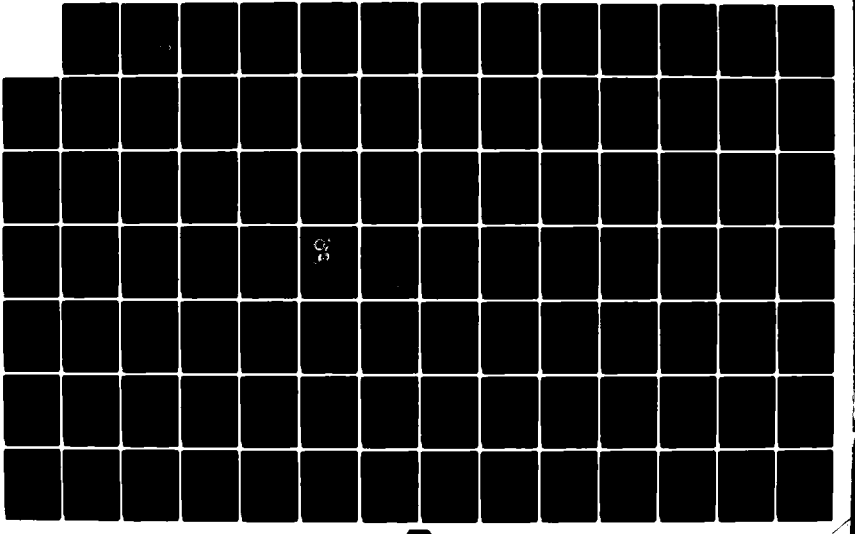
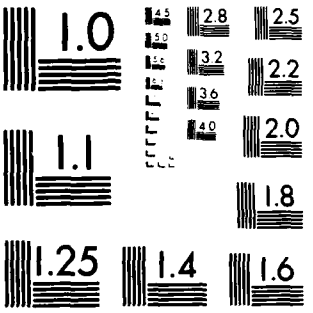
AD-A145 106 MODULATION OF THE CHOLINERGIC MECHANISMS IN THE BRONCHIAL SMOOTH MUSCLE(U) NORWEGIAN DEFENCE RESEARCH ESTABLISHMENT KJELLER P AAS JUN 84 NDRE/PUBL-84/1001 1/2

UNCLASSIFIED F/G 6/16 NL





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MODULATION OF THE CHOLINERGIC MECHANISMS IN THE BRONCHIAL SMOOTH MUSCLE

A THESIS SUBMITTED TO THE UNIVERSITY OF BERGEN
FOR THE DOCTOR SCIENTIARUM DEGREE

BY

PÅL AAS

NDRE/PUBL-84/1001

ISSN 0800-4412

FORSVARETS FORSKNINGSINSTITUTT
NORWEGIAN DEFENCE RESEARCH ESTABLISHMENT
P O Box 25 – N-2007 Kjeller – Norway

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June 1984

NORWEGIAN DEFENCE RESEARCH ESTABLISHMENT (NDRE)
FORSVARETS FORSKNINGSINSTITUTT (FFI)

POST OFFICE BOX 25
N-2007 KJELLER, NORWAY

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE
(when data entered)

REPORT DOCUMENTATION PAGE

1) PUBL/REPORT NUMBER	2) SECURITY CLASSIFICATION	3) NUMBER OF PAGES
NDRE/PUBL-84/1001	UNCLASSIFIED	118
1a) JOB REFERENCE	2a) DECLASSIFICATION/DOWNGRADING SCHEDULE	
FFITOX/465/001	-	

4) TITLE

MODULATION OF THE CHOLINERGIC MECHANISMS IN THE BRONCHIAL SMOOTH MUSCLE
(A thesis submitted to the University of Bergen for the Doctor Scientiarum degree)

5) NAMES OF AUTHOR(S) IN FULL (surname first)

AAS P

6) DISTRIBUTION STATEMENT

Approved for public release. Distribution unlimited
(Offentlig tilgjengelig)

7) INDEXING TERMS

IN ENGLISH:	IN NORWEGIAN:
a) Acetylcholine release	a) Acetylcholin frisetting
b) Presynaptic modulation	b) Presynaptisk modulering
c) Adenosine	c) Adenosin
d) Serotonin	d) Serotonin
e) Peptides	e) Peptider

THESAURUS REFERENCE: -

8) ABSTRACT (continue on reverse side if necessary)

In the present work it was shown that cholinergic nerves to the bronchial smooth muscle were modulated by several independent mechanisms. The release of acetylcholine (Ach) was regulated by presynaptic muscarinic receptors and by adenosine. The presynaptic regulation of release was shown to operate in addition to the postsynaptic stimulation of the bronchial smooth muscle. The function of the cholinergic nervous system in bronchi and lung is depending on the activities of acetylcholinesterase and cholinesterases, which exhibited rather high activities in the tissues. Serotonin, which is stored in and released from pleural mast cells, potentiated the release of Ach and thereby the contraction of the bronchial smooth muscle. In addition to the presynaptic effect there was also a postsynaptic stimulatory response to serotonin. Special attention was paid to the peptide neurotensin, which potentiated both

9) DATE	AUTHORIZED BY	POSITION
7 Jun 84	This page only E Klippenberg	Director

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE
(when data entered)

ABSTRACT (continued)

the release of Ach and stimulated the muscle to contract by postsynaptic receptors. Further, NT specifically induced release of serotonin and histamine from rat pleural mast cells.

ACKNOWLEDGEMENTS

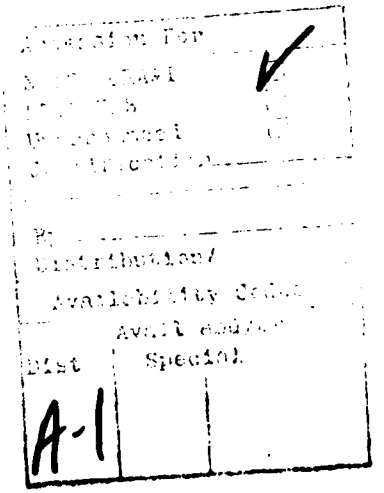
The present work has been carried out at the Department of Physiology, University of Bergen, 1980, and in the Norwegian Defence Research Establishment (NDRE), Division for Environmental Toxicology, Kjeller, during the years 1981 to 1984.

I would like to express my sincere gratitude to Professor Dr Philos Karen B Helle in the Department of Physiology, University of Bergen and to the Head of the Division for Environmental Toxicology, NDRE, Dr Philos Frode Fonnum for their excellent supervision and continuous support during my work.

Dr Karen B Helle and Dr Frode Fonnum constituted the advisory group for this thesis.

And my thanks are also extended to cand real Viggo M Fosse and cand real Sigrun H Sterri, Division for Environmental Toxicology, NDRE, for their collaboration and helpful discussions during my work.
At last I would also like to thank all my colleagues for a supporting and friendly attitude.

The work was supported by a one year Doctor Scient grant from the Norwegian Research Council for Science and the Humanities, 1983-84.



In memory of my mother and father.

CONTENTS

The thesis is based on the following publications, which will be referred to in the text by Roman numerals:

PAPER I: Aas, P. and Helle, K. B. 1982. Neurotensin receptors in the rat bronchi. Regulatory Peptides 3, 405-413.

PAPER II: Krüger, P. G., Aas, P., Onarheim, J. and Helle, K. B. 1982. Neurotensin-induced release of histamine from rat mast cells in vitro. Acta Physiol Scand 114, 467-469.

PAPER III: Aas, P. 1983. Serotonin induced release of acetylcholine from neurons in the bronchial smooth muscle of the rat. Acta Physiol Scand 117, 477-480.

PAPER IV: (manuscript)
Aas, P. and Fonnum, F. 1984. Presynaptic muscarine and adenosine receptors inhibiting evoked release of acetylcholine from nerves in the rat bronchial smooth muscle. Eur. J. Pharmacol.

PAPER V: (manuscript)
Aas, P. 1984. Neurotensin and serotonin modulation of acetylcholine release from cholinergic nerves in the rat bronchi. Br. J. Pharmacol. (submitted).

PAPER VI: (manuscript)
Aas, P., Sterri, S. H., Hjermstad, H. P. and Fonnum, F. 1984. A method for generating toxic vapours of soman; toxicity of soman by inhalation in rat. Toxicol. Appl. Pharmacol. (submitted).

SUMMARY

In the present work it was shown that cholinergic nerves to the bronchial smooth muscle were modulated by several independent mechanisms. The release of acetylcholine (Ach) was regulated by presynaptic muscarinic receptors and by adenosine. The presynaptic regulation of release was shown to operate in addition to the postsynaptic stimulation of the bronchial smooth muscle. The function of the cholinergic nervous system in bronchi and lung is depending on the activities of acetylcholinesterase and cholinesterases, which exhibited rather high activities in the tissues. Serotonin, which is stored in and released from pleural mast cells, potentiated the release of Ach and thereby the contraction of the bronchial smooth muscle. In addition to the presynaptic effect there was also a postsynaptic stimulatory response to serotonin. Special attention was paid to the peptide neurotensin, which potentiated both the relase of Ach and stimulated the muscle to contract by postsynaptic receptors. Further, NT specifically induced release of serotonin and histamine from rat pleural mast cells.

INTRODUCTION

Historical background

The intercellular communication in the nervous system involves the release of chemical neurotransmitters, suggested first by Elliot (1904), Langley (1905) and by Dale (1906). The concept of chemical transmission was established somewhat later by Loewi (1921), discovering that the vagus nerve released a substance, "Vagusstoff", later to be identified as acetylcholine (Ach) (Loewi and Navratil, 1926). Further support for his hypothesis was given by Dale and Dudley (1929) who found Ach to be a naturally occuring substance in mamalian tissues. The observation of Dale (1914) that the actions of Ach was mimicked by muscarine and nicotine, was the first indications of two different types of Ach receptors.

Nachmansohn (1940) proposed that the cholinergic nervous system required cholinesterases (ChE) for its normal function and that cholinesterases was located in parallel to Ach. The enzyme later identified as acetylcholinesterase, hydrolyse the ester-bond in acetylcholine with higher specificity than non-specific cholinesterase (also called pseudo-cholinesterase) (Augustinsson, 1948; Whittaker, 1951). Somewhat later Gray and Whittaker (1962) were able to isolate and identify nerve terminals as small discrete particles, later named synaptosomes, after homogenization of brain tissue under carefully controlled conditions. When synaptsomes were submitted to hyposmotic conditions, they lysed and from the lysate it was possible to isolate a large number of synaptic vesicles which was shown to contain Ach (Whittaker *et al.*, 1963; Whittaker and Sheridan, 1965). The discovery of the synaptosomes has played a major role in many of the subsequent studies on Ach release. Whether Ach is released from the synaptic vesicles (Whittaker *et al.*, 1963) or from the synaptosomal cytoplasm (cf. Israël *et al.*, 1979) is still a matter of much controversy. On the

other hand the synaptsomes were found to contain the synthesizing capacity for Ach (Fonnum, 1967), and the enzyme choline acetyltransferase was shown to be localized to the synaptosomal cytoplasm of the nerve terminals.

MacIntosh and Oborin (1953) were the first to demonstrate an increase in the output of Ach from cat cerebral cortex in vivo by injection of atropine. This was the first indication for an autoregulation via muscarinic receptors of Ach release, although other explanations were also plausible. Later, presynaptic muscarinic receptors have been detected and studied in great detail in several cholinergic synapses which also contain postsynaptic muscarinic receptors.

The inhibitory presynaptic autoreceptors occur on cholinergic nerves in myenteric plexus, parasympathetic nerves of heart and iris (Sawynok and Jhamandas, 1977; Kilbinger and Wagner, 1979; Gustafsson et al., 1980; Alberts et al., 1982) as well as on central cholinergic neurons in cortex, hippocampus and striatum (Polak, 1971; Szerb, 1979; Molenaar and Polak, 1980). Several of the studies, comprising a number of observations, show an increase in the release of Ach when the muscarinic antagonists atropine or scopolamine are present, while on the other hand a decrease in transmitter release is apparent after addition of the muscarinic agonist oxotremorine.

Presynaptic Ach receptors were first reported to occur on noradrenergic terminals. Stimulation by Ach and other muscarinic agonists decreased the evoked release of noradrenaline from noradrenergic terminals (cf. Löffelholz and Muscholl, 1970; Stjärne, 1975; Muscholl, 1979).

In order to quantitate the actual output of endogenous Ach from cholinergically innervated tissues it is necessary to inhibit the cholinesterase activites of the tissue. Cholinesterase inhibitors, however, cause an accumulation of Ach in the synaptic cleft, which in turn decreases the stimulus evoked release of Ach (Szerb, 1979). It was therefore a step forward, when Szerb suggested that Ach release could be studied in the absence of a cholinesterase inhibitor from the radioactive release after preincubation cholinergically innervated tissue in [^{3}H]-choline (Szerb, 1976).

In general acetylcholinesterase is specific for neural tissue, while non-neural tissue usually contain non-specific cholinesterases. However, this is a generalization, some neural tissues contain both esterases as do some extraneural organs, e.g. lung and liver. Carboxylesterases (synonymous to aliesterases) are on the other hand present in neural and non-neural tissues, but are not involved in the hydrolyzis of Ach. However, they have in general a broad specificity towards carboxylesters and they, as well as cholinesterases, are inhibited by organophosphorus compounds (cf. Heyman, 1980). The carboxylesterases have shown to be involved in several detoxification pathways and they are specially important in the inactivation of the organophosphorus anticholinesterases. This mechanism is presumably a part of the detoxification process in lung.

Following the discovery of non-adrenergic, non-cholinergic inhibitory nerves (cf. Burnstock, 1972; Fredholm and Hedqvist, 1980) it has been proposed that the neurotransmitter of these nerves, also present in lung, may be ATP or a closely related purine. The nature of the neurotransmitter(s) have been a matter of much controversy. However, adenosine and the adenine nucleotides have shown to stimulate postsynaptically but recently they have also shown to be active presynaptically (Sawynok and Jhamandas, 1976; Vizi and Knoll, 1976) to decrease the spontaneous release of Ach from Auerbach's plexus of guinea-pig ileal longitudinal muscle in a dose-dependent manner. The origin of ATP and the related purines has been a subject of discussion, but adenosine is probably derived from ATP, which is costored and released with Ach into the synaptic cleft, in several peripheral nerve terminals (Silinsky, 1975). On the other hand ATP has also been looked upon as a transsynaptic modulator, originating from the postsynaptic cell (Israël et al., 1980). Burnstock (1979) proposed that adenosine may have its function in parallel to Ach, acting as a neuromodulator on a presynaptic level. In vitro studies have confirmed previous hypothesis and purines, especially adenosine, reduce the contraction induced by cholinergic nerve stimulation in the

guinea-pig ileum (Gustafsson et al., 1978). The inhibition is most likely presynaptic, since it was accompanied by diminished release of Ach (Hayashi et al., 1978; Silinsky, 1984).

The adenosine antagonist, theophylline, enhance the release of Ach induced by stimulation (Cook et al., 1978) from Auerbach's plexus and from brain cortical slices, indicating that endogenous adenosine might modify the release of Ach. Although theophylline antagonizes several of the responses to adenosine, it is yet to be clarified whether theophylline is a specific antagonist for the adenosine receptor (McIlwain, 1972), or merely an inhibitor of phosphodiesterase activity (Fredholm, 1980).

Other neurotransmitter candidates for non-cholinergic, non-adrenergic neurotransmission are the increasing number of biologically active peptides, present in lung (Said et al., 1980). These include vasoactive intestinal polypeptide (VIP), substance P, bradykinin, cholecystokinin, somatostatin and others. Carraway and Leeman (1976) have also by radioimmunoassay characterized the tridecapetide neurotensin (NT) (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-COOH) in the rat, and only 10% of the peptide was located to the brain, while 90% was found in extracts of the body. NT was mainly located to neuroendocrine cells in the small intestine, but NT immunoreactivity was also present in the lung. Whether NT is present in neuroendocrine cells or in nerve cells, as is shown in the subcortical limbic forebrain including the nucleus accumbens (Jennes et al., 1982) or reach the lung via plasma as a hormone, has to be elaborated. Specific binding sites for NT in lung tissue are found on the pleural mast cells and a NT induced release mechanism of biogenic amines from mast cells has been suggested (Lazarus et al., 1977; Selbekk et al., 1980; Ouirion et al., 1980b; Sydbom, 1982; Paper II).

