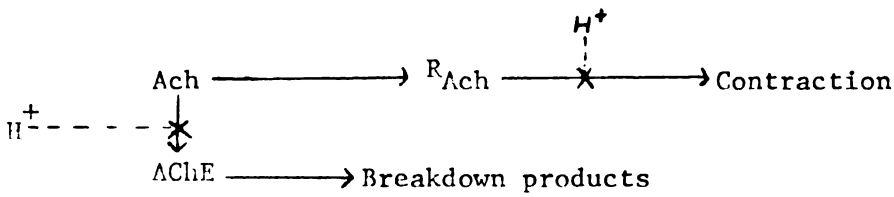


Figure 18: Schematic diagram to illustrate possible action of pH on acetylcholinesterase (AChE) activity. - - - X indicates inhibition. R = drug receptor.

Acetylcholinesterase has been shown to be present in bronchi (Ellman et al, 1961; Mann et al, 1971), and the activity of this enzyme is dependent on pH within the range (pH 6.9-7.6) under present study (Bergmann et al, 1958). As pH decreases, acetylcholinesterase activity also decreases. A decrease in activity of acetylcholinesterase could produce an increased concentration of acetylcholine at receptor sites. Thus, if increased CO_2 (decreased pH) decreased the activity of acetylcholinesterase leading to an increased effectiveness of acetylcholine while at the same time decreasing the contractile effect of acetylcholine, the net result could be no change in the acetylcholine-induced contraction.

Thus, the hypothesis of this dissertation would be disproved.

To test this possibility, the effect of CO_2 on acetylcholine-induced contractions was studied in the presence of physostigmine, an acetylcholinesterase inhibitor. As a control the effect of physostigmine on serotonin-and carbachol-induced contractions and on their sensitivity to CO_2 was investigated. Acetylcholine concentration-effect curves were shifted in the presence of physostigmine; a given acetylcholine concentration produced a greater effect which can be attributed to inhibition of acetylcholinesterase by physostigmine (Figure 19). The effect of 10% CO_2, however, was not increased in the presence of physostigmine.

The effect of physostigmine on half-maximal serotonin-induced contractions was studied in 5 muscles and on half-maximal carbachol-induced contractions in 3 muscles. Serotonin-induced contractions in 5% CO_2 were not altered by physostigmine ($p>0.5$) nor was the effect of 20% CO_2 changed ($p>0.1$). Half-maximal carbachol-induced contractions and the effect of CO_2 were also not influenced significantly by physostigmine ($p>0.3$) (Figure 20). Concentration-effect curves for carbachol in the presence of physostigmine indicated a tendency for contractions in 5% CO_2 to be potentiated slightly. Additionally the effect of 10% and 20% CO_2 tended to be reduced, although the effect of increased CO_2 was not completely blocked. None of these changes were statistically significant at the 0.05 level perhaps because of the large variability (Figure 21).

2. Analogs of Acetylcholine To further confirm that the cholinoreceptor was insensitive to pH change, the effect of CO_2 on contractions induced by several analogs of acetylcholine (Figure 22) was investigated in 10 bronchial rings. Contrary to our expectations, contractions induced

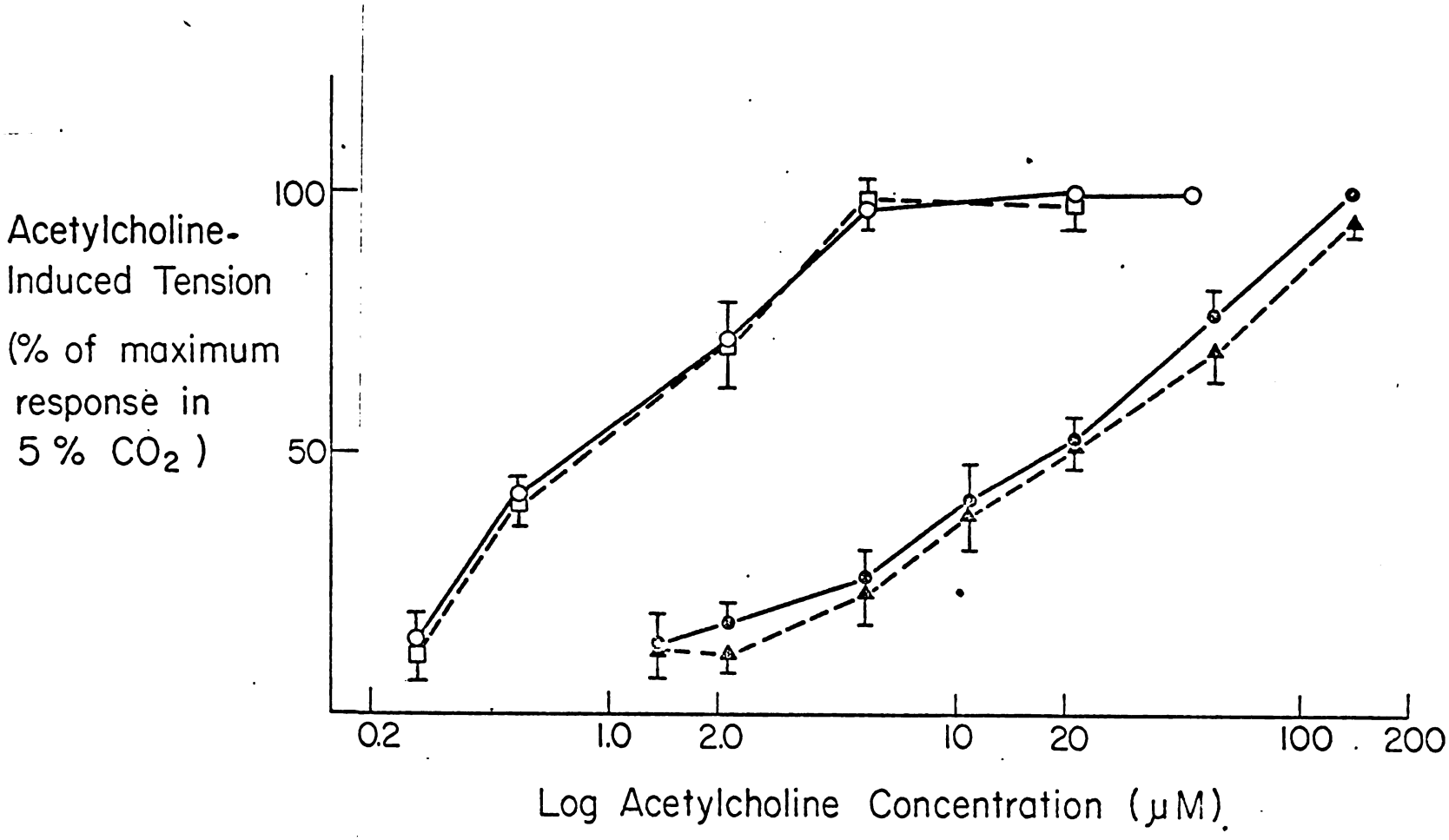


Figure 19: Concentration-effect curves for acetylcholine with and without physostigmine (10^{-7} M) with two different concentrations of CO_2 in the bath. Drug-induced tension (ordinate) is expressed as percent of maximal contraction with 5% CO_2. Without physostigmine: ●—●, 5% CO_2; ▲---▲, 10% CO_2; mean ± S.E. (n=4). With physostigmine: O—O, 5% CO_2; □---□, 10%; mean ± S.E. (n=2).

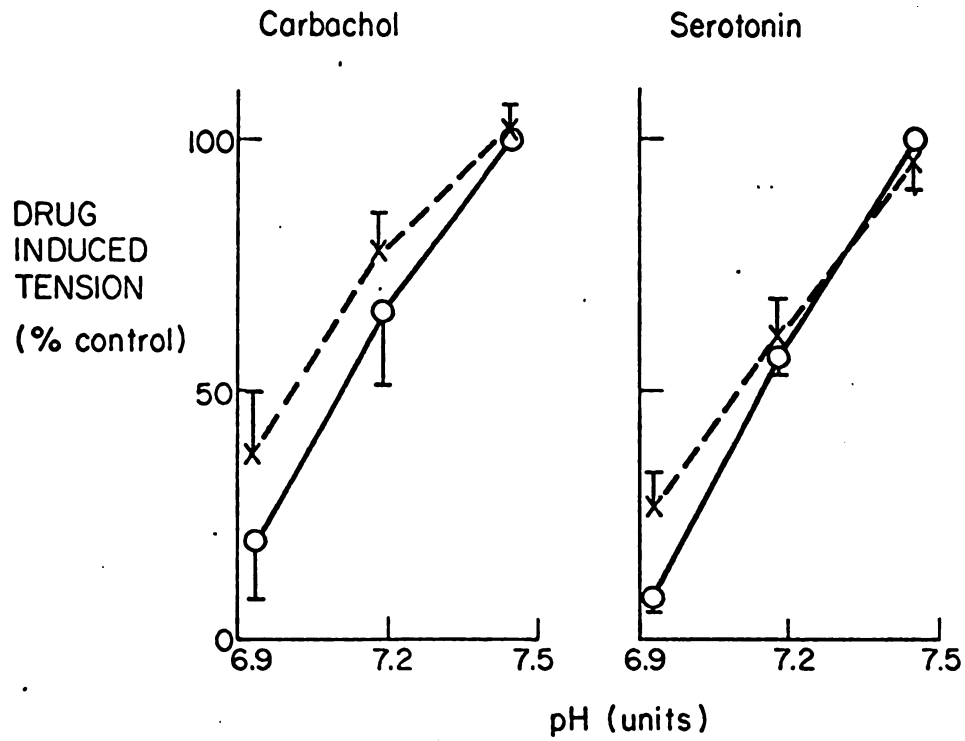


Figure 20: Effect of pH on contractions induced by carbachol (0.19 μM) and serotonin (0.54 μM) with and without physostigmine (10^{-7} M). Carbachol and serotonin concentrations were chosen to produce half-maximal contractions with 5% CO_2 in the bath. Drug-induced tension (ordinate) is expressed as percent of control contractile response (pH 7.45; without physostigmine) and plotted as a function of bath pH (abscissa). Values are means ± S.E. (n=3 for carbachol, n=5 for serotonin). O——O, without physostigmine; X---X, with physostigmine.