It has long been known that histamine induce severe bronchocontriction in man (Benson, 1978) and guinea-pig (Bhattacharya, 1955), while serotonin (5-hydroxytryptamine) has a smaller effect on the bronchi in these mammals. In other mammals the situation is different; Offermeier and Ariëns (1966) suggested that serotonin either induce broncho-

constriction through a reflex mechanisms or through a direct stimulation of the bronchial smooth muscle isolated from calf. Similar results were found for the dog (Hahn et al., 1978). Furthermore, serotonin may induce contractions in other smooth muscle preparations as in viceral smooth muscle such as ileum, by release of Ach through a tetrodotoxin sensitive mechanism (Ádám-Vizi and Vizi, 1978). Therefore, serotonin may serve as a modulator of cholinergic neurotransmission in several tissues of different mammals.

Aim of the present study

The present investigation was carried out with the aim to characterize the cholinergic mechanisms in the bronchial smooth muscle and particularly to study the modulation of Ach-release by other neurotransmitters and hormones. Previous investigations of the innervation of airway smooth muscle have mainly been performed on preparations in tracheal and bronchial tissues from the dog and the guinea-pig while virtually no information was available for the rat.

In addition I was interested in providing a basis for further studies of cholinergic function of the rat airway smooth muscle during inhalation studies. In this respect, the inhalation of the organophosphorus anticholinesterases and their inhibition of the cholinesterase activities and how the organophosphorus compounds are detoxified by carboxcylesterases were investigated. This is of importance since acetylcholine has shown to be the most potent constrictor of the rat airway smooth muscle (Paper I).

RESULTS AND DISCUSSION

The results presented in this study have revealed several mechanisms which may modulate the cholinergic induced bronchoconstriction in the rat (Figure 1, page 23). These modulators are chemically different and are either released from nerve terminals within the bronchi (Ach and adenosine), from pleural mast cells (serotonin), or blood-borne as hormones (neurotensin).

Acetylcholine

It is previously shown that the parasymphathetic nerve of the autonomic nervous system is the primary motor control nerve to the smooth muscle in the airways in mammals (Widdicombe, 1963) and this is also the case for the rat. The muscarinic acetylcholine (Ach) receptor appeared to be the only cholinergic receptor located postsynaptically in the rat bronchi (Paper I). Contraction of the circular preparation by exogenous Ach or the cholinergic agonist carbachol induced a concentration dependent and atropine-sensitive increase in the tension (Paper I). The contraction induced by electrical stimulation was sensitive to tetrodotoxin, but was also, as the response to the cholinergic agonists inhibited by atropine. This is a strong evidence for an electrically induced release of Ach from cholinergic nerves in the bronchi. The intrinsic activity (α) for Ach and carbachol was similar, but the potency (affinity) of carbachol (pD_2=6.1), was considerably higher than for Ach (pD_2=3.7). This difference is most probably due to hydrolysis of Ach by the relatively high cholinesterase activities measured in the bronchial tissue (Paper VI, Table 2). Although the potency of carbachol was high in the bronchi and similar to what was found by Vornanen and Tirri (1981), it was higher than the potency for the cholinergic agonists in the trachea and bronchioles.

The relatively high activities of the cholinesterases (acetylcholine-

esterase and unspesific cholinesterases) (Paper VI) also support our conclusion of the cholinergic nervous system being of main importance in the motor control of the rat bronchial smooth muscle.

Although Ach induced contractions in the bronchi, Ach also appeared to stimulate muscarinic receptors presynaptically, thereby autoregulating the release of Ach from the cholinergic terminals (Paper IV, Figure 4). This autoinhibition of the Ach release in the rat bronchi was demonstrated by techniques similar to those used for other preparations (cf. Stjärne, 1975; Szerb, 1976,1979; Vizi, 1979; Gustafsson et al., 1980; Molenaar and Polak, 1980; Alberts et al., 1982; Kilbinger, 1982). In order to avoid the unphysiologically high concentrations of extracellular Ach resulting from the presence of a cholinesterase inhibitor, the release of Ach was best determined as [^{3}H]-choline released from tissue preincubated in [^{3}H]-choline in the absence of a cholinesterase inhibition according to Szerb (1976,1979).

The muscarinic antagonist, scopolamine, potentiated the calcium dependent potassium evoked release of [^{3}H]-Ach by nearly 60%, whereas the muscarinic agonist, oxotremorine, reduced the output of [^{3}H]-Ach by only 20% (Paper IV, Figure 4). This is a strong indication for the existence of presynaptic muscarinic receptors, which modulate the release of Ach. The oxotremorine reduced release of [^{3}H]-Ach upon stimulation was not mediated by a diminished synthesis, but was presumably induced by stimulation of presynaptic muscarinic receptors. [^{3}H]-Ach was synthesized in the cholinergic terminals during the preincubation, and not during the superfusion in the presence of hemicholinium-3. On the other hand, an increase in potassium stimulated release of [^{3}H]-Ach by scopolamine was expected if there was a functional muscarinic receptor present. However, it is impossible to decide from these experiments whether the muscarinic receptors were located on the axons or on the cholinergic terminals.

It has previously been shown (Alberts et al., 1982) that the potassium evoked, calcium-sensitive increase in [^{3}H] efflux mainly consisted of [^{3}H]-Ach when [^{3}H]-choline was used as a precursor for the synthesis of [^{3}H]-Ach in the guinea-pig myenteric plexus.

Hemicholinium-3 was present in the superfusing medium to block the reuptake of [^{3}H]-choline, i.e. the hydrolysis product of [^{3}H]-Ach if no esterase inhibitor was present. Under these conditions, the fractional increase in efflux of [^{3}H], i.e. (evoked increase in [^{3}H] efflux/total [^{3}H] in the tissue) was used to estimate the evoked secretory response. When the esterase inhibitor soman is present, the total increase in Ach efflux is a direct measure of the potassium induced transmitter release. However, in the presence of soman, one would expect a presynaptic autoregulation reducing the output of Ach from the cholinergic terminals.

In the present study (Paper IV), the release of [^{3}H]-Ach was reduced 85% when changing the concentration of Ca^{2+} from 2 mM to 0.1 mM, demonstrating a Ca^{2+}-dependent release mechanism. Tetrodotoxin (TTX), which blocks the electrical excitation of the axons but not the release of transmitter substance from the depolarized nerve terminal, did not significantly lower the potassium evoked release of [^{3}H]-Ach. The release of [^{3}H]-Ach was also induced by veratridine, which acts through activation of sodium channels. TTX, by blocking the sodium channels, inhibited the veratridine induced release without affecting the K^+ depolarized release (Paper IV, Figure 3).

These findings strengthen our assumption that the stimulation of Ach occurs from a physiological releaseable pool of transmitter, also suggested by Szerb (1976). Therefore the modulation of Ach release from cholinergic nerves can be studied by measuring the fractional increase in [^{3}H] efflux as a relevant measure of [^{3}H]-Ach secretion, both because of its sensitivity and reproducibility, and because it can be used to assess the Ach release also in the absence of an esterase inhibitor (Szerb, 1979). Therefore the present results strongly suggest that the cholinergic terminals in the preparation were intact and functionally active, by fulfilling several of the most common requirements for an intact cholinergic system.

Choline- and carboxylesterases

The activities of acetylcholine-, choline- and carboxylesterases were shown to be relatively high in both lung and bronchi (Paper VI, Table 2). This reflects the importance of the cholinergic nervous system in this tissue, and lung as a primary target organ for volatile anticholinesterase vapours. Previous work (Fonnum et al., 1984) have shown that even very small concentrations (nM) of the organophosphorus anticholinesterase compound, soman, increased substantially the response to cholinergic stimulation and therefore the contraction of the bronchial smooth muscle.

The importance of the carboxylesterases for inactivation of cholinesterase inhibitors during inhalation experiments with organophosphorus compounds are also clearly shown by the 70% decrease in lethal dose by inhalation of soman in the presence of tri-ortho-cresyl-phosphate (TOCP) (Paper VI, Table 3). TOCP has shown to be a specific inhibitor of the carboxylesterase activities (Myers, 1959; Sterri et al., 1981). During inhalation of a cholinesterase inhibitor there is a dose-dependent decrease in the total cholinesterase and carboxylesterase activities (Paper VI, Table 2). At high concentrations of soman the acetylcholine- and cholinesterase activities were inhibited and the carboxylesterase activities were also substantially reduced by binding of the organophosphorus compound. This is in accordance with the general effect of this toxic agent (Heymann, 1980), and carboxylesterases as important enzymes in detoxification of anticholinesterase.

Adenosine

Modulation of cholinergic neurotransmission by exogenous adenosine on responses to potassium induced release of Ach from cholinergic nerves in the rat bronchi were also assessed.

ATP presumably coreleased with Ach (Silinsky, 1975) may act as a

neurotransmitter, or as its hydrolysis product adenosine, also operate as a modulator of the cholinergic transmission at a presynaptic level (Burnstock, 1979). With adenosine present in the superfusing medium the reduction in the potassium evoked release of [^{3}H]-Ach was nearly 30% (Paper IV, Table 1). Although theophylline was without an effect on the resting release of Ach in the bronchi, theophylline (1.0 mM) reduced slightly (10%) the response to adenosine on the potassium induced release of transmitter. This indicates that there might be a control of Ach release by adenosine. This antagonizing effect of theophylline on the effect to adenosine and adenine nucleotides has also been shown in a number of other tissues (Daly, 1977; Gustafsson et al., 1981). These findings are contradictory to earlier results presented by Ginsborg and Hirst (1972) for the rat phrenic nerve showing that the adenosine effect was completely antagonized by theophylline (1.8 mM). Whether the effect of theophylline is that of a receptor blocker or resulting from the inhibition of the phosphodiesterase activity is uncertain. Fredholm et al. (1979) demonstrated, however, a tracheorelaxant effect of theophylline. The effect was dose-dependently inhibited by adenosine and increased in the presence of dipyridamol, possibly by inactivating the uptake and thereby the inactivation of adenosine (Daly, 1977). This therefore implies an action of adenosine on the cell membrane rather than inside the cell.

Adenosine induced an increase in the contraction in the circular preparation of the rat bronchi in vitro (Paper IV, Table 2). The onset of the response was rapid and disappeard almost immediately upon washing. A similar effect was observed by adenosine in the guinea-pig trachea (Fredholm et al., 1979), where the concentration of adenosine giving maximal response was 50 µM. When adenosine was added repeatedly to the preparations, a tachyphylactic effect was observed. On the other hand when stimulating the bronchial smooth muscle to contract electrically in the presence of adenosine, there was a large increase in the response compared to a control stimulation. The results therefore suggests two opposing effects of adenosine, one presynaptic adenosine receptor mechanism, which reduce the output of Ach during

nerve activity and another postsynaptic potentiation of the Ach-induced contraction. The responses operate most likely in parallel to the pre- and postsynaptic effect of Ach. The dual effect of adenosine is analogous to that described for the gastric smooth muscle by Gustafsson (1981). The postsynaptic potentiation may be due to a synergism between the muscarinic and adenosine receptors on the bronchial smooth muscle cells or that adenosine enhances the depolarization induced by Ach. Since adenosine only to a minor degree altered the response of the bronchial smooth muscle to stimulation with exogenously added Ach (Paper IV), in agreement with results from other cholinergic synapses (Vizi and Knoll, 1976), the results therefore indicate that adenosine interfers with nerve stimulation and the Ach released from the cholinergic nerves.

Serotonin

Contrary to previous studies on isolated airway smooth muscle preparations in guinea-pig and dog (Hahn et al., 1978) serotonin, but not histamine induced contration in the bronchial smooth muscle of the rat (Paper III). It is known from earlier work that under conditions in which histamine is released, the rat mast cells also release serotonin (Parrat and West, 1957; Carlsson and Rizén, 1969). The intrinsic activity of serotonin was low (20%) compared to carbachol (Paper I, Table 1) but the potency (pD_2=5.7) was somewhat higher than for Ach (pD_2=3.7). The contractions were partly atropine sensitive (Paper III, Table 1) and potentiated by inhibiting the acetylcholinesterase and cholinesterase activities with physostigmine. Physostigmine induced a two-fold increase in the intrinsic activity and enhancement of the potency of serotonin in the unstimulated preparations. The potentiation by physostigmine of the serotonin response was blocked by atropine. In addition, there was a potentiation by serotonin of the electrically induced contraction.

The results strongly indicate that a serotonin effect was present on the cholinergic terminals in the bronchial smooth muscle. This

specific serotonin induced release of Ach was further shown by the potentiation of the potassium evoked release of [^{3}H]-Ach (Paper V, Table 1). This potentiation was blocked by the serotonin antagonist methysergide and the methysergide sensitive serotonin induced *in vitro* contraction of the smooth muscle was on the other hand only partially blocked by atropine. The results therefore indicate the existence of specific postsynaptic serotonin receptors which was stimulated in parallel to the presynaptic receptors. A similar presynaptic serotonin effect was previously also shown on cholinergic nerves in the isolated guinea-pig ileum (Brownlee and Johnson, 1965).

Methysergide did, on the other hand, not have any inhibitory effect on the stimulation induced by Ach or by carbachol (Paper III, Table 2) and serotonin did not induce any tachyphylaxis in the tissue, providing further evidence for a specific serotonin receptor mechanism.

Therefore, the present findings indicate that serotonin is the only amine of the rat mast cells to have an effect on the tonus of bronchial smooth muscle and that the effect of serotonin appears to be mediated via pre- and postsynaptic receptors.

Peptides

The potentiation of the Ach-release and stimulation of the smooth muscle by specific serotonin receptors is probably also potentiated indirectly via neurotensin (NT). The tridecapepeptide NT induced release of the amines, serotonin and histamine, from the rat pleural mast cells whereas only a small release-induced effect on mast cells isolated from the peritoneum was found (Paper II). The release seems to be specific for NT. The release of histamine as a measure of the total mast cell release was 4 times larger from pleural mast cells than from peritoneal mast cells (Paper II, Table 1).

A difference in response to NT on degranulation of mast cell granules was found by studies in the light-microscope and in the electron-microscope. The morphological changes of the electron dense granules appeared to be smaller than the histamine response. On the other hand,

the degranulation of the mast cells was larger in the cell population isolated from the pleural cavity compared to the degranulation of cells from the peritoneal cavity. The results also indicate a difference between the mast cells in the two populations towards NT-sensitivity. This may be due to differences in the NT-receptor coupling in the two subpopulations of mast cells. However, similar potency of NT on the isolated pleural mast cells as in the rat bronchi were observed (Papers I,II), but somewhat higher than for the peritoneal mast cell population.

NT appeared not only to stimulate the pleural mast cells to release serotonin and histamine, but also to a significant extent, potentiate the release of Ach from the cholinergic nerves in the bronchi. This presynaptic effect occured in parallel to the postsynaptic effect of neurotensin (Papers I, V). Although bradykinin (BK) and angiotensin II (ANG II) as well as NT stimulated the electrically induced release of Ach, NT and ANG II were the most potent of these peptides. By itself this effect is not sufficient evidence for the existence of a presynaptic receptor mechanism, but the NT induced enhancement of potassium induced [^{3}H]-Ach release (Paper V, Table 1) strengthen our postulation of presynaptic receptors for NT in this tissue.

A presynaptic effect of NT on cholinergic terminals has also been reported in guinea-pig ileum. NT potensiated the electrically induced contraction and the effect was observed in parallel to a contraction induced via postsynaptic NT receptors (Regoli, 1982). An inhibitory effect of NT on the release of noradrenaline was on the other hand observed in rat vas deferens (Magnan, 1979).

The potentiation of potassium evoked release of [^{3}H]-Ach was not inhibited by (D-Trp^{11})-NT. This peptide has previously shown to have an antagonistic effect on the response to NT in the μM range in rat portal vein and heart (Quirion et al., 1980c; Rioux et al., 1980), but not in rat stomach smooth muscle or in atria isolated from guinea-pig hearts (Rioux et al., 1980; Quirion et al., 1980a). Although (D-Trp^{11})-NT was without an effect on the NT potentiated Ach release, there is no reason to exclude a specific NT receptor on the cholinergic terminals.

Although atropine reduced the response to NT, neither atropine nor methysergide blocked the response to NT. This provides further indications of an independent receptor for NT in the bronchi. The reduction of the NT induced response by atropine could be due to tachyphylaxis under *in vitro* conditions, but it may also be a result of a partly atropine sensitive contraction induced by NT.

Despite the marked tachyphylaxis to the peptides (NT, BK, ANG II) the effects were additative, when maximal concentrations of NT and the two other peptides were added in concert. Moreover Sar^1-Ala^8-ANG II (saralasin), inhibited the ANG II induced contraction without blocking the response to NT. This also strongly suggest separate receptor mechanisms for these systemic peptides.

In contrast, substance P and vasoactive intestinal polypeptide (VIP) had no effect in the bronchial smooth muscle (Paper I), although their presence in lung have been shown (Polak and Bloom, 1982). But substance P may on the other hand have important functional implications in view of the postulate that substance P is a sensory neurotransmitter (Otsuka *et al.*, 1975).

The peptides had low intrinsic activities in the bronchial smooth muscle compared to carbachol (NT,α=0.1), but the intrinsic activity of NT was 40% of the activity of serotonin (Paper I, Table 1). The potency for NT was, on the other hand, higher than for both cholinergic agonists. At the present time it is therefore difficult to understand a role for NT in bronchi and lung under normal conditions. This is also due to the low NT-immunoreactivity that has so far been identified in the lung. But one cannot exclude that NT can be present in the vagus nerve among a number of other peptides already shown to be located in this nerve (Lundberg *et al.*, 1980) and thereby modulate the release of Ach and other neurotransmitters.

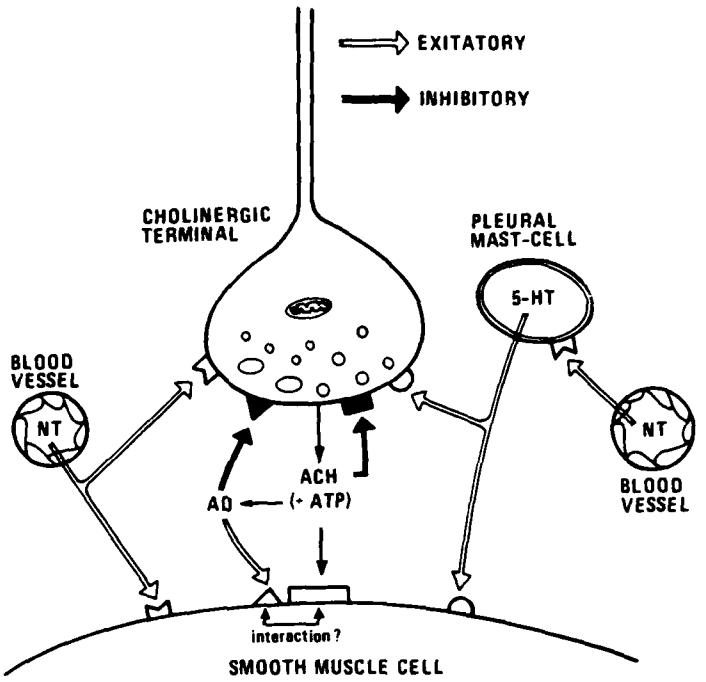


Figure 1 The figure summarizes the mechanisms regulating the cholinergic transmission in the bronchial smooth muscle of the rat. The cholinergic terminal has inhibitory receptors for acetylcholine (Ach) and adenosine (AD) (Paper IV), while exitatory receptors for serotonin (5-HT) (Paper III, V) and neurotensin (NT) (Paper I, V) have been found. NT may also stimulate the pleural mast cells to 5-HT release (Paper II). Exitatory postsynaptic receptors for NT are also present (Paper I) in addition to postsynaptic receptors for Ach (muscarinic receptors), 5-HT and adenosine.

CONCLUSIONS

1 The bronchial smooth muscle of the rat were without spontaneous activity and acetylcholine (Ach) was the primary neurotransmitter inducing constriction.

2 There was a high acetylcholinesterase and unspecific cholinesterase activity in the bronchi and lung and therefore a rapid hydrolysis of Ach. The low potency of Ach is probably due to the high activities of the enzymes.

3 Carboxylesterases in lung and bronchi play an important role in the detoxification of organophosphorus anticholinesterases and thereby modify the toxic effects of Ach during organophosphate intoxication.

4 There is evidence for a presynaptic muscarinic autoreceptor for Ach, reducing the output of Ach from cholinergic nerve terminals.

5 Exogenous adenosine reduced the release of Ach by a presynaptic action, but enhanced the contraction induced by Ach postsynaptically. The postsynaptic potentiation may be due to a coupling between the adenosine and muscarine receptors.

6 Serotonin, but not histamine, induced contraction of the bronchial smooth muscle by stimulating postsynaptically in addition to potentiate the release of Ach by a presynaptic effect. The effects of serotonin were methysergide sensitive.

7 Neurotensin (NT) increased the electrical and potassium evoked release of Ach. The results indicate therefore a presynaptic modulation of Ach release by NT. The potassium induced release of Ach was not antagonized by (D-Trp^{11})-NT, a possible NT-antagonist.

8 NT induced contraction in the bronchial smooth muscle by a postsynaptic receptor different from the receptors for angiotensin II and bradykinin. Substance P and VIP were without effects both pre- and postsynaptically.

9 There is also strong evidence for specific NT receptors on mast cells by which NT induce release of histamine and serotonin. The response to NT was larger for pleural mast cells than for mast cells isolated from the peritoneum.

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PAPER I

Regulatory Peptides, 3 (1982) 405–413
Elsevier Biomedical Press

Neurotensin receptors in the rat bronchi

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(Received 8 February 1982; accepted for publication 4 March 198.)

Summary

The isolated, circular preparations of the left and right bronchi of the rat were examined for mechanical responses to neurotensin (NT) and other vasoactive peptides. NT caused concentration-dependent increases in the isometric tension of the unstimulated preparations, with an apparent affinity higher than for the cholinergic agonists but with considerably lower intrinsic activity. Pronounced tachyphylaxis to NT was observed. NT potentiated the atropine-sensitive increase in tension resulting from electrical field stimulation. Neither atropine nor methysergide abolished the response to NT in the unstimulated preparations. Bradykinin and to a lesser extent angiotensin II contracted the unstimulated preparations and both systemic peptides enhanced the cholinergic output in response to field stimulation. Substance P and VIP on the other hand were without effects in the stimulated and unstimulated bronchi. The results are consistent with the presence of receptors for NT on the presynaptic cholinergic terminals as well as on the post-synaptic smooth muscles of the rat bronchi.

respiratory; smooth muscle; bradykinin; angiotensin; substance P; VIP; serotonin; cholinergic; presynaptic

Introduction

Neurotensin (NT) is a tridecapeptide which was first isolated from its hypothalamic location and later shown to be one of the conspicuous components of the intestinal mucosa [2,3]. In the rat about 95% of the total amount of NT occurs in the intestinal cells and this peptide is also a normal constituent of plasma [3]. Moreover,

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a number of rat organs besides the viscera respond to NT, indicating that receptors for NT are present in the atria [10,18] and coronaries [11] as well as in the portal vein [4,15]. NT also induces a release of histamine selectively from the pleural mast cells [7], indicating that NT may have indirect as well as direct effects in the lungs. It is however not yet established whether the bronchial smooth muscle has primary receptors for NT. A further survey of the distribution of NT receptors outside CNS and the visceral region is of particular interest in view of the postulated hormonal role for the circulating fraction of this peptide [16].

It has therefore been the aim of the present study to search for NT receptors in the isolated bronchi of the rat and to characterize these relative to other peptide receptors in this tissue. The results, briefly reported elsewhere [5], indicate that NT receptors occur not only on the bronchial smooth muscle but also on the postganglionic, cholinergic terminals in the bronchial wall.

Materials and Methods

Female Sprague-Dawley rats (250–300 g) were used. The animals were killed by a heavy blow on the head. The left and right bronchi (wet weight ca. 0.02 g) were mounted in parallel as circular preparations on hooks made from cannula (Fig. 1). The thermostatically controlled organ bath contained Krebs solution (50 ml, 37°C) of the following composition (in mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.6; $MgSO_4$, 1.2; $NaHCO_3$, 24.9; KH_2PO_4, 1.2; glucose, 11.1. The solution was gassed with 95% O_2 + 5% CO_2 (pH 7.4).

For electrical stimulations the bronchi were mounted between platinum electrodes

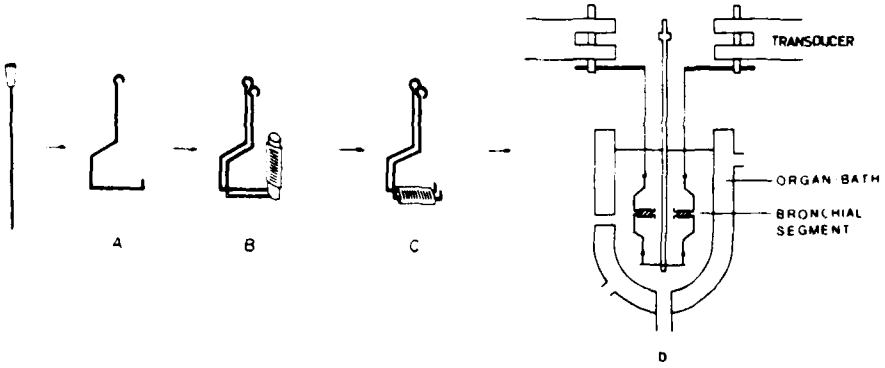

Fig. 1. The isolated, circular bronchial preparation. The following stages in mounting are schematically illustrated. (A) hooks made from cannula (0.7 mm diameter, 25 mm length); (B) a polyethylene catheter was inserted into each of the bronchial branches to support the tissue during transfer over the tips of the two hooks, (C) the bronchial segment in position; and (D) the left and right bronchial branches mounted in parallel in the organ bath. The lower hooks were fixed to side hooks on the supporting rod while the upper hooks were connected by fine silk sutures to the force displacement transducers.

(8 mm apart). These were stimulated via a Grass S44 Stimulator. The voltage across the preparations was 3.5–5 V. At a duration of the square wave pulses of 8 ms and the half maximally effective frequency was 8 Hz.

Each preparation was given a preload of 1.0 g and equilibrated for at least 60 min before the drug experiments. The contractile tension was recorded isometrically via a Grass Force Displacement Transducer (FT03) and monitored via a Grass Polygraph (RPS 7A8A) fitted with amplifiers (7P122) for recording of the mechanical activity.

The peptides were added in cumulative doses for determination of apparent affinity (pD_2) and intrinsic activity (α). Each dose was allowed to react for 2–3 min before the next addition. Receptor antagonists were added 6 min before the agonists.

Carbachol was taken as the reference agonist ($\alpha = 1.0$) for estimation of the maximal contractile response in each preparation.

The peptides were purchased as pure, synthetic compounds from Beckman Instruments and kept frozen in concentrated solutions (0.5–1 mg/ml in 0.1 M acetic acid). Addition of 1–300-μl aliquots to the 50 ml volume of Krebs solution had no effect on pH.

The following peptides were used: neurotensin (NT), bradykinin (BK), lysine-bradykinin (LB), angiotensin II (ANG), saralasin (SAA, sar^1-ala^8-angiotensin II), substance P (SP). Vasoactive intestinal polypeptide (VIP) was a gift from Dr. R. Jorde, Tromsø.

Carbachol was obtained from the British Drug House Ltd., atropine from The Norwegian Pharmaceutical Association and methysergide from Sandoz AG.

Means and standard deviations were calculated for all data representing 6 or more observations for each drug and dose. Significance for differences between the means was calculated by Student's *t*-test for independent and dependent groups.

Results

The circular preparations of the rat bronchi were without spontaneous contractile activity. There was on the other hand a marked and rapid rise in isometric tension after addition of acetylcholine or a cholinergic agonist such as carbachol. Optimal stability of the tension response was obtained at a preload of 1 g, and there was no difference in the affinity for carbachol added as single or cumulative doses. Cumulative doses were therefore employed. The affinity for carbachol was considerably higher ($pD_2 = 6.1$) than for acetylcholine ($pD_2 = 3.7$) although both agents exhibited the same intrinsic activity (Table I). Carbachol was therefore chosen as the reference agonist for estimation of the intrinsic activities of other compounds. The tension responses to the cholinergic agonists were completely abolished by atropine ($1.4 \cdot 10^{-6}$ M).

Serotonin elicited a slower rise in tension than the cholinergic agonists while histamine (10^{-7}–10^{-4} M) was without effect. As given in Table I the intrinsic activity of serotonin was low although the affinity for this amine was of the same order as for carbachol. The effect of serotonin was abolished by methysergide ($1.1 \cdot 10^{-5}$ M).

TABLE I

Affinities and intrinsic activities of agonists for the isolated rat bronchi

Agonists	n	Maximal conc. * ($\times 10^{-6}$ M)	Maximal tension increase (in g)	pD_2	α
Neurotensin	6	1.3	0.33 ± 0.11	6.9 ± 0.3	0.10 ± 0.04
Bradykinin	8	7.7	0.37 ± 0.29	6.8 ± 0.3	0.11 ± 0.10
Angiotensin II	9	10.6	0.15 ± 0.05	6.6 ± 0.3	0.04 ± 0.02
Acetylcholine	12	8200	3.26 ± 0.67	3.7 ± 0.4	0.92 ± 0.04
Carbachol	12	29	3.48 ± 0.69	6.1 ± 0.2	1.00
Serotonin	15	38	0.81 ± 0.41	5.7 ± 0.3	0.21 ± 0.12

* By cumulative dosage. The values are means ± S.D. for (n) experiments. The intrinsic activity (α) was obtained as the ratio of the maximal tension increase to the agonist and that to carbachol. Substance P and VIP (10^{-9}–10^{-5} M) were without detectable effects on the isometric tension of the rat bronchial preparations preloaded with 1 g. pD_2 = apparent affinity.

Neurotensin (NT) induced concentration dependent increases in the isometric tension (Figs. 2 and 3). By comparison with the time course of the carbachol response the peptide-induced rise in tension was slower. Moreover, the response to NT reached a maximum within 2 min, after which it rapidly declined (Fig. 2). The apparent affinity for NT by cumulative doses ($pD_2 = 6.9$, Table I) was higher than

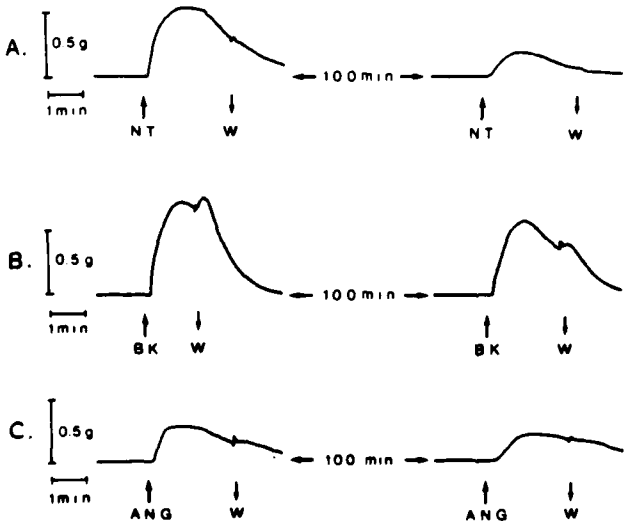

Fig. 2. Effects of peptides on the isometric tension in the isolated bronchial preparations. Tracings of the response to two subsequent doses of a maximally effective concentration of (A) neurotensin (NT), (B) bradykinin (BK), and (C) angiotensin (ANG). The first dose was allowed to act for 2 min before being washed out (W). The preparations were washed three times during the 100 min interval.