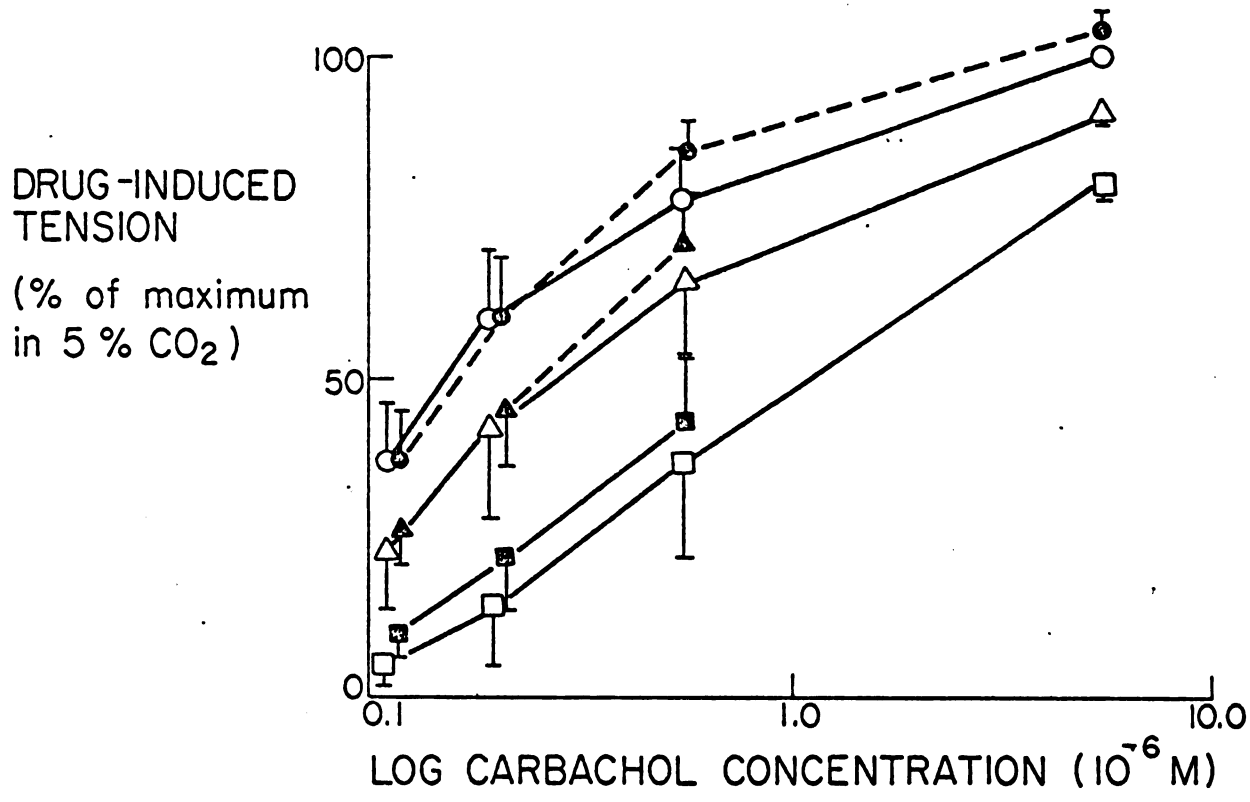


Figure 21: Concentration-effect curves for carbachol with and without physostigmine (10^{-7} M) with three different concentrations of CO_2 in the bath. Drug-induced tension (ordinate) is expressed as percent of maximal contraction with 5% CO_2. Values are means ± S.E. (n=3). The three lines represent results with different concentrations of CO_2 in the bath: circles, 5% CO_2; triangles, 10% CO_2; squares, 20% CO_2. Open symbols, without physostigmine; closed symbols, with physostigmine.

Acetylcholine Chloride	$(CH_3)_3 \equiv N^+ \cdot CH_2 \cdot CH_2 \cdot O \cdot COCH_3$	Cl^-
Methacholine Chloride	$(CH_3)_3 \equiv N^+ \cdot CH_2 \cdot CH \cdot O \cdot COCH_3$ CH_3	Cl^-
Carbachol Chloride	$(CH_3)_3 \equiv N^+ \cdot CH_2 \cdot CH_2 \cdot O \cdot CONH_2$	Cl^-
Bethanechol Chloride	$(CH_3)_3 \equiv N^+ \cdot CH_2 \cdot CH \cdot O \cdot CONH_2$ CH_3	Cl^-

Figure 22: Structures of choline esters.

by these acetylcholine analogs were all more sensitive to CO_2 than were acetylcholine-induced contractions (Figure 23). Slopes of regression lines for the relationship between pH and contractile response were calculated by analysis of covariance (Table 2).

TABLE 2

Comparison of sensitivities to pH of various drug-induced contractions. For each drug slopes of regression lines were calculated for the relationship of tension vs. pH (for data see figure 23) and compared with acetylcholine and serotonin. Drug concentrations were chosen to produce half-maximal contractions with 5% CO_2 in the bath.

Drug	Slope of Regression Line	Comparison of Slope With Acetylcholine	With Serotonin
Acetylcholine (22 μM)	59.4	____	$p<0.005$
Methacholine (1.02 μM)	105.0	$p<0.025$	$p<0.005$
Bethanechol (2.55 μM)	147.7	$p<0.005$	$p<0.005$
Carbachol (0.18 μM)	177.6	$p<0.005$	$p<0.025$
Serotonin (0.54 μM)	229.2	$p<0.005$	____

Carbachol was the most sensitive of the choline esters studied; both bethanechol and methacholine-induced contractions were also significantly more sensitive to pH than were acetylcholine-induced contractions.

3. Contraction Rates Rates of contraction in response to acetylcho-

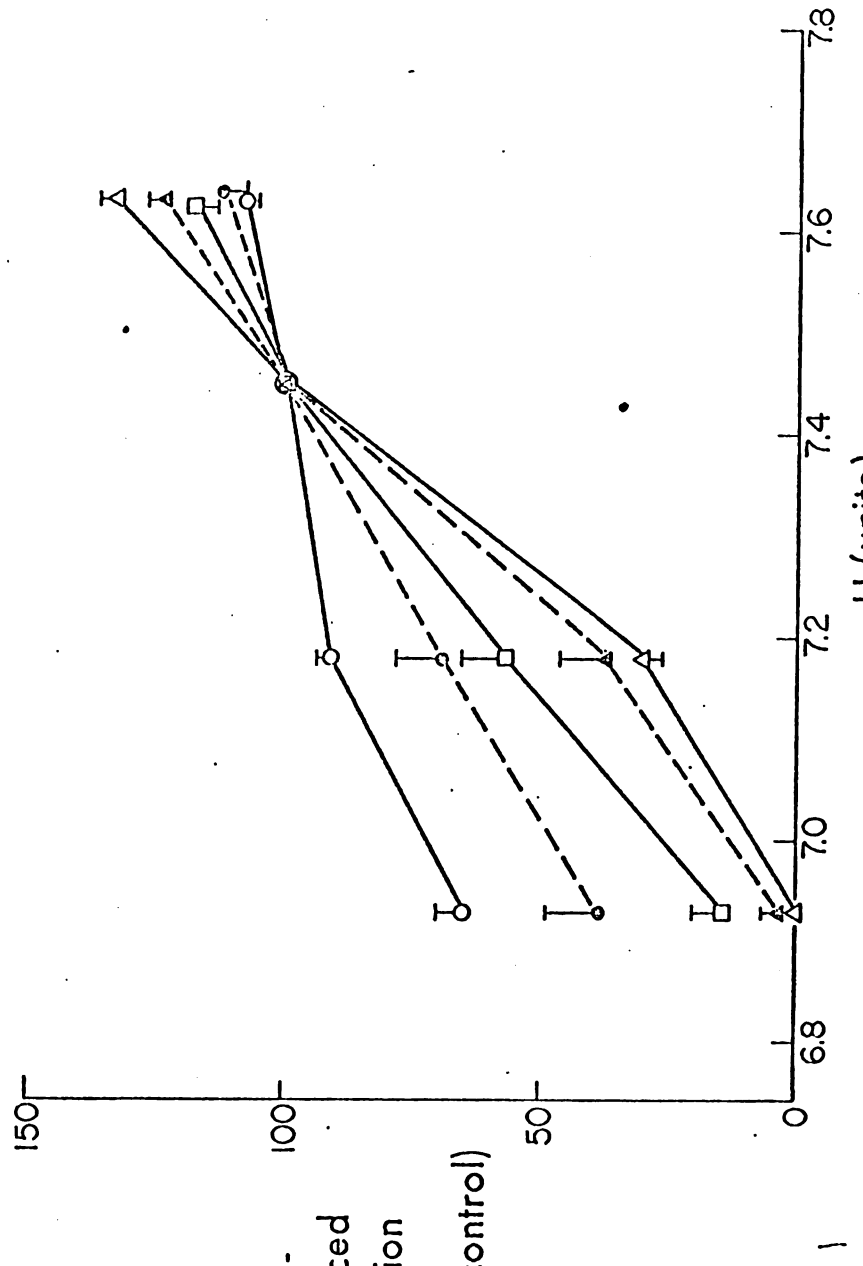


Figure 23: Effect of pH on contractions induced by various choline esters and serotonin. Drug concentrations were chosen to produce half-maximal contractions with 5% CO_2 in the bath. Drug-induced tension (ordinate) is expressed as percent of control contractile response (pH 7.45) and plotted as a function of bath pH (abscissa). pH was varied by changing CO_2 concentration. Values are means ± S.E. (n=4). O—O, acetylcholine (22 μM); ●---●, methacholine (1.02 μM); □—□, bethanechol (2.55 μM); ▲---▲, carbachol (0.18 μM); Δ—Δ, serotonin (0.54 μM).

line and serotonin were markedly different (Figure 6). Rates of contraction in response to the other choline esters studied were, however, not as fast as acetylcholine but more like serotonin. Since this difference in contraction rate could be caused by a difference in mode of interaction with the tissue this was quantified by measuring rates of half-maximal contractions produced by each of these agents. Indeed, carbachol and bethanechol contracted the muscle more slowly than did acetylcholine (Table 3).

TABLE 3

Rates of half-maximal contractions at pH 7.45

Drug	Contraction Rate* (grams/min)	Comparison of Rate With Acetylcholine	With Serotonin
Acetylcholine (22 μM)	2.47 $\pm$ 0.46	____	$p<0.03$
Methacholine (1.02 μM)	1.02 $\pm$ 0.21	$p>0.09$	$p>0.09$
Carbachol (0.19 μM)	0.54 $\pm$ 0.20	$p<0.03$	$p>1.0$
Serotonin (0.54 μM)	0.54 $\pm$ 0.12	$p<0.03$	____
Bethanechol (2.55 μM)	0.45 $\pm$ 0.04	$p<0.02$	$p>0.4$

* mean $\pm$ S.E. of 4 muscles

The rate of receptor combination as well as the rate of processes leading to shortening of the myofibrils will certainly determine the overall rate of muscle contraction. Another important consideration, however,

is the rate of diffusion of the drug to the receptor site. Diffusion rate is partly determined by drug concentration, and the concentrations of drugs necessary to achieve a half-maximal contractile response were widely disparate (22-0.19 x 10^{-6} M). To determine whether differences in concentrations could account for these differences in contraction rate, contraction rate was plotted as a function of concentration (Figure 24, data from 36 bronchial rings). This plot shows that contraction rate did, indeed, vary with drug concentration. The curves for carbachol and acetylcholine were roughly similar; the curve for acetylcholine in the presence of physostigmine (to extend doses of acetylcholine) also showed close correspondence to the curve for carbachol.

4. Effects of Methysergide and Atropine The possibility that carbachol and other choline esters act partly or completely by stimulating a CO_2-sensitive serotonin receptor was also considered. To test this possibility the effects of a serotonin receptor blocking agent, methysergide, were determined. A dose of methysergide (4.7 x 10^{-6} M) that completely blocked serotonin-induced contractions significantly increased acetylcholine-induced contractions and decreased carbachol-induced contractions in 5% CO_2 ($p<0.02$) (Figure 25). However, the effect of 10% CO_2 was unchanged in the presence of methysergide.

The distinctness of receptors for serotonin and carbachol was further confirmed by results previously reported. A dose of atropine that completely blocked acetylcholine-induced contractions reduced serotonin-induced contractions by only 6% and did not alter the sensitivity of serotonin-induced contractions to CO_2 (Figure 15). This dose of atropine also completely blocked carbachol-, bethanechol- and methacholine-induced contractions.

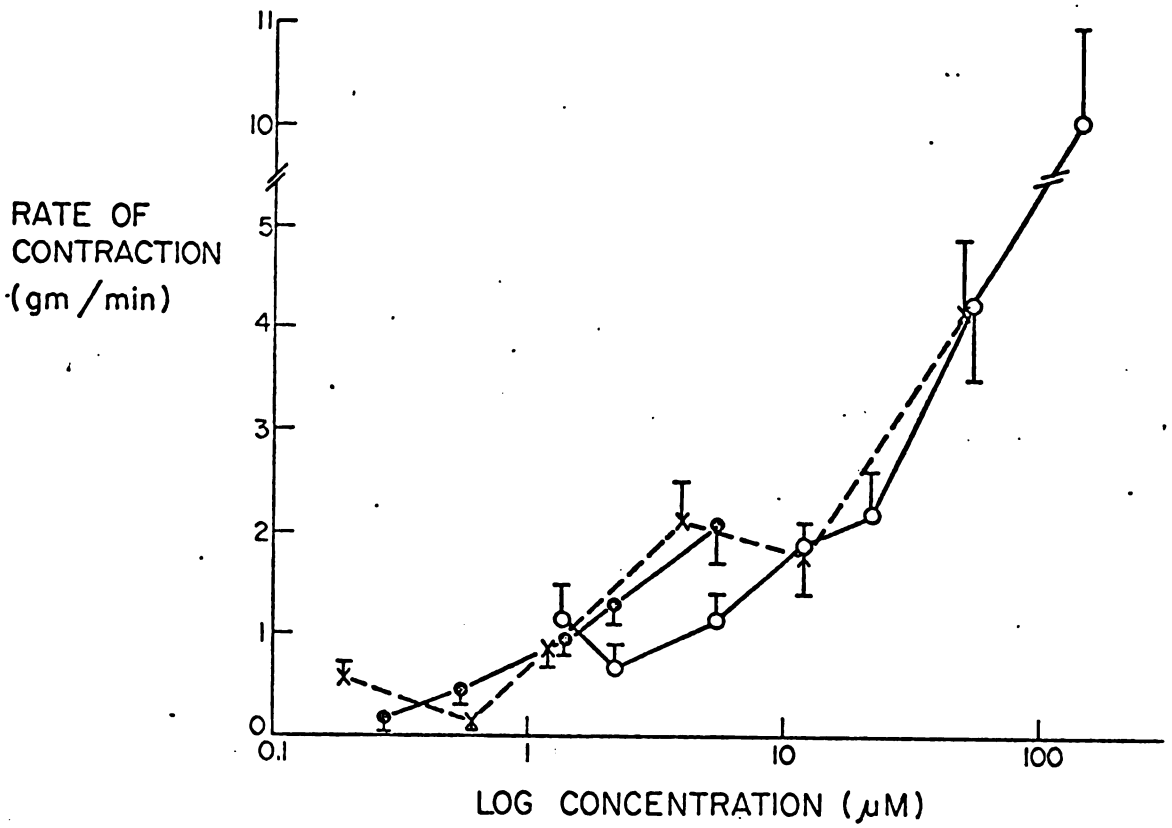


Figure 24: Rate of contraction with 5% CO_2 in the bath (ordinate) plotted as a function of log concentration of cholinergic agonists (abscissa). Values are means ± S.E. (n=3). O—O, acetylcholine; X---X, carbachol; ●—●, acetylcholine + 10^{-7} M physostigmine.

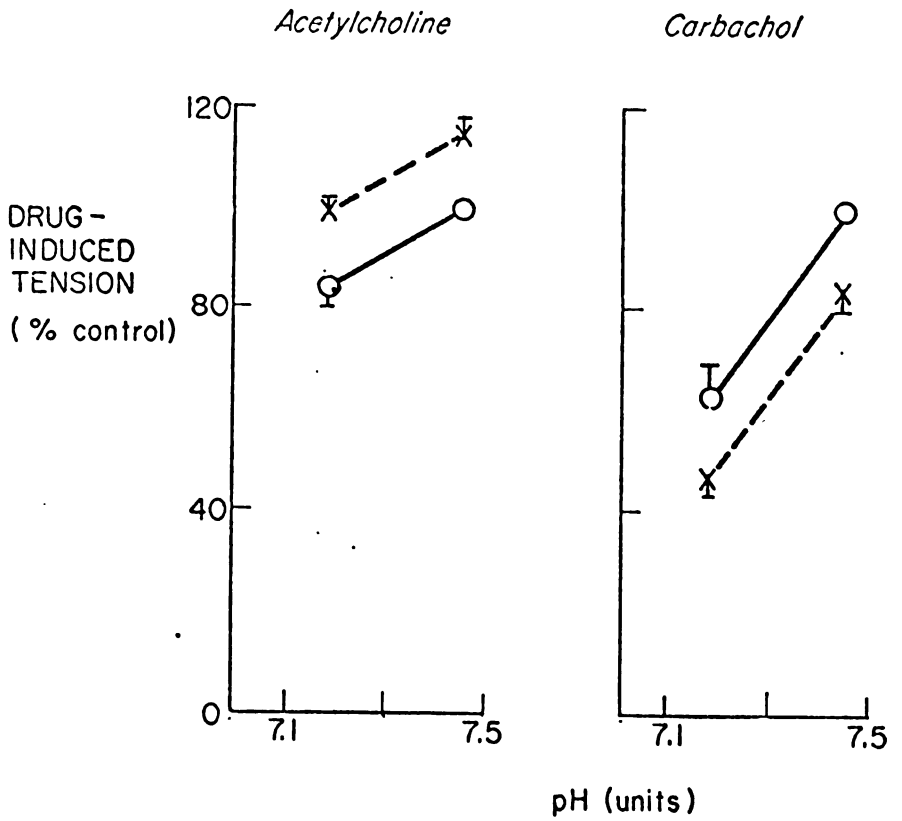


Figure 25: Effect of pH on contractions induced by acetylcholine (22 μM) and carbachol (0.19 μM) with and without methysergide (4.7×10^{-6} M). Acetylcholine and carbachol concentrations were chosen to produce half-maximal contractions with 5% CO_2 in the bath. Drug-induced tension (ordinate) is expressed as percent of control contractile response (pH 7.45; without methysergide) and plotted as a function of pH (abscissa). pH was varied by changing CO_2 concentration. Values are means ± S.E. (n=4). O—O, without methysergide; X---X, with methysergide.

5. Carbachol and Acetylcholine Interactions Since half-maximal carbachol-induced contractions were almost completely relaxed by 20% CO_2 whereas acetylcholine-induced contractions were not, the experiment illustrated in Figure 26 was suggested by Leon Hurwitz (personal communication) to determine whether the presence of carbachol would block acetylcholine-induced contractions at low pH. Four bronchial rings all gave similar results to the one illustrated in Figure 26. The contraction produced by acetylcholine at pH 6.93 with carbachol in the bath was 111.5 $\pm$ 10.1% of a control contraction at the same pH without carbachol present ($p>0.6$). Thus at low pH, carbachol did not block acetylcholine-induced contractions.

6. Effect of CO_2 During Washout of Carbachol Relaxation of supramaximal carbachol-induced contractions (5.5 μM) took approximately 20 min making it possible to investigate the effect of CO_2 on the washout phase. CO_2 had a small effect on this phase which appeared to be reversible (Figure 27). Thus although CO_2 concentration influenced tension during washout of carbachol, the rate of relaxation was unchanged.

B. CONCLUSIONS

Inhibition of acetylcholinesterase did not increase the effect of CO_2 on acetylcholine-induced contractions. Thus it does not seem likely that an effect of CO_2 on acetylcholinesterase can account for the insensitivity of acetylcholine-induced contractions to CO_2. However, if physostigmine blocked the effect of CO_2 by an action independent of its effect on acetylcholinesterase, this would put this conclusion in doubt. The effect of CO_2 on carbachol- and serotonin-induced contractions appeared to be reduced in the presence of physostigmine although further work would be necessary to clarify the significance of this effect. However, this con-

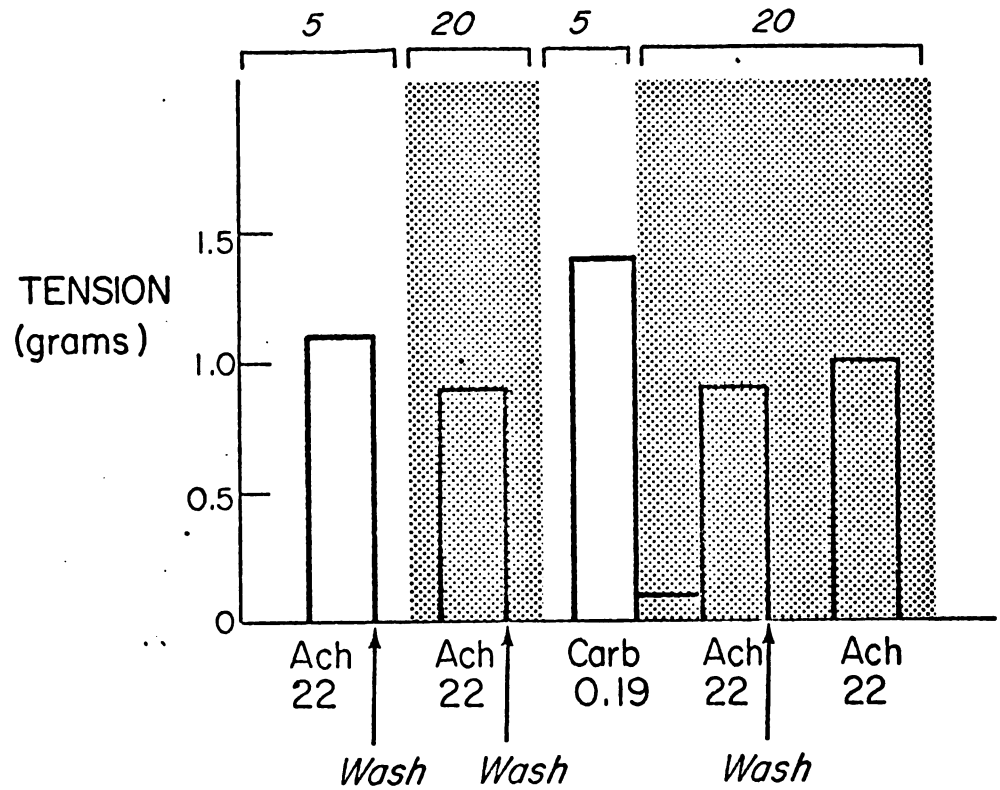


Figure 26: Effect of carbachol on acetylcholine-induced contractions with 20% CO_2 in the bath. Data is from one representative experiment; tension in grams is indicated by height of bars. Drug concentrations were chosen to produce half-maximal contractions with 5% CO_2 in the bath. CO_2 concentration is indicated at the top; drugs and concentrations in 10^{-6} M at the bottom. Ach=acetylcholine; Carb=carbachol. Shaded area indicates exposure to 20% CO_2.