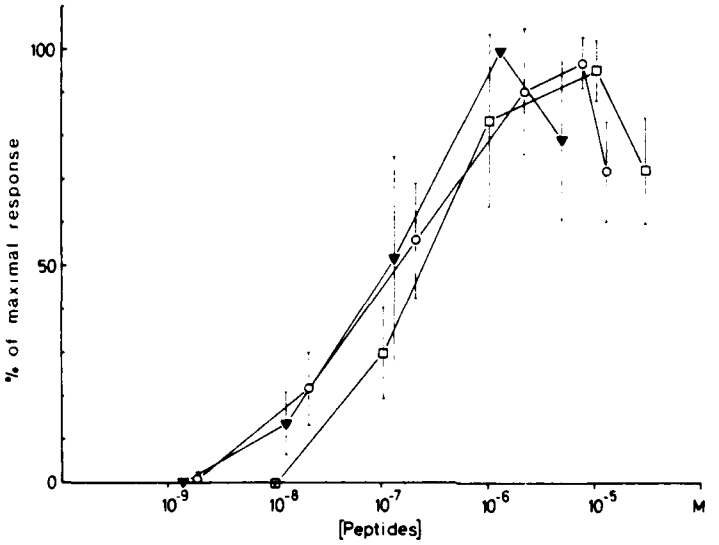

Fig. 3. Concentration-response curves for neurotensin, bradykinin and angiotensin II on the isolated rat bronchi. Values are means ± S.D. of n experiments with cumulative doses of neurotensin (▼——▼, $n = 6$), bradykinin (○——○, $n = 8$) and angiotensin II (□——□, $n = 9$).

for the cholinergic agonists and closely similar to that observed for two other, systemic peptides which also caused an increase in the contractile tension in the rat bronchial smooth muscle, namely bradykinin and angiotensin II. The intrinsic activities of NT and the two other peptides were however considerably lower than those of the cholinergic agonists (Table I). These peptide responses were, however,

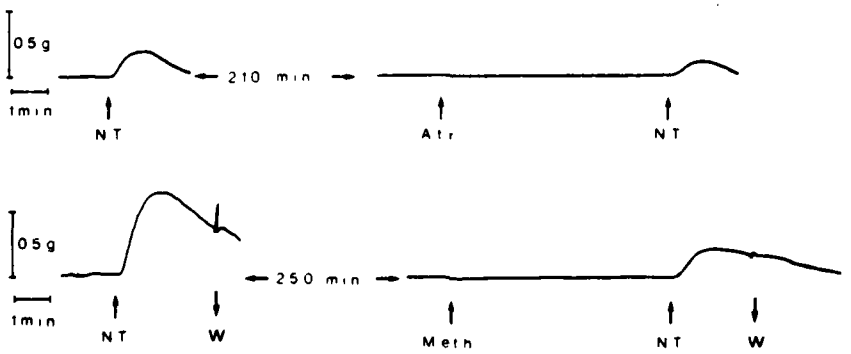

Fig. 4 Effects of atropine and methysergide on the tension response to neurotensin in unstimulated rat bronchi. Tracings of the responses to two subsequent doses of a maximally effective dose of neurotensin (NT). The first dose was allowed to act for 2 min before being washed out (W). After three additional washes during the 3–3.5 h intervals atropine (Atr., $1.4 \cdot 10^{-6}$ M) and methysergide (Meth., $1.1 \cdot 10^{-5}$ M) were added 6 min before the second dose of NT (upper and lower tracing, respectively).

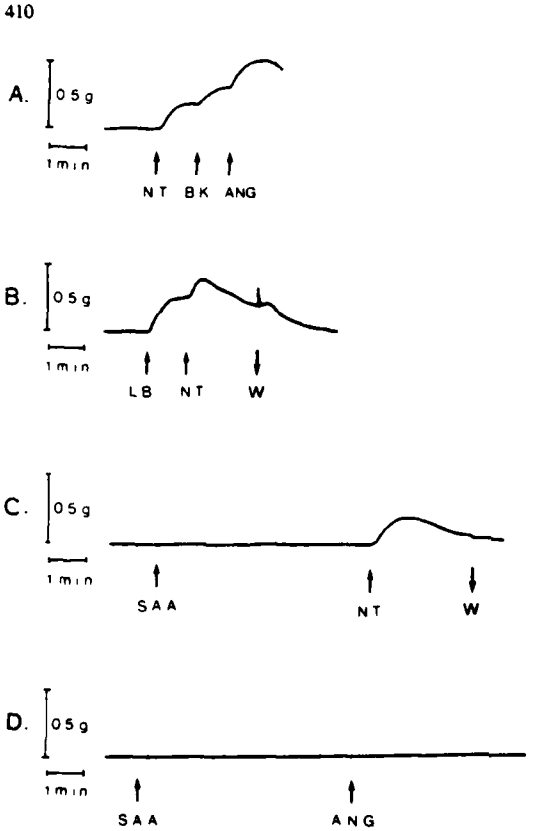

Fig. 5. Additive effects of neurotensin, bradykinin and angiotensin II on the tension in the isolated rat bronchi. The maximally effective doses of neurotensin (NT), bradykinin (BK) and angiotensin (ANG) were added (A) at 1 min intervals, (B) Lys-bradykinin (LB, $1.6 \cdot 10^{-6}$ M) 1 min before NT, (C) saralasin (SAA, $9.5 \cdot 10^{-6}$ M) 6 min before NT, and (D) SAA 6 min before ANG. The preparation was washed (W) after 2 min exposure to NT.

not abolished in the presence of atropine or methysergide, as shown for NT (Fig. 4). A pronounced degree of tachyphylaxis was observed after the first exposure to either of the three active peptides (Fig. 2). Only the angiotensin response was antagonized by saralasin (Fig. 5) while the presence of Lys-bradykinin greatly diminished the effect of bradykinin. The response to NT was on the other hand not blocked by pretreatment with either angiotensin II or bradykinin, their antagonist or agonist and the effects of NT, bradykinin and angiotensin II were additive (Fig. 5).

Substance P and VIP (10^{-8}–10^{-5} M) were without effect on the mechanical activity of the isolated rat bronchi.

Electrical field stimulation of the rat bronchial preparations resulted in frequency-dependent increases in tension. This response was completely abolished by atropine. The response to stimulation at half maximal frequency (8 Hz) and

TABLE II

Effects of peptides on the tension response to electrical stimulation of the isolated rat bronchi

Condition	Concentration ($\times 10^{-6}$ M)	Response in % of the control
Field stimulation (8 Hz, 8 ms)		
Control, no peptide	-	100
Experiment, + peptide		
Neurotensin	1.1	163 ± 49 *
Bradykinin	2.8	127 ± 24 *
Angiotensin II	9.5	169 ± 43 *
Substance P	1.5	114 ± 15 n.s.
VIP	4.5	94 ± 7 n.s.

The values are means ± S.D. of 6 experiments for each of the peptides. The preparations were first stimulated electrically (controls), then exposed to a single dose of the peptide (final concentration in organ bath is given) until the response was maximal before the electrical stimulation was repeated. The tension response to stimulation in presence of the peptide is expressed in percent of that in its absence (control). The responses to electrical stimulation in the absence or presence of peptide were abolished by atropine ($1.4 \cdot 10^{-6}$ M). * $P < 0.05$; n.s., $P > 0.05$.

optimal duration (8 ms) were potentiated by the presence of NT, bradykinin or angiotensin II while unaffected by substance P or VIP (Table II). The potentiating effects of NT and the two other peptides were, however, abolished by atropine.

Discussion

As presently shown, the rat bronchial smooth muscles respond to NT with concentration-dependent increases in contractile tension, indicating NT receptors in the preparation. Furthermore, the apparent affinity for NT in the bronchial preparation appears to be of the same order as previously reported for other nonvisceral tissues of the rat, such as the atria [10,18], the coronaries [11], the portal vein [4,15] and the pleural mast cells [7]. The relatively low intrinsic activity of NT in the bronchi suggests on the other hand a minor role for this peptide in the absence of parasympathetic activation or concerted exposure to systemic peptides such as bradykinin and angiotensin II.

The question is: To what extent are the observed effects of NT mediated via pre- or postsynaptic receptors, on the pleural mast cells or on the bronchial smooth muscle?

The bronchial walls of the rat are innervated by cholinergic fibers with ganglia close to the smooth muscle layers of the airway and arteries [14]. Our findings of atropine-sensitive potentiations of the responses to electrical stimulation by NT, bradykinin and angiotensin II suggest that the cholinergic terminals in the airway of the rat have modulating receptors for these three peptides. A role for NT in modulation of peripheral cholinergic terminals has not previously been reported for

the rat, although such an effect has been detected in the unstimulated guinea pig ileum [6]. Angiotensin II has on the other hand been shown to potentiate the release of the sympathetic neurotransmitter from electrically stimulated hearts [17] while NT was found to be without effect on the sympathetic terminals in the rat portal vein [15]. Neither bradykinin nor substance P appear to have specific effects on peripheral cholinergic elements [1]. The bronchial region of the rat may thus be unique in the sense that one here finds potentiating receptors on the cholinergic terminals, not only for NT but also for the two systemic peptides, bradykinin and angiotensin II.

The pleural mast cells of the rat seem to differ from the peritoneal mast cells in having functional receptors for NT [7]. The rat mast cells are known to release serotonin as well as histamine [9]. However, as shown in this study, only serotonin was able to increase the contractile tension in the isolated rat bronchi. An indirect effect of NT via mast cell release of serotonin might therefore be expected. The serotoninergic blocker, methysergide, did on the other hand not abolish the bronchial responses to NT, bradykinin or angiotensin II. This strongly suggests that there are additional, mast cell-independent receptor systems for these three peptides in the rat bronchi, on the cholinergic input and on the respiratory smooth muscle cells.

In the absence of electrical stimulation cholinergic blockade had no effect on the tension response to NT and this effect of NT must therefore be due to a direct activation of postsynaptic receptors. Analogous to the situation in the rat portal vein [4,15], the bronchial NT receptors exhibited a pronounced degree of tachyphylaxis. This was also the case for the bradykinin- and angiotensin II receptors. Despite the marked tachyphylaxis to each of these peptides, additive increases in tension were obtained when NT and the two other systemic peptides were added in concert. These observations strongly suggest that the bronchial smooth muscles contain independent receptors for these three peptides. Their low, individual intrinsic activities and additive effects suggest, moreover, that these peptide receptors must be rather loosely coupled to the transducer events in the cell membrane. Furthermore, the smooth muscles of the respiratory tract appear, contrary to those in the vasculature [4], unique in the sense that they do not dilate in response to VIP, neither in the absence nor in the presence of cholinergic activation.

The functional significance for the peripheral receptors for NT outside the viscera remains to be elucidated. In view of the postulated hormonal role for NT [16], it is of interest to note that the circulating levels of NT in the rat is relatively high ($0.05-1 \cdot 10^{-9}$ M, Ref. 3) and may increase further in response to a release from the intestinal mucosa after intake of fat [16]. In the rat the affinity for NT has been shown to increase after 24 h of fasting, most markedly for the hypotensive, mediated via mast cell stimulation, and coronary constrictor responses [7,8,12,13]. It is therefore not unlikely that the bronchial NT receptors, presently reported for the cholinergic terminals and smooth muscle, may have functional relevance for the rat under certain nutritional states, such as during postprandial regulations to a fatty meal after a period of starvation.

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PAPER II

Acta Physiol Scand 1982. 114: 467–469

Neurotensin-induced release of histamine from rat mast cells in vitro

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Intravenous injection of neurotensin (NT) in doses above 1 nmol/kg results in a dramatic fall in the arterial blood pressure concomitant with an increase in vascular permeability (Carraway & Leeman 1973), suggestive of a vasodilatory effect via the arterial smooth muscle. However, these actions of NT are prevented by pretreating the rats with compound 48/80 (Chahl 1979, Quirion et al. 1980) or antagonists to histamine and serotonin (Quirion et al. 1980), indicating that the vascular effects of the high doses of NT may to a large extent be mediated via the mast cells which in the rat release serotonin as well as histamine (Parrat & West 1957).

NT releases histamine from the human peritoneal mast cells in vitro (Selbekk et al. 1980) and binds to rat peritoneal and pleural mast cells at low peptide concentrations (Lazarus et al. 1977). It has however not yet been established whether the binding of NT is coupled to release from the rat mast cells and at what range of peptide concentration such a release is effective.

The present study described the effects of synthetic NT on the histamine release from rat mast cells derived from the peritoneal and pleural cavities. The results indicate that NT induces histamine release only from the pleural subpopulation under in vitro conditions.

Mast cells were prepared essentially as earlier described (Krüger 1976). Male Wistar rats (250–300 g) were used to obtain mixed cell suspensions containing 4–5% mast cells, or isolated suspensions from the pleural and peritoneal cavities. The mast cells were harvested in a balanced salt solution (BSS). $CaCl_2$ (1 mM) was present in all solutions.

Incubation procedures. Pleural and peritoneal cells were kept separate and run in parallel in each experiment. After harvesting the mast cells were resuspended and washed three times in BSS containing 1 mg/ml of bovine serum albumin (BSS-A).

Each cell type was resuspended in BSS-A, prewarmed at 37°C and aliquots of 20–50 µl were transferred to centrifuge tubes containing 2 ml BSS-A with or without NT (10^{-8}–10^{-5} M final concentration). Each tube contained approximately $5–10 \times 10^4$ or $20–40 \times 10^4$ mast cells/ml as counted in a Burker cell chamber. After incubating the cells for 10 min at 37°C the tubes were placed on ice and centrifuged at 300×g for 10 min at 4°C. The supernatants and pellets, resuspended in 2 ml H_2O, were assayed for histamine by the method of Shore et al. (1959), as described by Bergendorff & Uvnäs (1972). Histamine release is expressed in percentage of the total histamine content of the sample. The spontaneous release ranged between 0.2–8.2% (mean value 3.1±2.4, ± SD, n=33).

Preparation for light and electron microscopy. In experiments for microscopy, aliquots of 100–200 µl of the cell suspensions were transfered to 20 ml BSS-A with and without NT and incubated as described above. Aliquots of 2 ml were removed for histamine determination and the remaining 18 ml centrifuged in the cold for 10 min at 200×g. The pellets were fixed in 2% glutaraldehyde in 0.1 N cacodylate buffer (pH 7.0), dehydrated in alcohols and embedded in Spurr's medium (Spurr 1969). Semithin sections were cut for light microscopy and stained by toluidine blue at pH 3.5. Ultrathin sections were cut for electron microscopy, stained with lead citrate and uranyl acetate and observed in a Phillips EM 300 electron microscope.

Counting of altered mast cells. The semithin sections were observed in a Leitz Orthoplan microscope at a magnification of 1000. Approximately 100 mast cells were inspected from each sample. *Normal cells* were those with no more than one altered granule within the cell or any granule obviously expelled from that cell as observed from the section. *Altered cells* with signs of amine release were those with two or more altered granules within the cell or with granule matrices expelled from the cell. The number of altered cells are expressed in percentage of the total number of counted cells.

Neurotensin (NT) was obtained from Beckman Instruments Co.

Histamine release. As shown in Fig. 1 NT failed to induce a significant release from the peritoneal mast cells, whether in mixed or isolated cell suspensions. A different response was recorded for the pleural mast cells; these responded to NT (10^{-7} M) with 20% release of the total content and leveled off with a maximum release (25%) at 10^{-5} M NT. The values for experiments with $20–40 \times 10^4$ cells were similar to those containing $5–10 \times 10^4$ per ml.

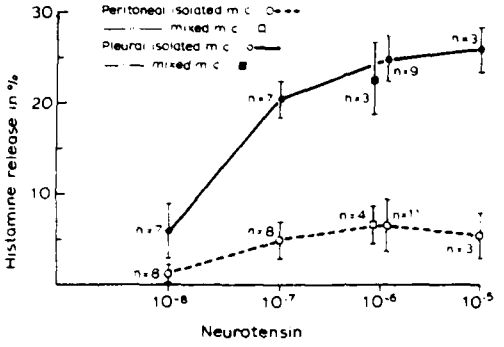

Fig. 1. Concentration-response curves for neurotensin on the histamine release from peritoneal and pleural mast cells of the rat in vitro. Neurotensin (M) in final concentration during incubation. Means ± SD.

Morphological changes by light microscopy. The effects of 10^{-6} M NT on the morphology of the isolated and mixed preparations were compared with the percentage of histamine release. As given in Table 1, half of the pleural and one fourth of the peritoneal mast cells showed morphological signs of release although the corresponding values for histamine release were considerably lower.

Electron microscopy. In the absence of NT the cells were densely packed with electron dense cored granules (Fig. 2*a*) and no difference could be observed in the morphology of peritoneal and pleural mast cells. In the presence of 10^{-6} M NT the pleural cells contained a varying number of altered granules in the intracellular vacuoles and altered granules were also exposed to the extracellular space (Fig. 2*b*). As also observed in the light microscope the ultrastructural study revealed pleural mast cells without signs of release.

The present results have shown that NT induces histamine release chiefly from a subpopulation of the pleural mast cells of the rat under in vitro conditions. This response occurs at relatively high concentrations of the peptide, as indicated from the half maximally effective concentration (3×10^{-7} M, Fig. 1). Such concentrations of the peptide may be reached during infusions of NT in the jugular vein in doses well above the threshold for the hypotensive response, 1 nmol/kg/min (Önarheim et al. 1981). Our findings thus strengthen the hypothesis forwarded by Chahl (1979) and Quirion et al. (1980) that the vascular effects of NT in rats, as first described by Carraway & Leeman (1973), are largely accounted for by mast cell discharge.

The observed release of histamine is a relevant expression of the total amine secretion from the rat mast cells since we know from earlier work that under conditions in which histamine is released, the rat mast cells also release serotonin (Carlsson & Ritzén 1969). Although the mixed population specifically binds NT in a reversible manner ($K_D = 1.54 \times 10^{-7}$ M, Lazarus et al. 1977), the pleural and peritoneal cells appear to differ with respect to coupling of the NT-receptor complex to the release-triggering events. Mast cells from the peritoneal cavity have been shown to differ from those of pleural origin in some other respects, such as in sensitivity towards antigen, concanavalin A, ACTH-C_{1-24} polypeptide, ATP and the calcium ionophore A 23187 (Batchelor & Stanworth 1980) and the anti-asthmatic drug disodium cromoglycate (Alm et al. 1981). Analogous to the case for the rat pleural mast cells only half of the human jejunal mast cell population degranulated when exposed to the maximally effective NT concentration (10^{-6} M NT, Selbekk et al. 1980). Moreover, the electron micrographs of the rat pleural cells revealed signs of moderate stimulation of each altered cell, with altered granules mostly located within the cellular vacuoles even at 10^{-7} M NT. These observations suggest that within the pleural population the activation of the NT-receptor appears to be less efficiently coupled to the events involved in amine discharge than common for more potent secretogogues.

Low intrinsic activities have also been reported for the NT receptors in other peripheral tissues of the rat, such as in the portal vein and bronchi where the apparent affinity for NT (10^{-8}–10^{-7} M, Helle et al. 1980, Helle & Aas 1981) is similar to that

Table 1. *Effect of neurotensin (NT) on the mast cell structure during histamine release under in vitro conditions*

Type of rat mast cells	Histamine release in % of total amine (n=7)	Cells with altered granules in % of total cells
Peritoneal cells		
Controls	1.3±1	5.2±1.2 (n=6)
+10^{-6} M NT	5.6±3.3	23.4±16.1 (n=5)
Pleural cells		
Controls	3.4±2.8	13.2±10.5 (n=7)
+10^{-6} M NT	22.4±7	52.5±23 (n=6)

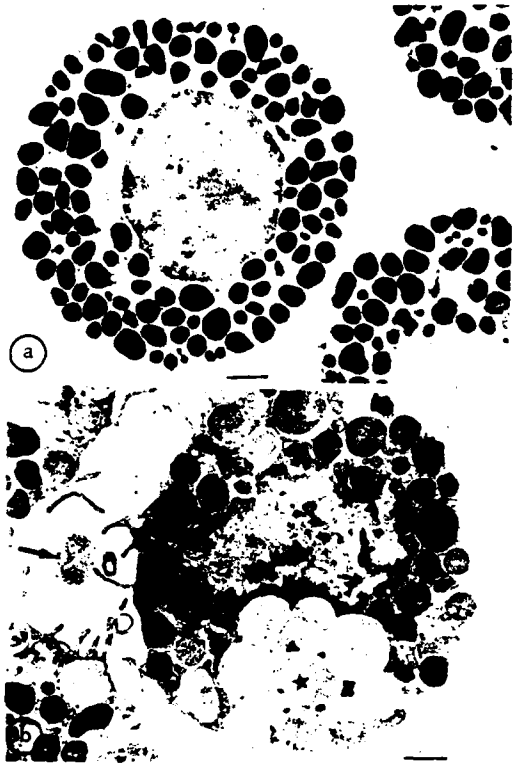

Fig. 2. Ultrastructure of the rat pleural mast cells. (*a*) Control cell with abundance of electron dense granules. Bar = 1 μm. (*b*) Cell after exposure to 10^{-6} M neurotensin (25% release of histamine). Altered granules in varying numbers are seen within cellular vacuoles (asterisk). Altered granules deposited free in the extracellular medium were frequently observed (arrow) Bar 1 μm.

in the pleural mast cells. Moreover, the vascular and bronchial NT receptors exhibited marked tachyphylaxis under in vitro conditions. Most of the rat NT derives from the intestinal mucosa and may reach the peritoneal and pleural mast cells via the circulation at levels ranging between $0.05-1\times10^{-6}$ M (Carraway & Leeman 1976). The inhomogeneity in the peritoneal and pleural subpopulations in response to NT may thus in part reflect different degrees of tachyphylaxis related to exposure to circulating NT in situ prior to these in vitro experiments.

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PAPER III

Acta Physiol Scand 1983, 117: 477–480

Serotonin induced release of acetylcholine from neurons in the bronchial smooth muscle of the rat

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Studies in intact and isolated preparations of the dog lungs have shown that serotonin can induce bronchoconstriction and vasoconstriction either through a reflex mechanism or via direct action on serotonin receptors on smooth muscle cells (Hahn et al. 1978). A cholinergic component in this reflex is suggested by the observations that atropine prevents the bronchoconstriction induced by serotonin in dogs (Islam et al. 1974). Several groups report on the possible release of acetylcholine by serotonin in guinea-pig ileum (Brownlee & Johnson 1965, Offermeier & Ariëns 1966) and lung (Bhattacharya 1955, Vaage 1976). It is, however, not yet established whether the contractions arising from the serotonin-induced acetylcholine release are of importance compared to the contractions elicited via postsynaptic serotonin receptors on the bronchial smooth muscle per se. These alternatives can be elucidated by studying the isolated rat bronchi. Unlike that of the guinea-pig (Mizrahi et al. 1982) this tissue shows atropine-sensitive potentiations of the responses to electrical stimulations by neurotensin, angiotensin II and bradykinin (Aas & Helle 1982) as well as to serotonin, indicating that cholinergic terminals with peptide receptors and receptors to serotonin are present in the preparation.

Moreover, the rat pleural mast cells, releasing both serotonin and histamine (Parrat & West 1957, Carlson & Ritzen 1969), is stimulated to amine release by neurotensin (Krüger et al 1982), suggesting that receptors for the mast cell amines may be located pre- and post-synaptically in parallel to that for neurotensin (Aas & Helle 1982).

The present experiments were designed to describe the diverse effecsts of serotonin on the isolated bronchial smooth muscle of the rat. The results indicate that serotonin stimulated the smooth muscles to contract via presynaptic receptors on cholinergic terminals in addition to postsynaptic receptors on the airway smooth muscles.

METHODS

Female Sprague-Dawley rats (250–300 g) were killed by a heavy blow on the head. The ventral body wall was opened along the midline and the lungs exposed. The left and right bronchi (w. weight ca 0.02 g) were dissected free and mounted in parallel as circular preparations on hooks made from cannula (Aas & Helle 1982) in a thermostatically controlled organ-bath containing Krebs solution (50 ml, 37°C) of the following composition (in mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.6, $MgSo_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2, Glucose 11.1. The solution was gassed with 95% O_2 and 5% CO_2 (pH=7.4).

Each preparation was given a preload of 1.0 g and equilibrated for at least 60 min before start of experiments. For electrical stimulation the bronchi were mounted between platinum electrodes (8 mm apart). These were stimulated via a Grass S 44 stimulator. The voltage across the preparations was 3.5–5.0 V.

Contractile tension changes induced by drugs or electrical stimulation was recorded isometrically via Grass force displacement transducers (FT 03) and monitored via a Grass polygraph (RPS 7A8A) fitted with amplifiers (7 P 122) for recording of the mechanical activity. Carbachol and acetylcholine were added in cumulative doses as reference agonists for determination of the affinity (pD_2) to and intrinsic activity (α) of serotonin. Each dose was allowed to interact with the preparation until maximal tension was reached before the next addition. Receptor antagonists were added 6 min before the agonists.

Carbachol was obtained from the British Drug House Ltd, histamine and atropine from the Norwegian Pharmaceutical Association, serotonin from Beckman, physostigmine from C H Boehringer Ingelheim and methysergide from Sandoz A G.

Means and standard deviations were calculated for all data representing 6 or more observations. Significance for difference between the data was calculated by Student's t-test for dependent groups.

RESULTS

Serotonin stimulated contractile activity in the smooth muscles of the rat bronchi in concentrations

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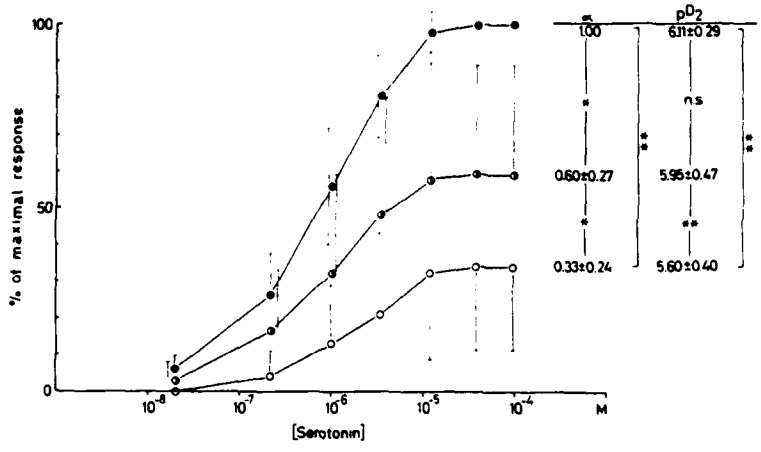

Fig. 1. The effects of physostigmine on concentration-response curves for serotonin. Serotonin: (○——○). Serotonin+physostigmine (1.3×10^{-7} M): (◑——◑). Serotonin+physostigmine (1.3×10^{-6} M): (●——●). The response to serotonin and serotonin+physostigmine (1.3×10^{-7} M) is plotted in per cent of the maximal response to serotonin+physostigmine (1.3×10^{-6} M). The values are means ± SD (n=7). The intrinsic activity (α) was obtained as the ratio of the maximal tension increase in each series and that to serotonin with physostigmine (1.3×10^{-6} M). pD_2=apparent affinity. $^{**}p<0.01$, $^{*}p<0.05$, $^{n.s}p>0.05$.

ranging from 10^{-8}–10^{-5}M as shown in Fig. 1. The affinity for serotonin (pD_2=5.6) was slightly lower than for the cholinergic agonist, carbachol (pD_2=6.1), while considerably higher than for acetylcholine in absence of acetylcholinesterase inhibitors (pD_2=3.7). The intrinsic activity of serotonin (0.21) was on the other hand considerably lower than for the cholinergic agonists (carbachol, 1.00, and acetylcholine, 0.92). The serotonin blocker, methysergid (10^{-5} M), which completely blocked the response to serotonin, had no effect on the responses to the cholinergic agonists (Table 2).

Inhibition of acetylcholinesterase by physostigmine (10^{-7}–10^{-6} M) enhanced the intrinsic activity of serotonin considerably (Fig. 1). On the other hand, when atropine was added, there was a signifi-

Table 1. *Effect of physostigmine and atropine on the response to serotonin in the bronchial smooth muscle*

The values are means ± SD (n=6). The intrinsic activity (α) is the relative tension increase compared to that in the presence of serotonin and physostigmine (1.3×10^{-6} M). $^{**}p<0.01$, $^{*}p<0.05$, $^{n.s}p>0.05$

Agonists	α	
Serotonin	0.33±0.24	* **
Serotonin+physostigmine (1.3×10^{-7} M)	0.60±0.27	*
Serotonin+physostigmine (1.3×10^{-6} M)	1.00	
Serotonin	0.33±0.24	*
Serotonin+atropine (1.4×10^{-6} M)	0.22±0.14	
Serotonin+physostigmine (1.3×10^{-7} M) +atropine (1.4×10^{-6} M)	0.22±0.14	n.s.
Serotonin+physostigmine (1.3×10^{-6} M) +atropine (1.4×10^{-6} M)	0.22±0.14	n.s.

Table 2. *The effect of methysergide on the response to serotonin, carbachol and acetylcholine*

The response that a maximally effective dose of serotonin (3.8×10^{-5} M), carbachol (3.0×10^{-5} M) and acetylcholine (8.0×10^{-3} M) induce after addition of methysergide (1.1×10^{-5} M) 6 min prior to the agonist is expressed in per cent ± SD (n=6) of that in its absence (control). $^{n.s}p>0.05$

Agonists	Response	
Serotonin	100	
Serotonin+methysergide	<1	
Carbachol	100.0	n.s.
Carbachol+methysergide	96.8±5.8	
Acetylcholine	100.0	n.s.
Acetylcholine+methysergide	97.8±7.5	

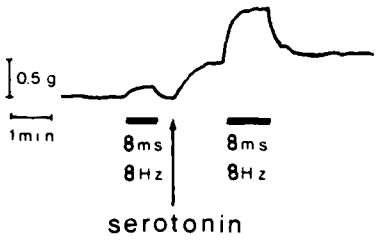

Fig. 2. Effects of serotonin on electrical stimulation. *Tracing of the response to electrical stimulation before and after the addition of serotonin* (3.8×10^{-5} M).

cant reduction in the intrinsic activity of serotonin (Table 1). In the presence of atropine there was no effect of physostigmine (10^{-7}–10^{-6} M).

Electrical field stimulation caused a contractile response in the rat bronchi which was completely abolished by atropine (Aas & Helle 1982). At the half maximal frequency (8 Hz, 8 ms) (Fig. 2), the presence of serotonin greatly facilitated the contractile response to electrical stimulation.

Neither the cholinergic agonists nor serotonin had any tachyphylactic effects in the rat bronchial preparation.

Histamine had no contracting or relaxing effects on the bronchial smooth muscle in concentrations ranging from 10^{-7}–10^{-4} M.

DISCUSSION

The methysergide-sensitive responses to serotonin are taken as evidence for serotonin receptors in the isolated bronchi of the rat. The intrinsic activity of serotonin was low compared to the cholinergic agonists and it is therefore reasonable to assume that serotonin plays a less important role than the parasympathetic neurotransmitter in regulating the tonus of the rat bronchi. However, the present results strongly suggest that serotonin participates as a pre-synaptic modulator, thereby augmenting the output from the parasympathetic terminals. These conclusions rest on the fact that the serotonin responses were partly blocked by atropine, in accordance with earlier work on calf tracheal preparations (Offermeier & Ariëns 1966). Mizrahi et al. (1982) reports on serotonin receptors in the guinea-pig trachea but do not provide data on atropine effects on the serotonin responses. Analogous to the situation in the guinea-pig ileum, in which serotonin modulates the electrically induced release of acetylcholine (Fosbraey & Johnson 1980), there seems to be a serotonin-potentiated release of acetylcholine in the electrically stimulated rat bronchi. The present conclusions on coexistence of pre- and post-synaptic receptors for serotonin in the rat bronchi are further strengthened by the potentiating effects of physostigmine, leading to a two fold increase in the intrinsic activity and enhancement of the affinity of serotonin in the unstimualted preparations. As these effects on the serotonin response were blocked by atropine, the present findings indicate that the serotonin-mediated potentiations of the parasympathetic output are of great importance. In this respect the rat resembles to some extent the dog where the response to serotonin was found to be abolished by atropine (Islam et al. 1974).

As the rat mast cells are known to release serotonin as well as histamine (Parrat & West 1957), it is of interest to note that only one of the mast cell amines is able to modulate the bronchial tonus in the rat. In this respect there is a marked species difference between the rat and the guinea-pig bronchi, which also show a much wider range of peptide receptors (Mizrahi et al. 1982).

In conclusion, the present experiments have provided additional evidence for our previous report on an intimate regulation of the bronchial tonus by the parasympathetic input (Aas & Helle 1982). Moreover, the present findings strongly suggest that serotonin is the only amine of the rat mast cells to have an effect on the bronchial tonus; an effect which appears to be largely elicitated via presynaptic receptors on the cholinergic terminals.

I wish to thank Dr K. B. Helle, Department of Physiology, University of Bergen for helpful discussions during the preparation of this manuscript.

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PAPER IV

PRESYNAPTIC MUSCARINE AND ADENOSINE RECEPTORS INHIBITING EVOKED RELEASE OF ACETYLCHOLINE FROM NERVES IN THE RAT BRONCHIAL SMOOTH MUSCLE

By

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ABSTRACT

P Aas and F Fonnum, European J Pharmacol. . Presynaptic muscarine and adenosine receptors inhibiting evoked release of acetylcholine from nerves in the rat bronchial smooth muscle.

The KCl (51 mM) evoked release of acetylcholine ($[^3H]$-Ach and Ach) from cholinergic nerves in the rat bronchial smooth muscle was studied in a superfusion system. The release was reduced 85% by lowering the Ca^{2+} concentration from 2 mM to 0.1 mM. The veratridine and electrically induced release of Ach were completely inhibited by tetrodotoxin. The muscarinic agonist oxotremorine reduced the potassium stimulated release by about 20%, without having any effect on the basal release. In contrast, scopolamine potentiated the potassium stimulated release by 58%. The inhibitory action of adenosine (28%) on the transmitter release, was only partially antagonized by theophylline. Although adenosine reduced the output of Ach, there was a potentiation of the Ach induced contraction of the bronchial smooth muscle. These results indicate that cholinergic terminals in the rat bronchi possess muscarinic receptors, which inhibits the release of Ach when stimulated. Adenosine receptors may have analogues effects, i.e. presynaptic inhibition and postsynaptic enhancement.