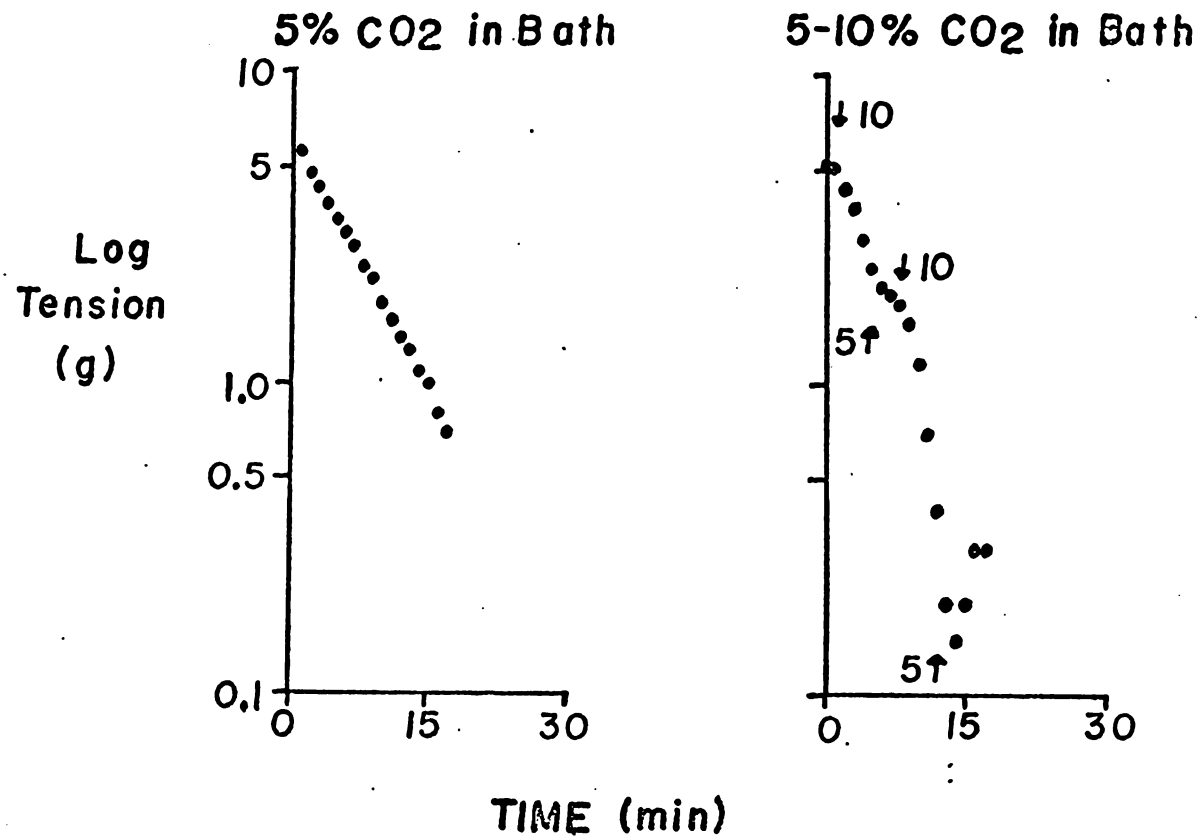


Figure 27: Rate of decrease in muscle tension during washout of carbachol following supramaximal contractions (5.5 μM). Log of muscle tension (ordinate) is plotted as a function of time (abscissa). The CO_2 concentration in the bath was maintained at 5% for one bronchial ring (left side) while for another the CO_2 concentration was alternated from 5% to 10% (right side). Both washouts began with 5% CO_2 in the bath. 5↑= change to 5% CO_2; 10↓= change to 10% CO_2.

centration of physostigmine did not completely block the effect of CO_2 on serotonin- and carbachol-induced contractions. Therefore, it seems likely that the insensitivity of acetylcholine-induced contractions to CO_2 was not the result of an effect of pH on acetylcholinesterase activity.

Physostigmine appears to have other types of actions as well, as evidenced by the tendency toward potentiation of carbachol-induced contractions. Carbachol is not hydrolyzed by acetylcholinesterase (Koelle, 1970) so the potentiation of the effect of carbachol cannot be attributed to inhibition of acetylcholinesterase unless carbachol releases acetylcholine. This point will be pursued in the discussion. The fact that physostigmine did not potentiate serotonin-induced contractions in 5% CO_2 is also evidence that serotonin does not release acetylcholine from tissue stores.

The hypothesis of this dissertation, that the site of action of CO_2 is the serotonin receptor, is supported by the finding that inhibition of acetylcholinesterase did not alter the CO_2 sensitivity of acetylcholine-induced contractions. However, the additional finding that contractions induced by analogs of acetylcholine were more sensitive to CO_2 than contractions induced by acetylcholine itself does seem to contradict that hypothesis. Differences in contraction rates among the choline esters could support the contention that there is more than one mechanism for interaction of choline esters with airway smooth muscle. However, these differences in contraction rate appeared to depend on the different drug concentrations necessary to produce a half-maximal contractile response.

The possibility that some choline esters might stimulate the serotonin receptor was also considered. However, the distinctness of the serotonin and carbachol receptors was demonstrated by use of specific

blocking agents, atropine and methysergide. Both these drugs did have other effects than blockade of specific receptors; however, the small magnitude of these effects and the maintenance of the effect of CO_2 argues against the hypothesis that carbachol stimulates the serotonin receptor.

Interactions of acetylcholine and carbachol at low pH were investigated. It was found that with 20% CO_2 in the bath carbachol did not block acetylcholine-induced contractions. By analogy to enzyme kinetics one can assume that a maximal response is equivalent to occupancy of all receptors. If one drug molecule combines with one receptor site and drug receptor binding represents the limiting step in the process to produce contraction then the magnitude of the response will be proportional to the number of receptors occupied (Goldstein et al, 1969). Since these concentrations of acetylcholine and carbachol each produced approximately 50% of a maximal contraction with 5% CO_2 in the bath, 50% of the receptors would be occupied. Although with 20% CO_2 carbachol was present in the bath it did not block the effects of acetylcholine. Therefore, it can be concluded that at low pH carbachol is not bound to the acetylcholine receptor in a manner that will block the contractile effect of acetylcholine. The implications of this finding will be considered further in the discussion.

During washout of carbachol, tension was influenced by changing pH, although the rate of relaxation was unchanged. This implies that, like serotonin, the binding of carbachol to its receptor is not altered by pH if the assumptions detailed previously are correct.

In summary: inhibition of acetylcholinesterase did not increase the sensitivity of acetylcholine-induced contractions to CO_2. However, con-

tractions induced by other choline esters were more sensitive to CO_2 than were contractions induced by acetylcholine itself. Differences in contraction rates among choline esters could be accounted for on the basis of differences in effective concentrations. Receptors for carbachol and serotonin were found to be quite distinct, and the presence of carbachol at low pH did not block the effect of acetylcholine.

V. EFFECTS OF CALCIUM AND POTASSIUM CONCENTRATIONS ON SENSITIVITY TO CO_2 OF DRUG-INDUCED CONTRACTIONS

A. RESULTS

1. Effects of Calcium A rise in intracellular calcium to concentrations of 1-2 x 10^{-4} M appears to activate smooth muscle contraction (Somlyo, et al, 1971) while a fall of intracellular calcium below this threshold level induces relaxation. The source of this activator calcium varies among different types of smooth muscle. Movement of calcium from the extracellular space across the cell membrane may be important in some cases whereas in other muscles, release of calcium from intracellular storage sites (sarcoplasmic reticulum, mitochondria or micropinocytocic vesicles) may play a role. It has been suggested (Somlyo and Somlyo, 1968) that the relative contributions of intracellular and extracellular sources of calcium may vary with the amount of sarcoplasmic reticulum or other intracellular binding sites in various types of smooth muscle, as well as with the means of excitation.

Drug-induced contractions of some types of smooth muscle may be maintained in very low concentrations of extracellular calcium, but are not maintained in calcium-free solutions (Hinke, 1965). Five bronchial rings were not contracted by either serotonin or acetylcholine in the presence of 2 mM EGTA added to calcium-free Kreb's solution. However, 0.12 mM calcium was sufficient to partially sustain serotonin- and acetylcholine-induced contractions. Furthermore, serotonin-induced contractions were still sensitive to CO_2 (Figure 28).

2. Effects of Potassium Since the report by Evans et al (1958) that several types of smooth muscle retain their ability to contract in response to drugs when depolarized by potassium solutions, there has been

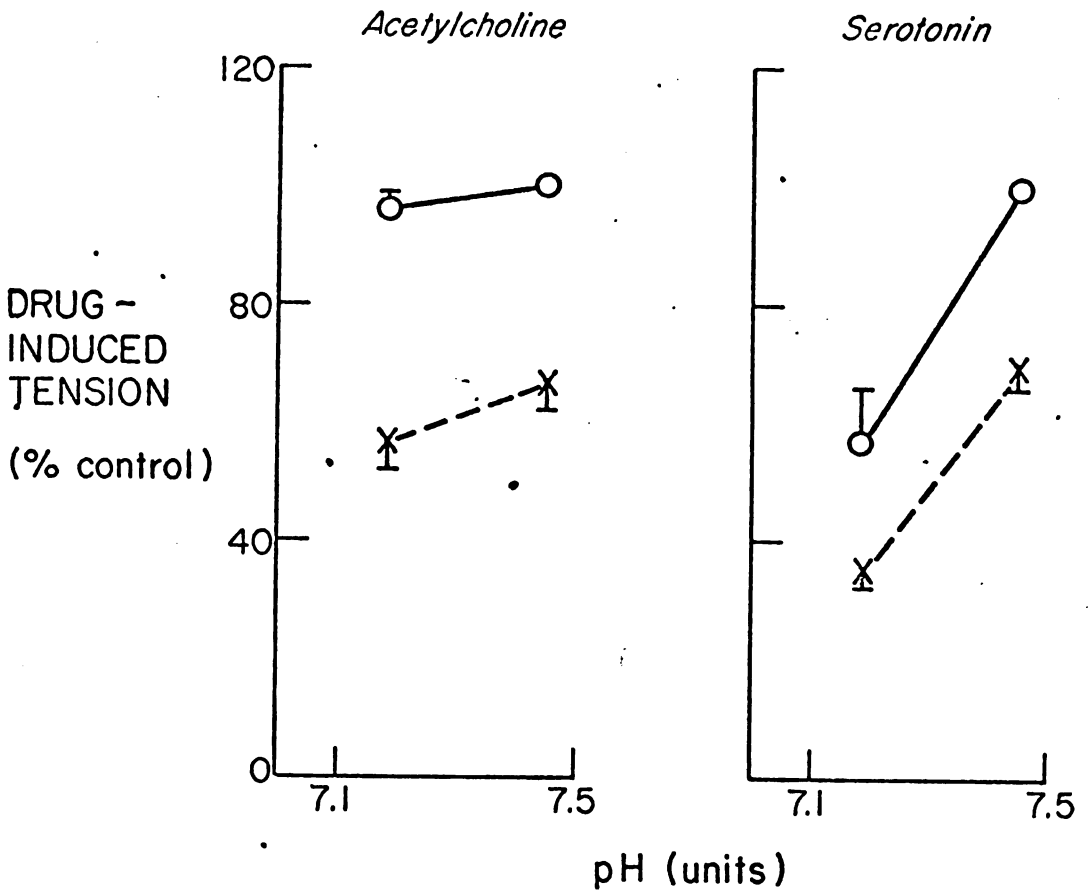


Figure 28: Effect of pH on contractions induced by acetylcholine (22 μM) and serotonin (0.54 μM) in the presence of low extracellular calcium. Drug concentrations were chosen to produce half-maximal contractions with 5% CO_2 in the bath. Drug-induced tension (ordinate) is expressed as percent of control contractile response (pH 7.45; 2.8 mM calcium) and plotted as a function of bath pH (abscissa). pH was varied by changing CO_2 concentration. Values are means ± S.E. (n=3). O—O, in the presence of 2.8 mM calcium; X---X, 0.12 mM calcium.

much speculation about the significance of the membrane potential in mediating contractions of smooth muscle. The relatively low resting membrane potential of many types of smooth muscle, the lack of action potentials, as well as the lack of consistent correlations between membrane potential and contractile response has led to the hypothesis that nonelectrical processes may mediate drug-induced contractions in some cases. Somlyo and Somlyo (1968) have termed this process pharmacomechanical coupling to distinguish it from electromechanical coupling. It seems possible that the membrane potential may be important in mediating conduction of impulses in muscles where electromechanical coupling is important, such as cardiac and skeletal muscle. In muscles where conduction of impulses may not be so important, such as some types of smooth muscle, the responses may also be elicited by direct effects of chemical mediators on each muscle cell membrane.

To determine whether the membrane potential is important for drug-induced contractions of airway smooth muscle, the effects of acetylcholine and serotonin were tested in the presence of increased concentrations of potassium. These experiments were carried out at 23.5°C because low temperature produces partial relaxation of potassium contractions (Evans et al, 1958). Acetylcholine- and serotonin-induced contractions in 5% CO_2 were not prevented in conditions of increased extracellular potassium (Figure 29). In addition the selective effect of 10% CO_2 was unchanged.