Index terms: Acetylcholine, Adenosine, Presynaptic, Bronchi.

INTRODUCTION

It is generally assumed that the parasympatetic innervation of the lung via the vagus is the motor control nerve for both airway smooth muscle and glands (Widdicombe, 1963). The existence of these cholinergic nerves in the bronchial smooth muscle have been shown in the rat (El-Bermain et al., 1970). The bronchomotor tone of the airway smooth muscle has usually been looked upon as a balance between parasympathetic, sympathetic and non-adrenergic, non-cholinergic inhibitory nerve activities (Diamond and Richardson, 1982). Although these mechanisms may dominate one cannot exclude an involvement of presynaptic modulatory receptors which either autoregulate the neurotransmitter release or regulate the release of other neurotransmitters.

The involvement of presynaptic receptors in the regulation of neurotransmitter release is well documented for nerves releasing norepinephrine i different mammalian tissues (cf. Stjärne, 1975; Starke et al., 1977). Regulation of Ach release by muscaric receptors is also well established for centrally located cholinergic nerves (Polak and Meeuws, 1966; Molenaar and Polak, 1970; Kato et al., 1975; Szerb et al., 1977; Nordström et al., 1982). Some studies also indicate the existence of autoregulation of transmitter release in the periphery by stimulation of presynaptic muscarinic receptors. Alberts and Stjärne (1982) and Alberts et al. (1982) have shown that presynaptic muscarinic receptors are present on cholinergic nerve terminals in the guinea-pig myenteric plexus. There was an inhibiting effect of oxotremorine on the release of Ach and this effect was competitively antagonized by atropine. Kilbinger and Wagner (1975) also demonstrated an inhibition of Ach release by oxotremorine and thereby showed the possible existence of receptors autoregulating the release of Ach from cholinergic nerves in the guinea-pig ileum longitudinal muscle. Presynaptic muscarinic receptors may be a fairly general regulation mechanism, by which the release of Ach is modulated. Whether this

mechanism only operates during inhibition of acetylcholinesterase when Ach release is depressed by excess Ach (Szerb and Somogyi, 1973) is still an open question, but more recent studies with radiotracer techniques suggest a moderate enhanchement of transmitter release during atropine inhibition in the absence of acetylcholinesterase inhibitors (Hadhazy and Szerb, 1977). This implies that Ach released from cholinergic nerves under normal physiological conditions may autoinhibit the release of neurotransmitter.

In parallel to the presynaptically stored Ach, considerable quantities of ATP have also been reported to be costored and released from terminals of the phrenic nerve in the rat diaphragm during stimulation (Silinsky and Hubbard, 1973; Silinsky, 1975; Fredholm and Hedqvist, 1980). Hydrolytic enzymes in the synapse hydrolyze ATP to adenosine, and the concentration of adenosine increases in the synapse after a stimulation. Therefore adenosine may have modulatory effects and stimulate both pre- and postsynaptically. These effects are probably due to stimulation of specific adenosine receptor sites. Several recent in vitro studies have demonstrated that purines, specially adenosine, inhibit the contraction induced by cholinergic nerve stimulation in guinea-pig ileum (Vizi and Knoll, 1976; Gustafsson et al., 1978). The inhibition was apparently presynaptic since it was found to be accompanied by diminished release of Ach from cholinergic nerves (Gustafsson et al., 1978; Hayashi et al., 1978). Adenosine probably also excert some of its effects through a postsynaptic receptor mechanism in the airway smooth muscle, shown by the contraction of the isolated guinea-pig trachea (Fredholm et al., 1979; Fredholm and Hedqvist, 1980). Adenosine induced, however only a slight contraction in the trachea smooth muscle preparation with a low intrinisic activity compared to cholinergic agonists.

The aim of the present study was to characterize the possible existence of modulating receptor mechanisms with particular interest in the effect of Ach and adenosine on cholinergic nerves in the bronchial smooth muscle of the rat. The study is of particular rele-

vance for interpretation of the toxic effects of inhaled cholinesterase inhibitors on the bronchial muscle tonus in vivo (Fonnum et al., 1984).

MATERIALS AND METHODS

Animals

Male Wistar rats (200-300 g) were used throughout and the rats were given a standard labroatory diet and water ad libitum. The animals (from Møllegaard, Copenhagen) were kept in standard laboratory cages on daily renewed sawdust for 1-2 weeks before start of experiment. The rats were healthy and they were without symptoms of infections in the lungs or in the respiratory tract. They were killed by decapitation and the lung and airways were dissected out and transferred to the physiological buffer.

Chemicals

The following chemicals were used:

Adenosine hemisulfate, Bovine serum albumine, Hemicholinium-3, Oxotremorine, Scopolamine hydrochloride, Theophylline and Veratridine sulfate salt, all from Sigma.

Additonal drugs were used: Atropine sulfate (Norsk Medisinal Depot, Oslo), [^{3}H](Methyl)-choline chloride, 80.0 Ci/mmol (New England Nuclear) and Tetrodotoxin (Sankyo, Tokyo).

Superfusion media

The basic superfusion medium (medium 1) had the following composition (in mM): NaCl 140.0, KCl 5.1, $CaCl_2$ 2.0, $MgSO_4$ 1.0, Na_2HPO_4 1.2, Tris-HCl 15.0, glucose 5.0. The depolarization medium (medium 2) contained 51 mM KCl with the concentration of NaCl reduced accordingly to keep the ionic strength constant. Medium 3 had a similar composition to medium 1 but 0.1 mM $CaCl_2$ and 10 mM $MgSO_4$. Medium 4 was similar to medium 2, but with the same changes as described for medium 3. The two latter media were employed in order to assess the calcium-dependent release. The media were continuously oxygenated with 100% O_2 (pH = 7.4, 25°C).

Superfusion conditions

Subsequent to decapitation and dissection the two main bronchi were opened along the ventral side and cut into pieces of approximately 1 mg wet weight. The pieces of bronchial smooth muscle tissue (4x1 mg wet weight, protein conc. mg/ml±SEM , 0.60±0.01, n=45) were superfused in perfusion chambers made from two disposable pipette tips. The chamber volume was approx 100 µl and the flow 200 µl/min generated by a peristaltic pump (Gilson, 4 channels). The dead volume of the flow system was very low (150 µl), thus permitting rapid responses when switching between the respective media. Nylon tubing (Portex, 0.25 mm I.D.) was employed to and from the superfusion chambers. The reservoirs containing the media and perfusion chambers were kept in a waterbath at 25°C during the experiments. Samples were collected at 5 min intervals (i.e. 1 ml fractions) after an initial wash-out period of 60 min. 500 µl of superfusing media was counted in 5 ml scintillation cocktail (Instagel, Packard Instrument Company). Homogenized tissue (1.5 ml) was counted in fractions of 500 µl.

Release of [^{3}H]-Acetyl choline

Prior to start of superfusion, the tissue was incubated for 60 min in 1.1 μM [^{3}H]-choline chloride (10 Ci/mmol) (medium 1) on a shaker (25°C), washed twice in medium 1 and superfused for 60 min prior to start of collecting samples. The superfusion media contained hemicholinium-3 (10 μM).

Isometric contraction measurements

The left and right bronchi were mounted in parallel as circular preparations and the tension was recorded isometrically via a Grass Force Displacement Transducer (FT03) and monitored via a Grass Polygraph (RPS 7A8A) fitted with amplifiers (7P122). For further details, see Aas and Helle (1982).

Data analysis

Fractional rate of release was calculated with a specifically designed computer program for the Apple II microcomputer. Peak areas as well as basal release during the depolarization period, ratios between peak areas and corresponding basal release could be calculated. Means and standard error of the mean (SEM) were calculated for all data. Significance for differences between the mean values were calculated by the Student's t-test for independent and dependent groups.

Protein was determined according to Lowry et al. (1951), with bovine serum albumine as standard.

RESULTS

General effects on the potassium evoked release of Ach

The results in Figure 1 show the release of [^{3}H]-Ach from cholinergic terminals in bronchial smooth muscle tissue from rat, when stimulated with high potassium (51 mM). The increase in transmitter release was rapid and decreased to the previous level of basal release after returning to the low potassium medium (medium 1). In the presence of hemicolinium-3, which inhibits the high affinity uptake of choline (Yamamura and Snyder, 1973) it was possible to stimulate the cholinergic terminals to release [^{3}H]-Ach repetitively, with only a minor decrease in the amount of [^{3}H]-Ach released.

There was a large decrease by 85% in the release of [^{3}H]-Ach when stimulating with high potassium in the presence of a low Ca^{2+}-concentration (0.1 mM) (Figure 2). This reduction in transmitter release was completely reversed when changing back to the 2 mM Ca^{2+}-medium. Tetrodotoxin (3 µM) had minor effects (19%) on the potassium stimulated release, but it totally inhibited the veratridine (75 µM) induced release of [^{3}H]-Ach as illustrated in Figure 3. Tetrodotoxin also completely inhibited the electrically induced in vitro contraction of the bronchial smooth muscle with no effect on Ach induced contraction (not shown).

The effect of presynaptic muscarinic receptor stimulation

The presence of the muscarinic receptor agonist oxotremorine (50 µM) induced a 20% reduction in the potassium stimulated release of [^{3}H]-Ach (Figure 4). Increasing the concentration of Ach in the synaptic cleft by superfusing the tissue with the cholinesterase inhibitor soman appeared also to reduce the spontaneously released [^{3}H]-Ach (not

shown). The opposite effect was observed by superfusing the bronchial tissue with a potassium medium (51 mM) containing the muscarinic antagonist scopolamine (30 μM). Scopolamine increased the amount of [^{3}H]-Ach released by 58%, without having any effects on the basal release of neurotransmitter. The modulatory effect of scopolamine which were seen on the stimulation with high potassium were not fully reversible within 20 minutes of superfusion in the absence of the antagonist.

Effects of adenosine

Adenosine (30 μM) decreased the potassium evoked release of [^{3}H]-Ach by 28% (Table 1). There was a small (10%) but significant inhibitory effect of theophylline (1 mM) on the adenosine induced reduction in transmitter release. The release of [^{3}H]-Ach slowly recovered to normal when omitting adenosine from the perfusion fluid. Although adenosine reduced the output of [^{3}H]-Ach from the cholinergic terminals, adenosine both stimulated the bronchial smooth muscle to contract and potentiated the contraction induced by electrically stimulated release of Ach from the cholinergic terminals in the tissue (Table 2). This potentiation exceeded 65% when the tissue was stimulated electrically, but was not significantly increased in the presence of exogenously added Ach. The electrically induced contraction of the bronchial smooth muscle was due to release of Ach (Aas and Helle, 1982). Adenosine showed a marked tachyphylactic effect in the tissue, but adenosine did on the other hand not induce any tachyphylaxis to repetitive stimulation by Ach.

DISCUSSION

The results presently described show that the cholinergic terminals in the bronchial smooth muscle in the rat are subject to autoregulation via inhibitory muscarinic receptors. Adenosine has been shown to modulate Ach release via an inhibitory presynaptic receptor in this pre-

paration in parellel to potentiate the postsynaptic stimulation by Ach. Our evidence rests on the assumption that [^{3}H]-Ach is formed from the radiolabeled choline taken up actively from the medium into the cholinergic terminals and subsequently released from the presynaptic stores in response to potassium evoked depolarizations.

Formation and release of [^{3}H]-acetylcholine

In the present experiments the neuronal stores of Ach were labelled by incubation of the bronchial smooth muscle with cholinergic nerves and terminals in 1.1 µM [^{3}H]-choline. High potassium (51 mM) released radiolabelled Ach in a Ca^{2+}-dependent manner (Figure 2). A similar release was obtained with veratridine, an effect that was largely antagonized by tetrodotoxin (TTX, 3 µM) (Figure 3A), in contrast to that of the potassium evoked release (Figure 3B). Our findings are consistent with those reported by Caterall (1984) of a TTX-independent potassium-evoked release of [^{3}H]-Ach, although TTX completely abolished the electrically induced in vitro contraction of the bronchial smooth muscle. We assume that the potassium evoked release of [^{3}H]-Ach was derived from the pool of neurotransmitter newly synthetized from [^{3}H]-choline taken up from the incubation medium (cf. Szerb, 1975,1976; Kilbinger and Wessler, 1980). In spite of the fact that most of the released [^{3}H]-Ach accumulated in the superfusate in the form of [^{3}H]-choline, the total rise in [^{3}H] (i.e. [^{3}H]-Ach and its hydrolysis products) efflux induced by depolarization of the nerves was taken as a relative measure of the released [^{3}H]-Ach. Moreover, our assumption that [^{3}H]-Ach derives from a functional cholinergic terminal network in the rat bronchi was strengthened by the TTX, veratridine and Ca^{2+}-effects.

Hence, our experiments are consistent with Ach being released from cholinergic nerve terminals. The release of Ach could also be quantitated by the contractile response in the postsynaptic effector, i.e. the bronchial smooth muscle.

Presynaptic muscarinic receptors

In the presence of oxotremorine (50 µM) there was a slight reduction (20%) in the potassium (51 mM) evoked release of Ach. This action of an exogenous muscarinic agonist indicates the existence of functionally presynaptic muscarinic receptors on the cholinergic terminals, whereby Ach mediate autoinhibition of transmitter release. Apparently, similar results have also been obtained in other peripheral cholinergically innervated smooth muscle tissues as in the myenteric plexus in the guinea-pig (Kilbinger and Wessler, 1980; Szerb, 1980; Alberts and Stjärne, 1982). The inhibition of Ach release is on the other hand probably more pronounced in the presence of an acetylcholinesterase inhibitor. Antimuscarinic drugs may, by blocking presynaptic muscarinic receptors, facilitate the evoked release of endogenous Ach determined both in the presence or in the absence of an Ach inhibitor.

The antimuscarinic drug scopolamine (30 µM) increased the potassium evoked release of Ach by nearly 60% which further indicate the existence of a muscarinic receptor. A similar enhancement of release by a muscarinic antagonist, in the absence of an acetylcholinesterase inhibitor, has also been shown by Kilbinger and Wessler (1980), Kilbinger *et al.*(1981) and Alberts *et al.*(1982) from guinea-pig myenteric plexus. Therefore, on the basis of the present results there is evidence for presynaptic muscarinic receptors which reduce the amount of Ach released upon stimulation. These findings therefore indicate a muscarinic receptor on the cholinergic terminals also in the rat bronchi which are subject to a moderate degree of autoinhibition.

Modulation of cholinergic neurotransmission by adenosine

The present results also provide evidence and confirms previous suggestions that adenosine reduces the evoked release of Ach from cho-

linergic terminals. The present findings indicate a similar mechanism in the isolated bronchial smooth muscle.

Adenosine reduced the output of [^{3}H]-Ach by 28% without having any effect on the basal release of [^{3}H]-Ach. This reduction of Ach-release is in agreement with results on the guinea-pig ileum presented by Vizi and Knoll (1976), Gustafsson et al. (1978) and Hayashi et al. (1978), which demonstrate that the modulation by adenosine may be a general effect.

Adenosine is most likely formed from ATP (Silinsky and Hubbard, 1973; Silinsky, 1975), but one cannot ignore the possible interaction from purinergic nerves which may be located in and stimulate the bronchial smooth muscle by the release of a purinergic neuroeffector (Bianchi et al., 1963; Coleman, 1976). Adenosine receptor sites seems to be located predominantly over the respiratory epithelium of the bronchi, shown by autoradiography in rabbit lung (Buckley and Burnstock, 1983). Although adenosine markedly diminished the output of [^{3}H]-Ach during potassium stimulation, it was only partly antagonized by the adenosine antagonist theophylline. This antagonism by theophylline has previously been shown by several others (Ginsborg and Hirst, 1972; Sawynok and Jhamandas, 1976), although theopylline has not shown to be a specific adenosine receptor antagonist in all the tissues so far studied. Therefore adenosine may have a function in regulating the release of Ach, by stimulating specific receptors.

Although adenosine reduced the release of Ach, adenosine potentiated contractions induced by electrical stimulation, which stimulated release of Ach (Aas and Helle, 1982). In addition to this potentiation of the cholinergic induced contraction adenosine also induced contraction probably by stimulating specific postsynaptic adenosine receptors on the smooth muscles. The postsnynaptic effect of adenosine was in the same range as was found for guinea-pig trachea (Fredholm et al., 1979), while Gustafsson et al. (1978) and Hayashi et al. (1978) found only a very small or no effect of adenosine on contractions induced by exogenously added Ach in the guinea-pig ileum in accordance to the present results. Gustafsson (1981) found on the other hand a poten-

tiation of Ach induced contractions by adenosine in the gastric circular muscle from rabbit. Therefore on the basis of these results adenosine seems to modulate cholinergic neurotransmission in vivo by a dual effect, prejunctional inhibition and postjunctional enhancement synergistically to Ach in the bronchial smooth muscle.

In conclusion, our results provide further evidence for a presynaptic action of Ach and adenosine. The release of Ach was reduced by stimulation of spesific presynaptic muscarine- and adenosine receptors respectively. Muscarinic drugs will therefore exert some of their effects by binding to presynaptic muscarinic receptors. Adenosine, probably produced by hydrolysis of ATP, which is co-released with Ach or released from purinergic nerves in the bronchial smooth muscle, may therefore also regulate the release of Ach. In addition to the presynaptic effect of adenosine, adenosine may stimulate postsynaptic adenosine receptors. These receptors are probably linked to the muscarinic receptors or receptor mechanisms and potentiate postsynaptic stimulation by ACh.

Acknowledgements:

Financial support from the Norwegian Research Council for Science and the Humanities is gratefully acknowledged.
We thank R Dahl (Norwegian Defence Research Establishment, Division for Environmental Toxicology) for designing a computer program for calculations of fractional release on the Apple II microcomputer.

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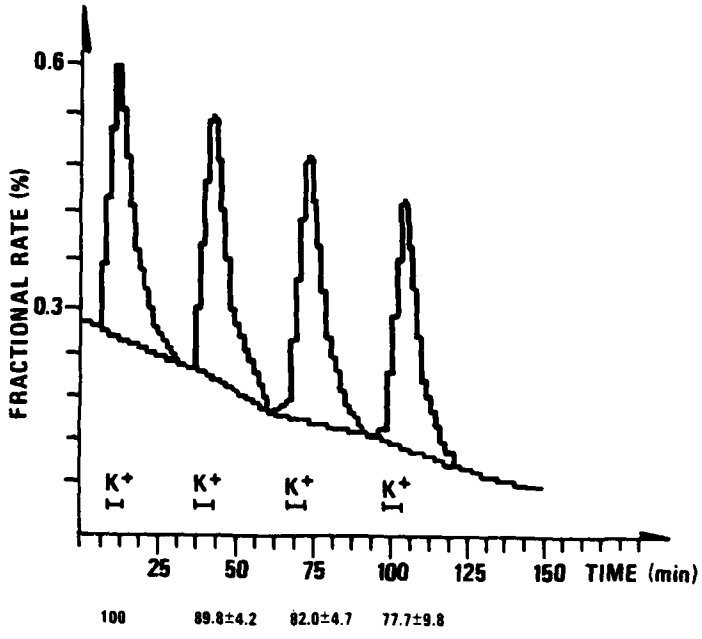

Fig. 1. Time course of release of Ach ([^{3}H]-Ach and [^{3}H]-choline) induced by sequential stimulation of pieces of bronchial smooth muscle with 51 mM potassium (K^+,⟷) in the precence of 2 mM calcium for a single experiment. Repetitive release in per cent of control (first stimulation, 100%).
Below the figure are the corresponding results expressed as mean ±SEM for 8 experiments.

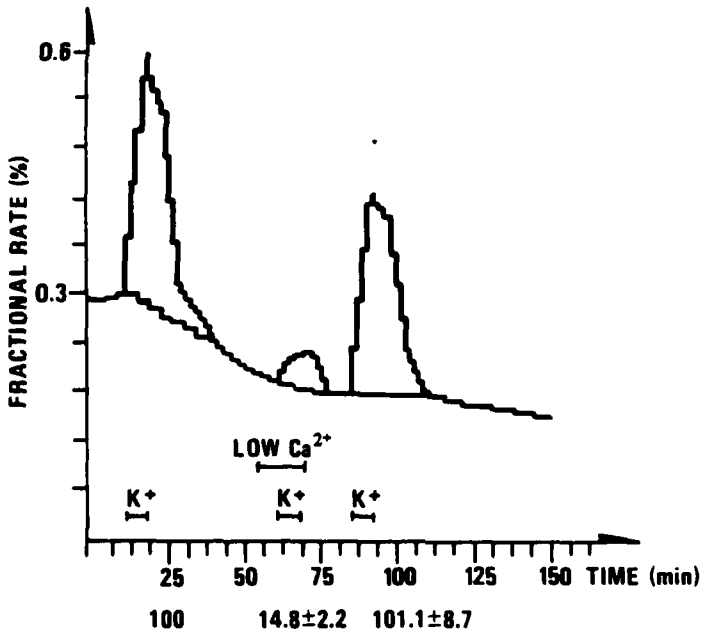

Fig. 2. The effect of low calcium (0.1 mM) on the potassium (K^+, 51 mM) induced release of [^{3}H]-Ach in per cent of control (100%).
Below the figure are the corresponding results expressed as mean ±SEM from 6 experiments.

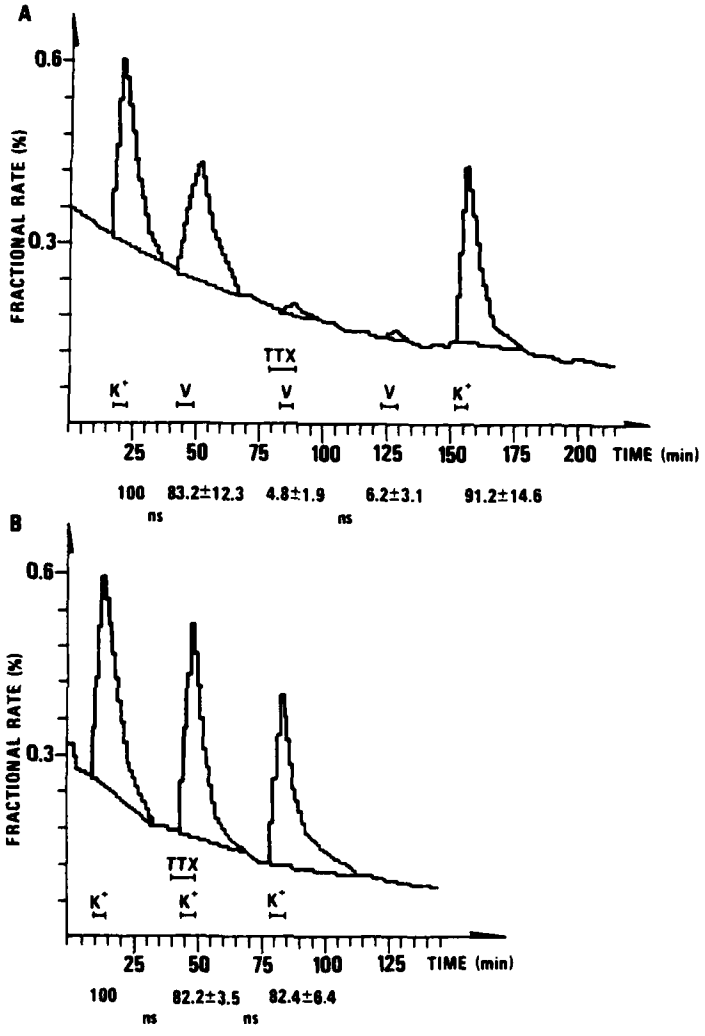

Fig. 3. A. [^{3}H]-Ach induced release by veratridine (V, 75 µM) in per cent of a control stimulation (100%) with potassium (K^+, 51 mM) and the response to tetrodotoxin (TTX, 3 µM).
B. The effect of TTX on sequential stimulations with potassium (K^+, 51 mM).
Below the figure are the correponding results expressed as mean ±SEM from 6 experiments. $^{n.s.}p>0.05$.

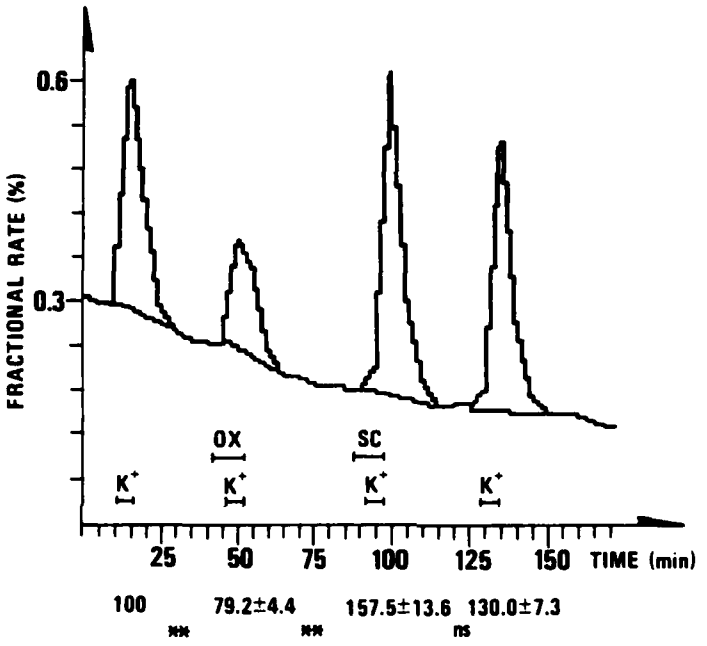

Fig. 4. The effect of oxotremorine (ox, 50 µM) and scopolamine (sc, 30 µM) on potassium (K^+, 51 mM) evoked release of [^{3}H]-Ach. The response values at each stimulation is in per cent of a control stimulation (100%).
Below the figure are the corresponding results expressed as mean ±SEM from 6 experiments. ** $p<0.01$, n.s. $p>0.05$.

Table 1 The effect of adenosine (32 μM) on potassium (51 mM) evoked release of [^{3}H]-Ach from cholinergic nerves in the bronchial smooth muscle. The effect of theopylline (1 mM) on the response to adenosine is shown in the lower part of the table. The response values at each stimulation with high potassium (K^+, 51 mM) were corrected for the normal decrease in evoked release. Values are means ±SEM in per cent of its control (1). $^{**}p<0.01$, $^{*}p<0.05$, $^{n.s.}p>0.05$.

	Control(1)		Adenosine		Control (2)	n
Time after start of superfusion (min)	20		100		150	
			*			
K^+	100	**	71,9±5,2	n.s.	84,3±8,9	7
	n.s.		**		**	
K^+ +						
Theophylline	100	*	81,8±4,9	n.s.	94,4±5,9	6
			n.s.			

Table 2 The effect of adenosine (50 μM) on the contraction induced in the bronchial smooth muscle by Ach (0.3 mM) and the electrical (6 Hz, 6 ms, supramax. voltage)induced release of Ach from cholinergic nerves in the tissue. The effect of adenosine on stimulations are potentiations in excess to the effect of adenosine. Values are means ±SEM (n=8), $^{**}p<0.01$, $^{n.s.}$ $p>0.05$.

	Per cent of control	
Ach (control)	100.0	┐
Adenosine	16.7±3.9	n.s.
Adenosine + Ach	125.0±6.1	┘
Electrical stim.(control)	100.0	┐
Adenosine	54.0±18.9	**
Adenosine + electrical stim.	165.9±9.8	┘

PAPER V

NEUROTENSIN AND SEROTONIN MODULATION OF ACETYLCHOLINE RELEASE FROM CHOLINERGIC NERVES IN THE RAT BRONCHI

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ABSTRACT

Neurotensin and serotonin potentiated the Ca^{2+}-dependent KCl evoked release of [^{3}H]-acetylcholine ([^{3}H]-Ach) from cholinergic terminals in the bronchial smooth muscle of the rat. The augmented response induced by neurotensin was approximately 26% and completely reversible. The neurotensin analogue [D-Trp^{11}]-neurotensin did not inhibit the effect of neurotensin. The serotonin potentiation was more pronounced, approximately 50%, and the induced response was blocked by methysergide, a specific serotonin antagonist.

INTRODUCTION

Neurotensin is a tridecapeptide originally isolated from the hypothalamus (Carraway & Leeman, 1973) and has a central as well as peripheral distribution in the rat (Carraway & Leeman, 1976). Different viceral smooth muscles are influenced by the peptide (cf. Uhl & Snyder, 1981). Many of the effects of neurotensin seems to be mediated directly through neurotensin receptors in the smooth muscles. However, blockers of neural transmission such as tetrodotoxin and anticholinergic drugs have been shown to alter the neurotensin effect on the guinea-pig ileum, suggesting a neuronal, probably a cholinergically mediated effect (Kitabgi & Freychet, 1979 a,b). Although neurotensin induced contraction in the bronchial smooth muscle of the rat, this response was only partly blocked by atropine (Aas & Helle, 1982). The contraction of rat bronchial smooth muscle by neurotensin appeared also to involve a specific neurotensin induced release of serotonin from pleural mast cells (Krüger et al., 1982; Sydbom, 1982). Stereospecific neurotensin receptors have previously also been shown on rat mast cells isolated from the peritoneal cavity (Lazarus et al., 1977a,b) and amine release from mast cells induced by neurotensin was lower after exposure to compound 48/80, a well known mast cell depletor (Ouirion et al., 1980b).

Therefore, it is possible that serotonin as well as neurotensin in part mediate some of their contractile effects via modulation of Ach release from nerve terminals in addition to activation of specific postsynaptic receptors for serotonin and neurotensin (Aas, 1983).

This paper presents results which further substantiate the hypothesis (Aas & Helle, 1982; Krüger et al., 1982; Aas, 1983) that neurotensin and biogenic amines released from pleural mast cells modulate the cholinergic input to the bronchial smooth muscle of the rat.

METHODS

Primary bronchi from the albino rat (Male Wistar rats, 200-300 g, Møllegaard, Copenhagen) were dissected out after decapitation of the animals (Aas & Fonnum, 1984). The bronchi were opened along the ventral side and cut into pieces of approximately 1 mg wet weight. Prior to start of experiment the tissue (4 x 1 mg wet weight, prot. conc. mg/ml± SEM; 0.60 ± 0.02, n=34) were preincubated with [^{3}H]-choline chloride (1,1 µM, 10 Ci/mmol) on a shaker for 60 min, 25°C, and washed twice before transferring to superfusion chambers (volume of 100 µl). There bronchial tissue was superfused for 60 min before start of sampling (Aas & Fonnum, 1984). The perfusion medium (medium 1) had the following composition (in mM): NaCl 140.0, KCl 5.1, $CaCl_2$ 2.0, $MgSO_4$ 1.0, Na_2HPO_4 1.2, Tris-HCl 15.0, glucose 5.0. The release of [^{3}H]-Ach was obtained by depolarization with a high potassium medium (medium 2) in 5 min. Medium 2 had a similar composition to medium 1 but the concentration of KCl was 51 mM and the concentration of NaCl reduced accordingly. Both media contained hemicholinium-3 (10 µM) and were continuously oxygenated with 100% O_2 (pH=7.4, 25°C). In experiments with peptides the media contained 0.5% bovine serum albumine and a protease inhibitor, trasylol (250 U/ml). Samples were collected at 5 min intervals (i.e. 1 ml fractions) and 500 µl were counted in 5 ml Instagel scintillation cocktail (Packard

Instrument Company). Homogenized tissue (1.5 ml total) was counted in 500 µl fractions.

Chemicals used were: Hemicholinium-3, 5-Hydroxytryptamine (Serotonin), Bovine serum albumin, [D-Trp^{11}]-Neurotensin (all from Sigma), Neurotensin (Peninsula Laboratories), Methysergide (Sandoz AG), Trasylol (Bayer AG) and [^{3}H](Methyl)-choline chloride, 80.0 Ci/mmol (New England Nuclear).

Fractional rate of release was calculated on the Apple II microcomputer. Peak areas as well as basal release during the depolarization period and ratios between peak areas could be calculated. Mean and standard error of the mean were calculated for all data. Significance for difference between data was calculated by Student's t-test for dependent groups. Protein was determined according to Lowry et al., (1951) with bovine serum albumine as standard.