B. CONCLUSIONS

Drug-induced contractions of airway smooth muscle were maintained in the presence of low levels of extracellular calcium but were completely prevented in the presence of EGTA. These findings are comparable to

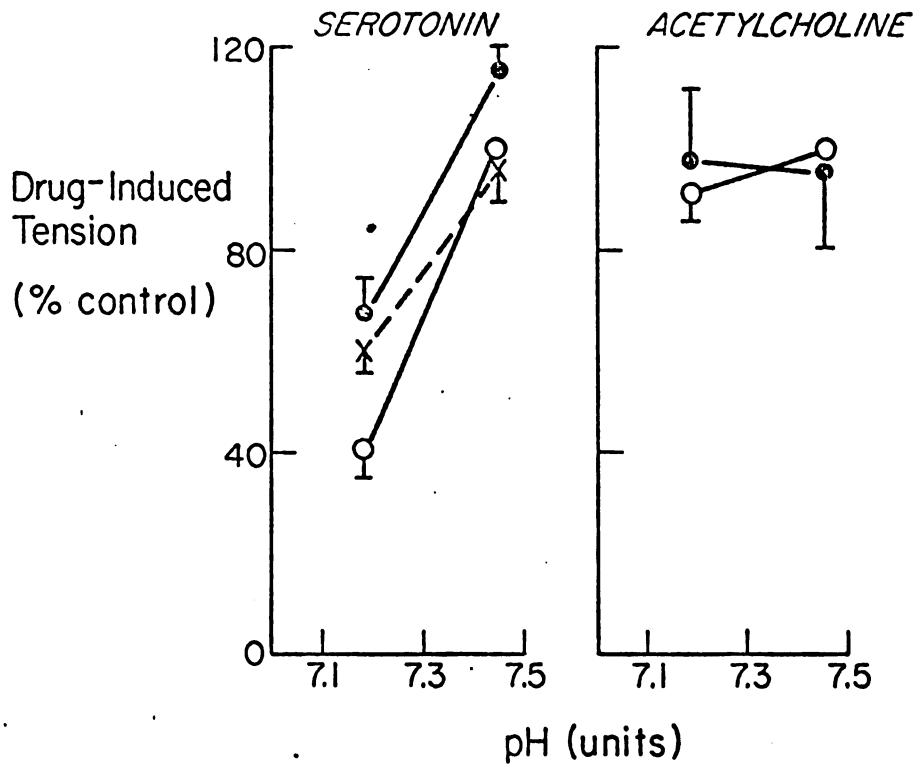


Figure 29: Effect of pH on contractions induced by serotonin (0.74 μM) and acetylcholine (22 μM) in the presence of increased extracellular potassium. Drug-induced tension (ordinate) is expressed as percent of control contractile response (pH 7.45; 5.9 mM potassium) and plotted as a function of bath pH (abscissa). pH was varied by changing CO_2 concentration. These experiments were carried out at 23.5°C to minimize the contractile effect of potassium itself. Values are means ± S.E. (n=2). O—O, in the presence of 5.9 mM potassium; X---X, 35 mM; ●—●, 64 mM.

what has been found in some types of vascular smooth muscle. Somlyo and Somlyo (1968) have proposed two barriers to calcium movement to account for such findings. An external barrier which contains high affinity sites for calcium could concentrate the cation from the external medium. An inner barrier to calcium would be the plasma membrane, whose permeability would be controlled by drugs. Thus in the presence of low-calcium-Kreb's, drug-induced contractions could be maintained by influx of calcium through the cell membrane from extracellular calcium binding sites. However, in the presence of EGTA, which has high affinity for calcium, these calcium binding sites would be depleted, and drug-induced contractions would be prevented. Whether such a model accurately reflects the situation for airway smooth muscle would require much more exhaustive study. Localization of calcium by microscopy and study of calcium fluxes can contribute to elucidation of the role of calcium. The preliminary work reported here is suggestive at best.

Airway smooth muscle was capable of drug-induced contractions in the presence of increased extracellular potassium. Extracellular potassium concentrations of 64 mM result in a depolarization of the membrane potential of taenia coli to -20mV (Casteels and Kuriyama, 1966). Although no direct evidence is available to confirm that airway smooth muscle is also depolarized by increased extracellular potassium, it seems likely that it would be.

It was recently reported that the intracellular resting membrane potential of the canine trachealis muscle is 45.3 $\pm$ 0.7 mV (Mitchell and Naimark, 1973). Such a low membrane potential coupled with the findings that drug-induced contractions were well maintained in the presence of increased extracellular potassium makes it likely that change of membrane

potential is not a necessary step in drug-induced contractions of airway smooth muscle. The membrane potential probably does not play a role in the effect of CO_2 either, since serotonin-induced contractions were still just as sensitive to CO_2 in the presence of increased potassium concentrations.

VI. OTHER STIMULANTS

A. RESULTS

1. α-sympathomimetic Receptor Stimulation The presence and significance of α-sympathomimetic receptors in airway smooth muscle has been a matter of some controversy. Some authors have been unable to find evidence for α-receptors (Cabezas et al, 1971; Foster, 1966); whereas others have demonstrated both α and β receptors (Fleisch et al, 1970; Castro de la Mata et al, 1962; Turker and Kiran, 1965; Everitt and Cairncross, 1969; Mathé et al, 1971; Simonsson et al, 1972).

To determine whether cat bronchial rings were sensitive to α-receptor stimulation two approaches were used. In 3 bronchial rings the effects of methoxamine, an α-receptor stimulant were tested. Doses of methoxamine up to 0.6×10^{-3} M had no effect. The effects of methoxamine and norepinephrine after treatment with 10^{-5} M propranolol were also investigated. This dose of propranolol completely blocked the β-receptor relaxation produced by norepinephrine. In each of 4 bronchial rings methoxamine at a concentration of 0.6×10^{-3} M had no effect in the presence of propranolol. Norepinephrine (0.25×10^{-3} M) had no effect in one bronchial ring in the presence of propranolol.

2. Increased Extracellular Potassium To produce stimulation of muscle contractile elements, 10 bronchial rings were bathed in Kreb's solutions containing increased potassium concentrations. In 5% CO_2, 123 mM potassium gave 99.7 ± 19.1% of a maximal serotonin-induced contraction; 64 mM potassium, 33.9 ± 6.8% and 35 mM potassium, 27.6 ± 6.3%. The effect of CO_2 on potassium-induced contractions was significantly less than the effect of CO_2 on serotonin-induced contractions (Figure 30). The effect of CO_2 on 123 mM potassium-induced contractions was

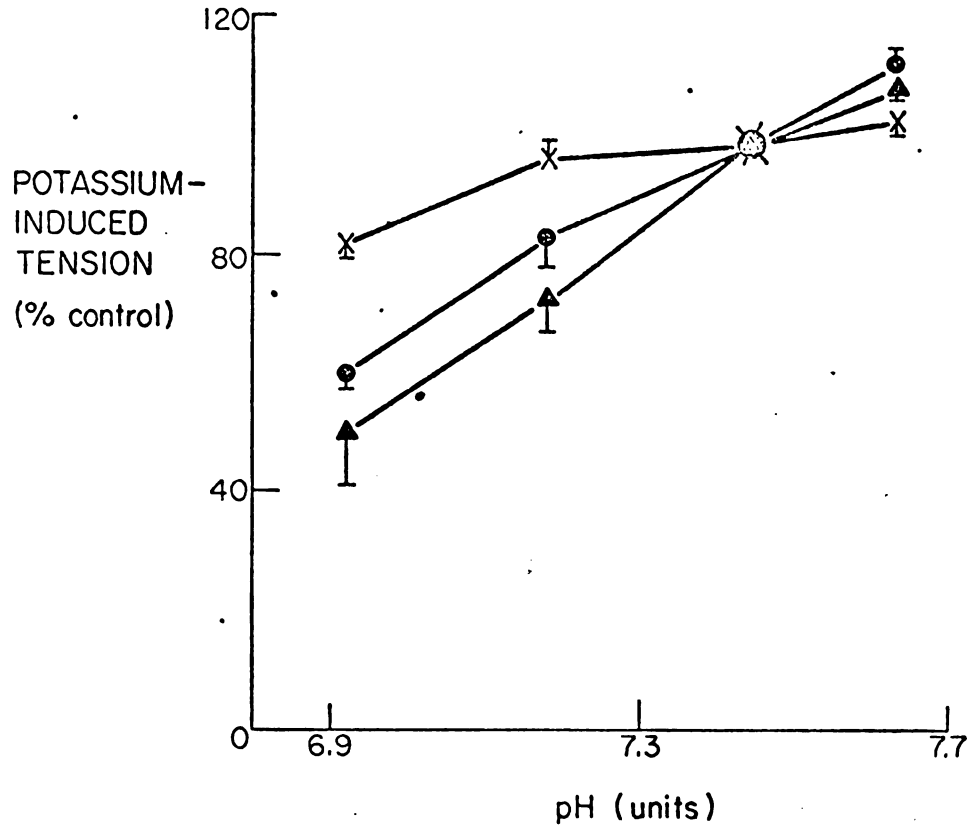


Figure 30: Effect of pH on potassium-induced contractions. Potassium-induced tension (ordinate) is expressed as percent of control (pH 7.45) and plotted as a function of pH (abscissa). pH was varied by changing CO_2 concentration. Values are means ± S.E. (n=3). X—X, 123 mM potassium; ●—●, 64 mM; ▲—▲, 35 mM.

also significantly less than the effect of CO_2 on acetylcholine-induced contractions while the effect of CO_2 on 64 and 35 mM potassium-induced contractions was not significantly different from the effect of CO_2 on acetylcholine-induced contractions (Table 4).

TABLE 4

Comparison of sensitivities to pH of various drug- and potassium-induced contractions For each drug slopes of regression lines were calculated for the relationship of tension vs. pH (for data see Figures 23 and 30) and compared with acetylcholine and serotonin. Drug concentrations were chosen to produce half-maximal contractions with 5% CO_2 in the bath, and three concentrations of potassium were studied.

Drug	Slope of Regression Line	Comparison of Slope With Acetylcholine	With Serotonin
Acetylcholine (22 μM)	59.4	---	$p<0.005$
Potassium (123 mM)	28.3	$p<0.006$	$p<0.005$
Potassium (64 mM)	72.4	$p>0.2$	$p<0.005$
Potassium (35 mM)	82.5	$p>0.1$	$p<0.005$
Serotonin (0.54 μM)	229.2	$p<0.005$	---

3. Ganglionic Stimulants Since it was shown that ganglion cells were present in the feline bronchial ring preparation used in this work, the effect of ganglionic stimulants was investigated. In 10 bronchial rings 0.09 mM tetramethylammonium gave 26.1 $\pm$ 4.0% of a maximal

acetylcholine-induced response. In 3 bronchial rings, 0.36 mM tetramethylammonium produced 51.7 $\pm$ 4.4% of a maximal acetylcholine-induced response. Sensitivity to CO_2 of tetramethylammonium-induced contractions was significantly greater than the sensitivity of acetylcholine-induced contractions (Figure 31). The feline bronchial ring showed very little sensitivity to nicotine. In 2 bronchial rings nicotine (3.8×10^{-5} M) produced only very small transient contractions. Lower doses of nicotine had no effect.

4. Histamine Histamine stimulates isolated bronchial and tracheal rings in a variety of species: man (Rosa and McDowall, 1951), guinea pig (Jamieson, 1962), and dog (Akcasu, 1959; Sollmann and Gilbert, 1937). In 9 feline bronchial rings concentrations of histamine as high as 5 mM had no effect. Possible relaxant effects of histamine were investigated in 3 bronchial rings that were contracted by carbachol. Concentrations of histamine of 2.7×10^{-4} M relaxed carbachol-induced contractions by an average of 29.8 $\pm$ 11.8%.

5. Bradykinin Bradykinin has been reported to constrict some preperations of human bronchi and relax others regardless of the dose (Mathé et al, 1971). Other workers have found no effect on similar preparations (Brocklehurst, 1953). Lynn James (1969) found that bradykinin decreased dynamic compliance of guinea pig lungs and slightly decreased pressure in a tracheal segment. Sheep bronchi were very weakly contracted by 19×10^{-6} M bradykinin (Eyre, 1969). In the present study, bradykinin (0.38×10^{-6} M) had no effect on tension of two feline bronchial rings.

6. Prostaglandins The isolation of a number of prostaglandins from many tissues has sparked a great deal of speculation about the biological significance of these compounds. The lung has not been exempt from such

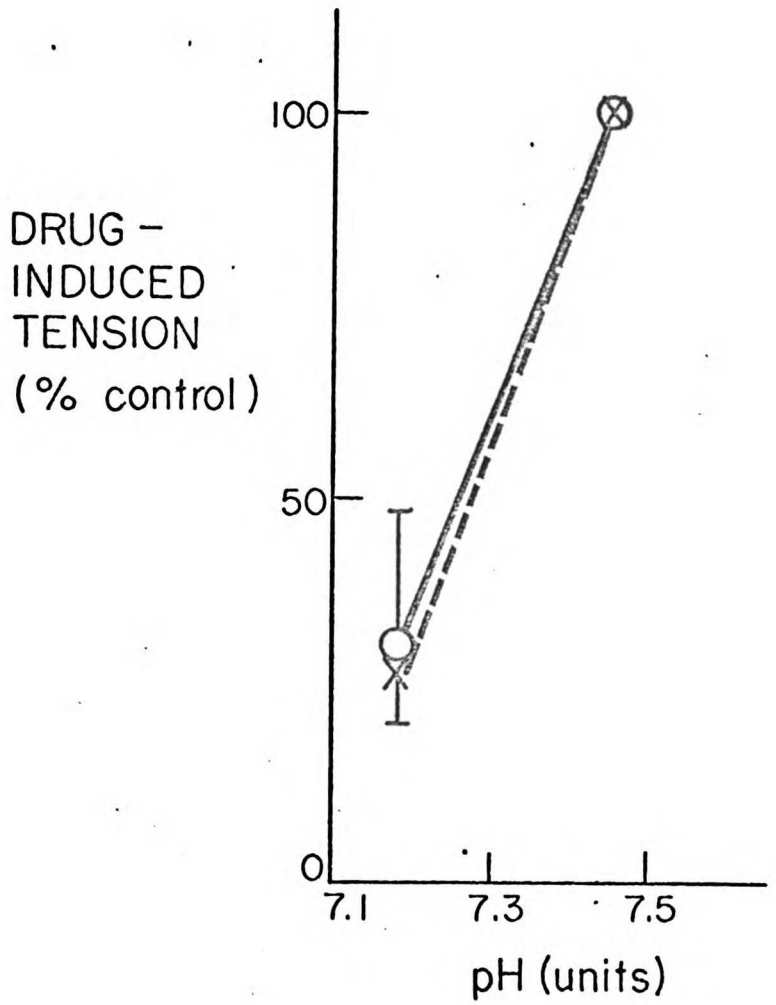


Figure 31: Effect of pH on tetramethylammonium-induced contractions. Drug-induced tension (ordinate) is expressed as percent of control contractile response (pH 7.45) and plotted as a function of pH (abscissa). pH was varied by changing CO_2 concentration. Values are means ± S.E. O——O, 0.09 mM tetramethylammonium (n=4); X---X, 0.36 mM (n=2).

speculation. Prostaglandins E_2 and $F_{2\alpha}$ (PGE_2 and $PGF_{2\alpha}$) have been shown to occur in cat lungs (Karim et al, 1968), and both bronchoconstricting and bronchodilating effects of various prostaglandins have been demonstrated in a number of species. Human bronchial strips are contracted by $PGF_{2\alpha}$ (Mathé et al, 1971; Sweatman and Collier, 1968) as are cat tracheal strips (Angaard and Bergstrom, 1963). Dynamic compliance of the cat and guinea pig lung is decreased by $PGF_{2\alpha}$ (Villanueva et al, 1972; Lynn James, 1969); however, Angaard and Bergstrom (1963) reported that $PGF_{2\alpha}$ had no effect on isolated cat bronchi. In the present study $PGF_{2\alpha}$ (2.1-174 x 10^{-6} M) was without effect on tension of 4 bronchial rings.

B. CONCLUSIONS

It seems clear that in the presence of propranolol, methoxamine or norepinephrine can stimulate airway smooth muscle in a number of species. This α-sympathomimetic effect, however, is relatively weak; maximum α-receptor stimulation appears to be only 10-20% of a maximal carbachol-induced contraction (Simonsson et al, 1972). In addition, without propranolol, norepinephrine always produces a relaxation. This indicates that β-receptors predominate over α-receptors.

Simonsson et al (1972) recently reported that treatment with endotoxin potentiated the maximum effect of α-receptor stimulation 2 to 10 times in human bronchial muscle preparations that were not treated with a β-blocking agent. Endotoxin supposedly reduced the content of cyclic AMP of these bronchial preparations. However, it is difficult to see how this can account for potentiation of an α-sympathomimetic effect. It is conceivable that α-sympathomimetic receptor stimulation may be due to release of constricting mediators, such as histamine or slow reacting sub-

stance, from mast cells. Release of these substances is enhanced by stimulation of α-sympathomimetic receptors (Orange et al, 1971).

An increase in extracellular potassium produces contraction in a manner that circumvents drug receptors. Thus it is possible to differentiate between effects on electromechanical coupling and effects on pharmacomechanical coupling (Somlyo and Somlyo, 1970). The effect of CO_2 on potassium-induced contractions was found to be relatively small, significantly less than the effect of CO_2 on serotonin-induced contractions. Pharmacomechanical and electromechanical coupling presumably produce contraction via a common final path, an increase in intracellular calcium which activates contractile proteins. Thus it can be concluded that the effect of CO_2 occurs at a step before this common pathway, and that CO_2 does not act by altering the activity of contractile proteins.

The effect of ganglionic stimulants on airway smooth muscle has been carefully studied by Hawkins and Paton (1958). They found that nicotine produced only very small, transient contractions of cat tracheal and bronchial rings. These contractions were blocked by hexamethonium and atropine. According to these workers, the effects of ganglion cell stimulation by nicotine are expected to be transient and blocked by hexamethonium or atropine. When bronchial or tracheal rings were contracted by pilocarpine, however, Hawkins and Paton (1958) found that nicotine produced a transient relaxation. Whether this indicates the presence of ganglion cells of the sympathetic system or whether nicotine stimulates some other site, such as nerve endings mediating a local axon reflex, is unknown. In the present study, nicotine produced only small transient contractions. Although ganglion cells were found to be present in bronchial rings, it is worth considering that nervous connections to smooth

muscle may be cut. Possible relaxant effects of nicotine were not investigated in the present study.

According to Hawkins and Paton (1958), in feline bronchi tetramethylammonium probably has some other site of action than ganglia. These workers found concentrations of hexamethonium of 3×10^{-6} M sufficient to block the effects of nicotine, but these concentrations did not block tetramethylammonium-induced contractions. The insensitivity of tetramethylammonium-induced contractions to hexamethonium was confirmed in this study. The lowest concentrations of hexamethonium that could block tetramethylammonium-induced contractions in the present work were 100 fold larger than concentrations sufficient to block nicotine-induced contractions in the same tissue (Hawkins and Paton, 1958). Thus the significance of the sensitivity to pH of tetramethylammonium-induced contractions is difficult to determine since the mechanism of these contractions is not well defined.

There have been a number of reports that histamine aerosol or injection of histamine produces bronchoconstriction in the cat (Nisell, 1950; Daly and Mount, 1951; Nagao et al, 1971). However, the measure of bronchoconstriction used in these studies was a change in the dynamic pressure volume relationship of the lungs. Either a change in pressure with constant volume inflations or a change in volume with constant pressure inflations was measured. As discussed by Widdicombe (1963) dynamic compliance is much more sensitive to changes in lung stiffness and relatively less sensitive to changes in lung resistance to flow. Lung compliance, the reciprocal of elastance or stiffness, is the slope of a static volume-pressure curve expressed in liters/cm H_2O. Lung resistance to flow is the ratio of pressure to flow and is expressed in

cm H_2O/liter/sec. At low frequencies of ventilation, dynamic compliance is much more sensitive to small changes in compliance than it is to large changes in resistance (Figure 32). Thus dynamic pressure volume measurements are more sensitive to changes in lung stiffness due to pulmonary edema, pulmonary vascular congestion, or collapse of alveoli due to bronchiolar constriction than to changes in large airway diameter.

Indeed, Colebatch et al (1966) showed that the effects of histamine on airways in the vagotomized cat were predominantly due to constriction of alveolar ducts and small bronchioles, rather than constriction of larger airways. They compared the effects of injecting histamine into the right and left sides of the heart. Pulmonary arteries perfuse the respiratory bronchioles and alveolar ducts, whereas bronchial arteries perfuse the conducting airways proximal to the respiratory bronchioles. Thus right heart injections will reach the pulmonary circulation first; left heart injections will reach the bronchial arteries first. Histamine injected into the right side of the heart of cats had a more rapid onset of action than histamine injected into the left side. Compliance decreased and resistance increased. Right-sided injections of acetylcholine and serotonin produced much greater increases in resistance with less decrease in compliance. Left-heart injections of histamine had a slower onset of action and required larger doses to produce similar effects. In addition, histological evidence of alveolar duct constriction was obtained. These authors concluded that the primary effect of histamine in the cat was on "peripheral airways", the respiratory bronchioles and alveolar ducts. Effects of histamine on larger airways *in vivo* appear to be mainly due to vagal reflexes, as demonstrated in the dog by De Kock et al (1966).

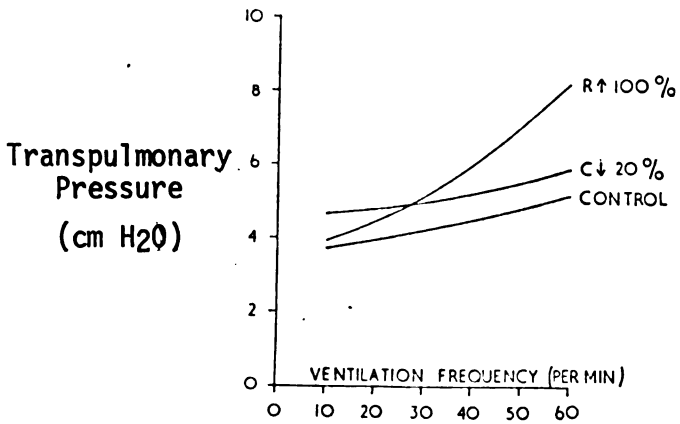


Figure 32: Relationship between pump ventilation frequency (abscissa) and dynamic transpulmonary pressure swings (ordinate). The lowest line corresponds to a total lung resistance of 0.192 cm H_2O/liter/min, a lung compliance of 0.0134 liter/cm H_2O, and a tidal volume of 0.05 liter. The upper lines correspond to a 100% increase in lung resistance, and a 20% decrease in compliance. From: Widdicombe (1963).

In vitro studies of feline bronchial or tracheal rings have indicated that histamine does not produce contraction; histamine may in fact cause relaxation (Akcasu, 1959; Maengwyn-Davies, 1968). In the present study histamine did not contract feline bronchial rings but did produce relaxation in high concentrations. High concentrations of histamine dihydrochloride can produce a change in pH; thus the possibility that the relaxant action of histamine could be attributed to such a change in pH should be considered.

That effects of histamine may differ on various levels of the airways was illustrated by Eyre (1969). He showed that sheep tracheal rings were contracted by histamine while bronchial rings were relaxed. Careful studies of the effects of histamine *in vitro* on different airway levels and in different species are necessary to better understand the effects of histamine on airways. However, as de Kock et al (1966) pointed out, since histamine may act either locally or reflexly or both, the effects of drugs on isolated airways may not be directly related to their effects on airways in the intact animal.

In the present study both bradykinin and prostaglandin $F_{2\alpha}$ were without effect on feline bronchial rings. As mentioned above, in other studies bradykinin has been found either to contract or relax bronchi or to have no effect. The effect of prostaglandins depends on the particular prostaglandin as well as the animal species studied. $PGF_{2\alpha}$ contracts some species but not others; whereas PGE_1 and PGE_2 appear to decrease airway resistance of cats (Rosenthale et al, 1971). To elucidate the effects of these substances, careful studies of different species as well as different levels of airways will have to be undertaken.

In summary: potassium-induced contractions were found to be rela-

tively insensitive to CO_2. This indicates that CO_2 does not have a generalized effect on the muscle contractile system. No evidence for the existence of α-adrenergic receptors was found; histamine, $PGF_{2\alpha}$ and bradykinin were also found to be ineffective in contracting feline bronchial rings. Although tetramethylammonium contracted this muscle, such contractions were probably not due to stimulation of ganglia. Nicotine was found to produce only small, transient contractions.

DISCUSSION

A. THE SHORT-TERM EFFECT OF CO_2 ON AIRWAY SMOOTH MUSCLE

Serotonin-induced contractions were rapidly and reversibly relaxed by increased concentrations of CO_2; acetylcholine-induced contractions were much less affected. This effect of short-term exposure to CO_2 appeared to depend on changes of extracellular pH since the effect of CO_2 was mimicked by addition of strong acid to the bathing medium.

The insensitivity to CO_2 of acetylcholine-induced contractions was unchanged in the presence of the acetylcholinesterase inhibitor, physostigmine, eliminating the possibility that an effect of CO_2 on acetylcholine-induced contractions was masked by a simultaneous alteration of acetylcholinesterase activity by changes in pH.

A number of possible mechanisms for the effect of short-term exposure to CO_2 have been investigated. The possibility that CO_2 acts by altering the intrinsic contractile apparatus was considered since pH is known to alter the activity of the actin-myosin system in vitro (Goodall and Szent Gyorgi, 1953). However, potassium- and acetylcholine-induced contractions, which presumably activate contractile elements, were relatively insensitive to CO_2, thus ruling out this hypothesis. On the other hand, the effect of long-term exposure to CO_2, which appears to depend on alteration of intracellular pH (Stephens et al, 1968), may involve a change in the activity of contractile elements.

The mechanism of the CO_2 bronchodilator effect could not be accounted for by involvement of tissue nervous elements since propranolol, sotalol, atropine, tetrodotoxin, and hexamethonium did not influence the effect of CO_2 on acetylcholine- or serotonin-induced contractions.

The membrane potential probably does not play a role in the effect of CO_2, since serotonin-induced contractions were still as sensitive to

CO_2 in the presence of increased potassium concentrations. Furthermore, Mitchell and Naimark (1973) recently reported that the resting membrane potential of tracheal smooth muscle cells was not altered by exposure to CO_2.

The possibility that CO_2 acts by changing the form of drugs used in this study was considered. The pKa of serotonin is 10.0 (Vane, 1959), so there is little change in the proportion of ionized to unionized drug in the range of pH studied. Acetylcholine and the other choline esters studied are quaternary ammonium compounds which remain completely ionized over a wide pH range. Thus the effect of CO_2 cannot be explained by a change in the ionization of these agents. The possibility that formation of carbamate compounds between CO_2 and amine functions of carbachol and serotonin could be occurring was also considered. However, this does not occur to any appreciable extent in the pH range concerned (E.C. Jorgensen, personal communication). In addition, formation of a carbamate compound would only increase with increased CO_2 concentration and not when pH was lowered by addition of strong acid. Since both these methods of changing pH produced comparable effects on drug-induced contractions of airway smooth muscle, the effect of CO_2 does not depend on an alteration of the form of these drugs.

The hypothesis that the effect of CO_2 could be explained by a change in soluble calcium was considered. For practical reasons it is difficult to measure the amount of soluble calcium in a bicarbonate buffer system. Centrifugation cannot be carried out while maintaining conditions of constant P_{CO_2}, since pressures will alter greatly during centrifugation. Filtration will not guarantee that free and associated calcium will separate effectively. However, there are several reasons that make it

highly unlikely that alteration of available extracellular calcium accounts for the effects of CO_2 and pH. First of all, drug-induced contractions were not very sensitive to changes of extracellular calcium: low calcium concentrations were sufficient to maintain partially drug-induced contractions. Thus, small changes in soluble calcium produced by changes in CO_2 or pH are not likely to have much effect on drug-induced contractions. In addition, sensitivity to CO_2 of serotonin-induced contractions was maintained in the presence of low concentrations of calcium in the bathing medium. Furthermore as pH falls, the amount of calcium that can dissolve increases. If it is assumed that $CaCO_3$ will be the primary precipitant, formulas describing the ionization of bicarbonate and the precipitation of $CaCO_3$ can be used.

$$[H^+]\,[CO_3^=] = K_2\,[HCO_3^-]$$

$$[H^+]\,[HCO_3^-] = K_1\,[CO_2]$$

$$[CO_3^=]\,[Ca^{++}] = K_{sp} \qquad \text{(Watton, 1964)}$$

Combining these formulas a relationship between soluble calcium and hydrogen ion and carbon dioxide concentration can be obtained.

$$[Ca^{++}] = \frac{K_{sp}\,[H^+]^2}{K_1\,K_2\,[CO_2]}$$

As the hydrogen ion concentration falls, if CO_2 concentration remains constant, the amount of soluble calcium will increase. If the CO_2 concentration is increased and the hydrogen ion concentration falls, the soluble calcium concentration will still increase because the hydrogen ion concentration is raised to the second power. Thus, as pH falls the solubility of calcium increases even though serotonin- and carbachol-induced contractions decrease. The reversibility of the effect of CO_2

also appears to contradict the hypothesis that changes in soluble calcium account for this effect. $CaCO_3$ will not redissolve easily once it precipitates, but the effect of CO_2 is rapidly and completely reversible.

Although serotonin- and acetylcholine-induced contractions were markedly different in their response to pH, sensitivity to pH was not a unique property of the serotonin system. Contractions induced by other choline esters were more sensitive to pH than were contractions induced by acetylcholine itself. The mechanism of the effect of pH must account for the difference in sensitivity between contractions induced by acetylcholine, other choline esters, and serotonin. Two sites of action of pH can be considered: pH could act by altering binding of drug to receptor or by affecting the specific transmission process from the receptor to the general contractile apparatus.

pH could act by altering the conformation of the serotonin receptor, thus reducing the likelihood of serotonin binding. In this case the binding of acetylcholine to its receptor would be unchanged by pH, whereas the binding of other choline esters would be affected. If acetylcholine and the other choline esters stimulate the same receptor, this implies that their binding sites are different enough that a change in conformation will affect the binding of one but not the other (Figure 33). This concept is consistent with the observation that with 20% CO_2

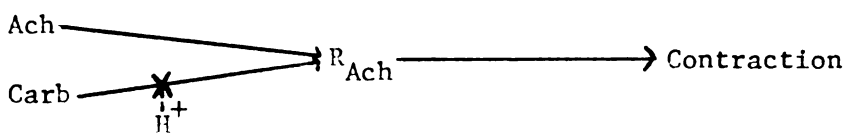


Figure 33: Schematic diagram to illustrate the hypothesis that pH alters the binding of carbachol (one of the pH sensitive choline esters) to the acetylcholine receptor but does not alter the binding of acetylcholine. ---X indicates inhibition. R=drug receptor.

in the bath carbachol did not inhibit the contractile effect of acetylcholine (Figure 26). This implies that at low pH carbachol was not bound to the acetylcholine receptor in a manner that altered the effect of acetylcholine.

The reversibility of the effect of CO_2 during washout of serotonin- or carbachol-induced contractions suggests that pH does not act by altering drug-receptor binding. As discussed previously, this conclusion depends on two assumptions. 1. The relaxation phase is due to slow release of serotonin. 2. If serotonin is released from receptor binding the rate of serotonin release from the muscle will be accelerated. The effect of CO_2 on drug binding should be directly measured. However, the small percentage of smooth muscle in the bronchial wall makes it probable that in this case non-specific binding will make up a very large proportion of total tissue binding. Thus possible changes in specific binding produced by $[H^+]$ would be obscured. It is possible that a more suitable tissue for binding studies could be used (e.g. trachealis muscle).

pH could act by influencing the specific transmission process from the receptor to the contractile elements. This implies that the transmission process is not identical for contractions induced by acetylcholine and the other choline esters. As discussed in the introduction, although some parts of this transmission process have been identified, all the steps are not understood. If pH does act by altering some step in the transmission from receptor to contractile elements it may be a useful tool to investigate the nature of this process since pH influences contractions induced by some drugs but not by others.

Just because pH has an effect on contractions induced by both serotonin and some choline esters does not imply that the mechanism of the

CO_2 effect is the same for these agents. It is possible that pH relaxes serotonin-induced contractions by altering binding of serotonin to its receptor and relaxes contractions induced by the choline esters by some other mechanism. Nevertheless, it is still difficult to understand why acetylcholine and the choline esters have such different sensitivities to pH. This problem must be resolved in either case, whether pH affects contractions induced by serotonin and the other choline esters by one or several mechanisms.

Whether pH acts by influencing drug binding or by altering the transmission process from the receptor to the contractile elements, acetylcholine and the other choline esters appear to interact with the muscle by somewhat different mechanisms. Carbachol-induced contractions were the most sensitive of contractions induced by the choline esters studied; contractions induced by other choline esters exhibited a range of sensitivities. If there are two mechanisms for interaction of choline esters with airway smooth muscle, carbachol and acetylcholine appear to represent the strongest stimulants of the pH-sensitive and -insensitive systems respectively. The other choline esters studied may stimulate both of these systems to varying degrees, thus producing a range of sensitivities to pH. Several hypotheses may be considered to explain why carbachol and acetylcholine stimulate airway smooth muscle by two separate mechanisms, one sensitive to pH and the other insensitive.

One possibility is that carbachol and other choline esters act in part by stimulating the serotonin receptor (Figure 34). However, the distinctness of these receptors was confirmed by use of specific blocking agents, atropine and methysergide.

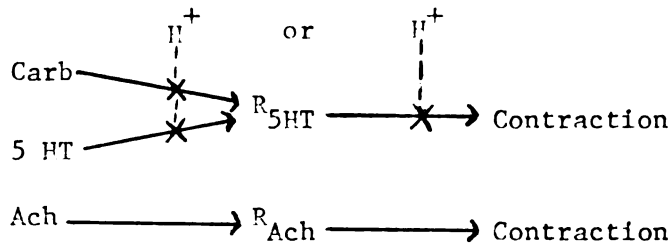


Figure 34: Schematic diagram to illustrate hypothesis that carbachol and serotonin stimulate the same receptor. As indicated, H^+ could act either by altering drug binding or by influencing the transmission process from receptor to the contractile elements. ---X indicates inhibition. R=drug receptor.

Another possibility is that carbachol stimulates the release of acetylcholine by a mechanism that is sensitive to pH (Figure 35).

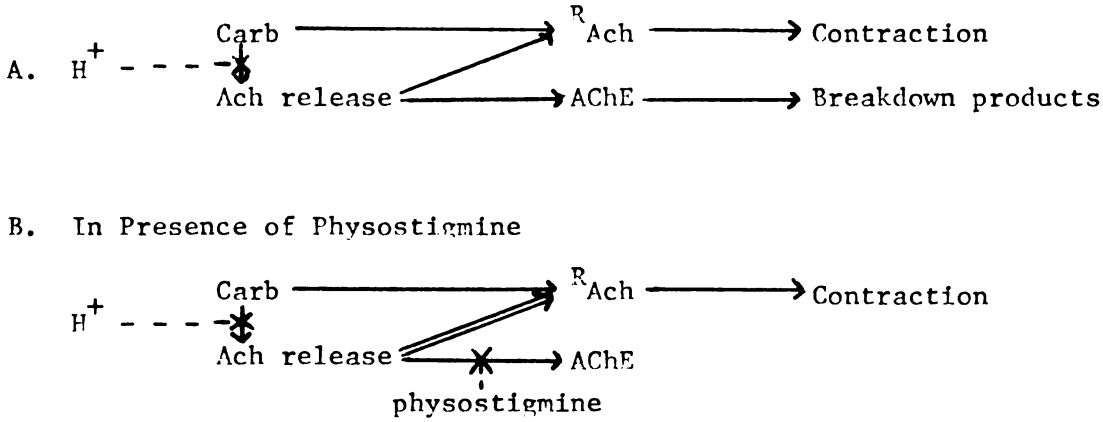


Figure 35: Schematic diagram to illustrate the hypothesis that carbachol stimulates release of acetylcholine, a release mechanism that is inhibited by H^+ (A). The consequences of acetylcholinesterase inhibition by physostigmine are also illustrated (B). ---X indicates inhibition. R=drug receptor. AChE=acetylcholinesterase.

If it were true that carbachol stimulates the release of acetylcholine from tissue stores, then the consequences of acetylcholinesterase inhibition with physostigmine should be predictable. If carbachol acted <u>entirely</u> by release of acetylcholine (no arrow from Carb to ^{R}Ach in Figure 35) then the effect of physostigmine on carbachol-induced contractions should be at least as large as the effect on acetylcholine-induced contractions.

However, physostigmine had much less effect on carbachol-induced contractions than on acetylcholine-induced contractions (Figures 19 and 21). If carbachol acts only partially by release of acetylcholine and partially by directly stimulating the same receptor that acetylcholine stimulates, then physostigmine should potentiate carbachol-induced contractions in 5% CO_2. This is consistent with the findings detailed in the Results and Conclusions. If CO_2 acts by inhibiting the release of acetylcholine the effect of 10% CO_2 would be decreased by physostigmine because in the presence of physostigmine the acetylcholine released would be more effective (Figure 35B). Thus, increased CO_2 would have less effect when acetylcholinesterase was inhibited. However, if this hypothesis were true 20% CO_2 would not be able to completely relax carbachol-induced contractions. Part of the effect of carbachol must be due to direct combination with the acetylcholine receptor; however, the acetylcholine receptor is not sensitive to CO_2 according to this hypothesis. Thus increased CO_2 should never completely relax carbachol-induced contractions. That this is not the case can be seen from the data of Figure 23.

A third possibility is that acetylcholine and carbachol combine with two separate receptor mechanisms. In this case pH could affect either the binding of drug to receptor (Figure 36A) or the transmission process from the receptor to the contractile elements (Figure 36B). Another possibility not illustrated is that acetylcholine and carbachol stimulate the same receptor but the transmission process from the receptor to the contractile elements is different for these two different drugs.

The effect of CO_2 on serotonin-induced contractions was most striking over the physiological pH range. The pH of bronchial muscle could vary

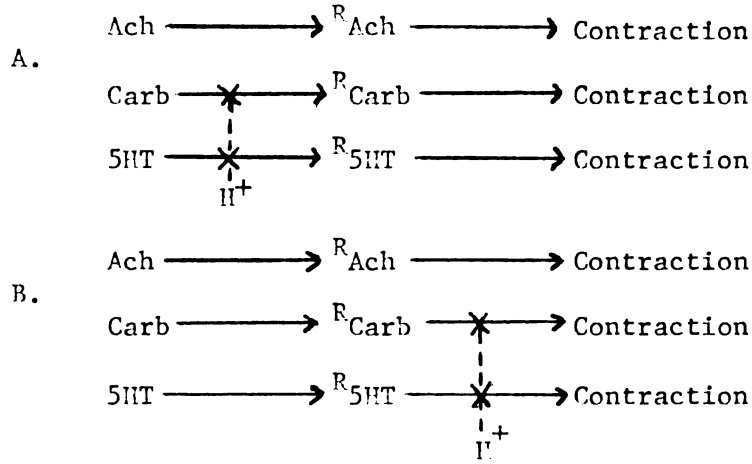


Figure 36: Schematic diagram to illustrate hypothesis that acetylcholine and carbachol combine with two separate receptor mechanisms. ---X indicates inhibition. R=drug receptor.

widely under different conditions of ventilation and perfusion since it may be influenced by both bronchial arterial and intraluminal P_{CO_2}. Muscle pH may have a permissive effect on some drug-induced contractions: when pH is low a stimulating drug may be present but without effect; only when pH increases can the drug produce contraction.

CO_2 has been postulated to play a role in adjustments of ventilation to perfusion (Nisell, 1950; Widdicombe, 1963). Thus if perfusion via the pulmonary arterial system to a part of the lung is interrupted, the CO_2 concentration in the alveoli and small airways of that portion will fall. Since CO_2 can diffuse through the thin airway wall, smooth muscle pH may increase. Thus the effects on smooth muscle of bronchoconstrictor substances released from nerves or other sites will increase, and the small airways and alveolar ducts will constrict. This decreased compliance in the unperfused region will divert ventilation to better perfused areas of the lungs to maintain efficient breathing. Severinghaus et al (1961) have demonstrated such a mechanism in anesthetized dogs. After unilateral pulmonary artery occlusion, ventilation was shifted

away from the unperfused side of the lungs. Such shifts were prevented by inhalation of 6% CO_2 or isoproterenol aerosol on the occluded side which suggests that they were due to increased smooth muscle tone produced by hypocapnia. The rapidity of these effects indicates that short-term effects of CO_2 were probably responsible. The cause of prior airway tone was not clear in these experiments making it difficult to determine if these observations are similar to those reported in this dissertation. Furthermore, peripheral airways are likely to be most involved in adjustments of ventilation to perfusion whereas larger airways were studied in this dissertation. Thus it would be important to determine whether small airways react to CO_2 in the same manner as larger airways.

Studies in man suggest that inhalation of CO_2 affects airway smooth muscle tone. Hypocapnia produced by hyperventilation may increase airway resistance (Newhouse et al, 1964; Sterling, 1968). CO_2 inhalation has also been shown to reverse the bronchoconstriction which follows exercise in certain asthmatics (Fisher et al, 1970). The cause of exercise-induced bronchoconstriction remains unknown: however, it does not seem to be relieved by atropine (Fisher et al, 1970; Chan-Yeung et al, 1971) which suggests that exercise-induced asthma is not mediated by acetylcholine but perhaps by some other bronchoconstrictor substance. Perhaps bronchoconstriction produced by this mediator is sensitive to the direct effect of CO_2.

The selectivity of the CO_2 effect implies that CO_2 will be an effective bronchodilator only in some forms of increased muscle tone. Therefore, the selective effect of short-term exposure to CO_2 may become an effective tool for investigating the control of airway tone in physiologic as well as pathologic states.

B. EFFECTS OF CO_2 ON OTHER TYPES OF MUSCLE

As in airway smooth muscle, CO_2 may act by more than one mechanism in any given tissue. Besides reflex effects mediated by nerves, CO_2 may act directly at several different sites. Activity in vitro of most enzymes is profoundly affected by pH (Webb, 1963); these effects can be due to a number of different mechanisms. Since enzymes or other macromolecules probably make up each of the multiple steps leading to muscle contraction, each of these steps is certainly liable to alteration by change of pH.

Indeed, a wide variety of effects of pH and CO_2 have been demonstrated in various muscles. The spontaneous activity of a muscle can be altered by pH; for example, increased pH decreases the resting potential and conduction velocity of rabbit atria (Vaughan Williams, 1955; Cingolani et al, 1970). Spontaneous activity of other muscles, for example the rabbit ileum, is not sensitive to pH in the physiological range (Reynolds and Hardman, 1972).

The effects of drugs on muscle can also be altered by pH. This can be due to change of ionization of the drug (for example, the effect of epinephrine on the isolated rabbit ileum; Reynolds and Hardman, 1972) or it may be caused by alteration of the process by which the bound drug interacts with the muscle (for example the effects of serotonin on isolated pulmonary arterial strips; Lloyd, 1967).

Since short-term exposure of airway smooth muscle to CO_2 relaxes contractions induced by some drugs but not contractions produced by others, the literature was examined to determine if similar selective effects of CO_2 or pH have been demonstrated in other tissues.

CO_2 relaxes contractions induced by histamine on guinea pig intestine

and by oxytocin on uterus from several species but does not affect contractions produced by acetylcholine, electrical stimulation, or potassium (Halpern et al, 1959; Rocha e Silva, 1960). Rocha e Silva (1960) speculated that an imidazol ring (pKa~7.0) probably formed the active center of the histamine receptor in the guinea pig intestine because of the sensitivity of histamine contractions to pH. However, such speculation seems to be unfounded since it was not established that changes of pH altered the binding of histamine to its receptor.

Selectivity of the effect of CO_2 has also been noted on isolated pulmonary arterial strips (Lloyd, 1967). Serotonin-induced contractions were found to be the most sensitive to CO_2 concentration; although angiotensin- and acetylcholine-induced contractions were affected slightly. Unfortunately the magnitude of these effects was not indicated making it impossible to determine whether they are similar to the findings of this dissertation. Only a few studies have been designed to uncover selective effects of CO_2, and in no case are the reasons for such selectivity understood.

Inhalation of CO_2 has been shown to modulate airway tone in humans in two conditions: voluntary hyperventilation and exercise-induced bronchoconstriction in asthmatics. In addition, CO_2 has been implicated as a controlling factor in peripheral airways, bronchioles and alveolar ducts, in adjustments of ventilation to perfusion. Since CO_2 is known to influence muscle tone in other tissues, the possible physiological significance of some of these effects has been reviewed.

Increased CO_2 concentration can increase blood flow by a direct effect (Kontos et al, 1967; McGinn et al, 1967). Furthermore, sensitivity to stimulating drugs has been shown to decrease with decreased pH

(McGinn et al, 1967). There appears to be general agreement, however, that changes in P_{CO_2} and pH cannot account for local regulation of peripheral blood flow. Reduced pH or increased P_{CO_2} have too weak a dilator effect to be of much significance in functional hyperemia. Other factors, such as extracellular potassium, possible release of vasoactive chemicals, or regional hyperosmolarity, play a more important role in local regulation of peripheral blood flow (Mellander and Johansson, 1968; Haddy and Scott, 1968).

The cerebral vasodilator effects of CO_2 have been emphasized and employed clinically (Fazekas and Alman, 1965). Furthermore changes in cerebral blood flow in man at high altitude have been attributed to changes in P_{CO_2} and pH of cerebrospinal fluid (Severinghaus et al, 1966). In these studies in vivo, it was not determined whether constricting substances were present in the cerebral vascular tissue. Thus it is not known whether pH altered the tone of resting vascular tissue or modified the effects of a vasoconstricting substance in a manner analogous to the effect of pH on airway smooth muscle.

The function of the sensitivity to pH of cerebral vascular smooth muscle may be to preserve the blood flow of the brain in cases of restricted perfusion. Thus build-up of CO_2 from tissue metabolism will decrease pH resulting in dilation of vascular smooth muscle and increased blood flow. This same mechanism could operate rather differently in the lungs. Since lung tissue metabolism may be very small and may contribute relatively little CO_2, decreased perfusion may result in increased rather than decreased pH. A drop in luminal CO_2 concentration because of restricted perfusion via the pulmonary artery would result in increased pH and increased airway smooth muscle tone. Thus inspired air would be

diverted to better perfused portions of the lung. It is interesting that a similar effect of pH may function in two different ways in different tissues.

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