RESULTS

The effect of neurotensin and serotonin on potassium evoked release of [^{3}H]-Ach from cholinergic terminals were studied in the isolated preparation of rat bronchial smooth muscle. Neurotensin induced a small (26%), but significant potentiation of potassium stimulated release (Table 1). This potentiation could be reversed by removing neurotensin from the superfusion medium. To avoid the marked tachyphylactic response to neurotensin in the tissue (Aas & Helle, 1982), each preparation was only exposed once to each peptide. The neurotensin analogue [D-Trp^{11}]-neurotensin, suggested by Ouirion et al. (1980c) to be a neurotensin antagonist, did not prevent the neurotensin induced potentation of potassium induced [^{3}H]-Ach release (Table 1). Serotonin increased the potassium induced release of [^{3}H]-Ach by approximately 50% (Table 1). Superfusion for 30 minutes in the absence of serotonin

Table 1 The modulation of potassium evoked release of [^{3}H]-acetylcholine from cholinergic nerves in rat bronchi by neurotensin and serotonin.

Stimulation	1	2	3	4	n
Time after start of superfusion (min)	20	60	100	140	
A. K^+ + NT	100 *	126.5±12.0	n.s. 109.7±7.3	-	7
B. K^+ + NT + [D-Trp^{11}]-NT	100 **	131.3±7.5	** 93.0±3.9	-	11
C. K^+ + 5HT	100 **	151.7±11.7	** 118.5±10.5	-	8
D. K^+ + 5HT + Meth.	100 **	149.8±10.2	** 95.3±10.9	n.s. 92.4±8.2	8

n.s. brackets: A: 100 vs 109.7±7.3; B: 100 vs 93.0±3.9; C: 100 vs 118.5±10.5; D: 100 vs 95.3±10.9; D: 149.8±10.2 * 92.4±8.2

Experiment A: Neurotensin (NT, 6 µM) was added 5 min prior to stimulation with 51 mM potassium (stimulation 2). Stimulation 1 and 3 are control stimulations with potassium (51 mM) only.

Experiment B: The NT analogue [D-Trp^{11}]-NT (6 µM) was added 5 min prior to NT and was present during potassium stimulation (51 mM) (stimulation 2). Stimulation 1 and 3 are control stimulations with potassium (51 mM) only.

Experiment C: Serotonin (5HT, 47 µM) was added 5 min prior to stimulation with 51 mM potassium (stimulation 2). Stimulation 1 and 3 are control stimulations with potassium (51 mM) only.

Experiment D: In stimulation 3, methysergide (Meth.,10 µM) was added 5 min before serotonin and was present during stimulation with potassium (51 mM). In stimulation 2 serotonin (47 µM) was added 5 min prior to potassium (51 mM). Stimulation 1 and 4 are control stimulations with potassium (51 mM) only.

The responses are given in per cent ±SEM of its control stimulation with potassium (K^+, stimulation 1), and corrected for the unavoidable decline in [^{3}H] release with successive stimulation.
**$p<0.01$, *$p<0.05$, n.s. $p>0.05$.

was necessary to restore normal responsiveness in the tissue to potassium depolarization. The potentiation induced by serotonin was on the other hand completely blocked by the serotonin antagonist methysergide (Table 1).

DISCUSSION

The present results indicate that both neurotensin and serotonin potentiate the potassium evoked release of acetylcholine from cholinergic nerve terminals in the primary bronchi of the albino rat. This is in agreement with our previous results on the atropine sensitive potentiation by serotonin and neurotensin of the electrically induced in vitro contractions of the bronchial smooth muscle preparation (Aas & Helle, 1982; Aas, 1983).

The presence of cholinergic nerves in the rat bronchi preparation was shown by the fact that the electrically induced contraction of the bronchial smooth muscle was abolished by atropine and tetrodotoxin. The effect of electrically induced release of Ach was restored by omitting the two blockers from the physiological buffer indicating release of Ach, presumably from cholinergic terminals in the bronchial wall (Aas & Helle, 1982; Aas & Fonnum, 1984).

In the present model (Aas & Fonnum, 1984) the release of $[^3H]$-Ach was shown to be Ca^{2+}-dependent and the veratridine effect was blocked by tetrodotoxin. The results show that the bronchial preparation may be used in studying the modulation of transmitter release.

A presynaptic site for the inhibitory effect of neurotensin in the cholinergic nervous system has previously also been suggested by Kitabgi & Freychet (1978, 1979b) observing an atropine sensitive inhibitory effect of neurotensin in guinea-pig ileal longitudinal smooth muscle. A modulatory role for neurotensin in cholinergic neurotransmission has further been proposed by Regoli (1982) reporting that neurotensin potentiated the electrically induced contraction of guinea-pig ileum in addition to stimulating the ileal muscle to

contract. This is analogous to the situation presently observed for the rat bronchi. On the other hand a neurotensin inhibition of the release of the symphathetic neurotransmitter noradrenaline was observed in the rat vas deferens (Magnan, 1979). Therefore the responses in the rat bronchi are in accordance to some viceral smooth muscles in having cholinergic nerves which are presynaptically modulated by neurotensin.

Moreover, the neurotensin analogue [D-Trp^{11}]-neurotensin had no antagonizing effect on the Ach release response to neurotensin in the bronchial preparation. This is in agreement to results on strips of smooth muscle from rat stomach (Quirion et al., 1980a), were the neurotensin analogue did not inhibit the postsynaptic effect of neurotensin. On the contrary this neurotensin analogue inhibited the effect of neurotensin postsynaptically in rat portal vein and heart (Quirion et al., 1980c; Rioux et al., 1980. Therefore these receptors are probably pharmacologically different from the receptors in rat bronchi and stomach smooth muscle.

Previously, a modulatory role for serotonin in the cholinergic neurotransmission in the guinea-pig ileum has been suggested by Brownlee & Johnson (1965) and by Ádám-Vizi and Vizi (1978). It was shown that the response to serotonin was partly atropine sensitive and potentiated by acetylcholinesterase inhibitors, which indicate that the effect of serotonin was at least partly mediated by Ach.

This is similar to what is found for the rat bronchi. The presence of presynaptic serotonin receptors on the cholinergic nerves rests on the results demonstrating an increase in the in vitro contraction of the circular bronchial smooth muscle on electrical stimulation (Aas, 1983). Therefore these results showing a potentiation of the potassium stimulated release of [^{3}H]-Ach by serotonin increase the evidence for the existence of a presynaptic modulation. The presynaptically serotonin effect was blocked by the serotonin receptor antagonist, methysergide, which further confirms these results.

In conclusion, the present findings strengthen our hypothesis (Aas & Helle, 1982; Aas, 1983) that neurotensin and serotonin modulate the cholinergic nervous system in the rat bronchi.

ACKNOWLEDGEMENTS:

Financial support from the Norwegian Research Council for Science and the Humanities is gratefully acknowledged. I wish to thank Dr Frode Fonnum for helpful discussions during the preparation of this manuscript.

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PAPER VI

A METHOD FOR GENERATING TOXIC VAPOURS OF SOMAN; TOXICITY OF SOMAN BY INHALATION IN RAT

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ABSTRACT

A METHOD FOR GENERATING TOXIC VAPOURS OF SOMAN; TOXICITY OF SOMAN BY INHALATION IN RAT. P Aas, S H Sterri, H P Hjermstad and F Fonnum, Toxicology and Applied Pharmacology 1983,

A method for administration of highly toxic chemicals by inhalation has been developed. The model has three features of special interest, 1) A diffusion cell for producing a constant gas-concentration for several hours or days, 2) A small rapidly equilibrated inhalation chamber (1100 ml), 3) The toxic chemicals are isolated completely from the atmosphere. The LC_{50} of the anticholinesterase soman [o-(1,2,2 trimethylpropyl)-methyl-phosphonofluoridate] was 400 mg min/m^3 (117 µg/kg) with a 24 hour observation period after the end of exposure. The lethal dose of soman was 520 mg min/m^3 (142 µg/kg) when exposing the animals until death in the inhalation chamber (less than 30 min). The inhibition of acetylcholinesterase, cholinesterase and carboxylesterase activities in different tissues were analyzed to study possible barrier mechanisms that might exist in the body to soman. There was a large inhibition of the carboxylesterase and cholinesterase activities in bronchi and lung as well as in blood. Carboxylesterase was important as a detoxifying mechanism, which was shown by the 70% decrease in the lethal dose of soman following s.c. pretreatment with TOCP (tri-ortho-cresyl-phosphate), a carboxylesterase inhibitor.

Index terms: Inhalation, soman, bronchi, acetylcholinesterase, carboxylesterase, TOCP.

INTRODUCTION

Intoxications with organophosphorus cholinesterase inhibitors used as insecticides constitute an increasing problem in industrial and agricultural toxicology. Similar compounds, e.g. soman, are considered as potential warfare agents due to their high toxicity and their suitable physio-chemical properties. A large number of animal experiments have accumulated in the past years concerning the pharmacological effects of such compounds following various routes of injection. However, very few experiments have been published in the open literature on inhalation of organophosphorus agents. The main route of entry to the body of toxic vapours are through the respiratory tract and through the skin. The skin offers some protection by stratum corneum, the respiratory tree is freely permeable to most toxic vapours.

Inhalation systems can be characterized as static, when the agent is introduced into a chamber as a batch and then mixed, or dynamic, when airflow and introduction (and removal) of agent are continuous. The duration of static exposure is limited by the accumulation of carbon dioxide, the accumulation of water vapour, the gradual increase in temperature and the gradual depletion of oxygen inside the chamber. In a dynamic system, as the one presented here, these problems are avoided. There are further less difficulties with adsorption of the agent to the walls in the inhalation chamber or to the skin and fur of the animals.

Vapours can be generated by using one of several flow-dilution devices (Cotabish et al., 1961; Drew & Lippman, 1971; Nelson 1971; Saltzmann 1971; Salzmann & Warburg 1965). If the gas to be studied is a liquid at room temperature, a vapourization step must be included. One procedure is to use a motor-driven syringe and to apply the liquid to a wick or heated plate in a calibrated stream of air (Nelson & Griggs, 1968). Another method is to saturate the airstream with vapour and

AD-A145 106 MODULATION OF THE CHOLINERGIC MECHANISMS IN THE BRONCHIAL SMOOTH MUSCLE(U) NORWEGIAN DEFENCE RESEARCH ESTABLISHMENT KJELLER P AAS JUN 84 NDRE/PUBL-84/1001 2/2

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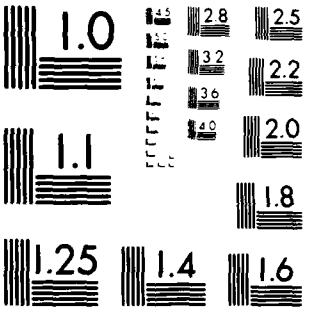

then dilute it with air to the desired concentration (Cotabish et al., 1961).

Most of the commercially available instruments for inhalation studies were developed for occupational hygiene, where the main problem is long exposure (days, weeks or months) of several animals in large cages to not very toxic chemicals. Our aim was to study the inhalation toxicity of the highly toxic compound soman in a system which made it possible to handle only small amounts of agent in a dynamic flow system. In addition the system had to be rapidly equilibrated and safe in operation. We have therefore developed an inhalation instrument which satisfies these criteria.

We have previously investigated the toxicity of soman after cutaneous, subcutaneous and intraperitoneal administrations and studied its inactivation by enzymes (Fonnum & Sterri, 1981; Sterri et al., 1980, 1981,1983). In the present paper we have studied the inhibition of acetylcholinesterase, cholinesterase and carboxylesterase activities of serveral organs after inhalation of soman. The role of carboxylesterases in detoxification of soman are elaborated.

METHODS

Inhalation apparatus

The aim of the present paper is to present a recently developed system for performing inhalation experiments with highly toxic vapours, such as organophosphorus agents, in a closed test system. The inhalation system is a dynamic system with a continuous introduction of the toxic vapour. The system consists of a diffusion cell for generation of the toxic vapour, the inhalation chamber and a carbon filter (Figure 1). The constant airflow through the system (3000 ml/min, prewarmed to 22°C) could be adjusted by needle-valves and flowmeters. One part of the airflow is passed through the diffusion cell and one part through a bypass.

Traces of oil residues and water vapour were removed from the air (100 ml/min) before entering the diffusion cell through a gas purifier packed with molecular sieve. The toxic vapour is generated by a diffusion cell. The cell is made of stainless steel and is divided by a polymer membrane, through which the toxic liquid from the vapour phase will diffuse at a constant rate. In the present experiments a high density polyethylene membrane with a thickness of 70 µm was fitted in the diffusion cell. The toxic liquid soman (100-200 µl) was applied through an orifice to a filterpaper in the upper part of the diffusion cell by using a disposable syringe. The penetration of the toxic vapour is dependent on the vapour pressure of the agent and the properties of the membrane, with lower diffusion through high density membranes. The gas concentration can therefore be varied by a factor of several thousand depending on the temperature and the material and the thickness of the membrane (Figure 2).

The toxic vapour are diluted and mixed with air from the airflow bypassing the diffusion cell before entering the inhalation chamber. In the inlet to the inhalation chamber the air and toxic vapour pass through a diffuser. The main function of the diffuser is to establish

a homogenous gas-concentration in the 1100 ml inhalation chamber. The toxic vapour from the outlet of the chamber passes through an activated charcoal bed. The instrument is kept in a fume hood to eliminate any risk of toxification of the personnel operating the instrument. To reduce the risk of wall loss, the diffusion cell and pipes were constructed of stainless steel and the sylindrical inhalation chamber of glass. To assure accurate measurements of the total inhaled dose, gas samples (100 ml/min, 30 min) were collected in isopropanol (Uvasol,10 ml, 0°C) before and after completion of the inhalation experiment, and analyzed on a gas chromatograph. Changes in pCO_2 were measured continuously in a capnometer by infrared spectroscopy (Hewlett Packard, 47210A).

Diffusion membrane

Diffusion velocity measurements with several different polymeric membranes (area; 12.56 cm^2) (Figure 2) were examined. The experiments were performed by connecting the thermostatically controlled diffusion cell, with membranes fitted, directly to a flame ionization detector (Carlo-Erba). The carrier-gas was N_2 (100 ml/min). The membrane temperature was controlled by a Cu-constantan thermocouple and the temperature variation was less than ±0.5°C. The diffusion barriers were prior to the experiments equilibrated at +20°C and 40% relative humidity. By selecting various membranes a large range of toxic vapour concentrations may be produced.

Animals

Male Wistar rats (200-300 g) (Møllegaard, Copenhagen) were kept in standard laboratory cages, 5 in each, for 1-2 weeks before start of experiment with free access to a standard laboratory diet and water. There was a light/dark cycle of 12 hours. They were kept on sawdust which was renewed every day to assure that the concentration of ammonia was low. The rats were without symptoms of diseases or infections in the lungs or in the respiratory tract.

Soman

Soman [o-(1,2,2 trimethylpropyl)-methyl-phosphonofluoridate], assessed to be more than 95% pure by Nuclear Magnetic Resonnance spectroscopy, was synthesized in this laboratory.

Tri-ortho-cresyl-phosphate

Tri-ortho-cresyl-phosphate (TOCP) 90-95% pure (K & K Laboratories) was dissolved in 1.2-propandiol (Fluka AG) and administered subcutaneously (100 mg/kg) 24 hours before the inhalation experiment. Injected volume was 0.1 ml/200 g.

Determination of
cholinesterase activity

Following decapitation, heparinized whole blood was centrifuged (1100 g, 10 min) and the supernatant (plasma) diluted 10-20 times in 20 mM sodium phosphate buffer pH 7.4 before assay. The erythrocyte pellet from 100 µl whole blood was washed twice, dissolved in 0.5 ml of 2.0% Triton X-100, and adjusted to 1.0 ml with 20 mM sodium phosphate buffer, pH 7.4 before assay. Homogenates of several organs were prepared with a Potter-Elvehjem or an Ultra Turrax homogenizer; bronchi (2%)(wt/vol), lung (10%), diaphragm (5%), liver (10%), duodenum (10%) and cerebrum (10%) in 20 mM sodium phosphate buffer pH 7.4. The homogenates were diluted with the sodium phosphate buffer containing 0.5-2% Triton X-100 before assay.

The cholinesterase activities were measured by the radiochemical method of Sterri & Fonnum (1978).

Determination of
carboxylesterase activity

Carboxylesterase activities were determined by a radiochemical method of

Sterri et al.(in preparation) measuring the hydrolysis of methyl[1-^{14}C]butyrat. 50 µl of 1% (wt/vol) bronchi, 0.25% lung, 2% diaphragm, 0.1% liver, 1% duodenum, 10% cerebrum or undiluted plasma were incubated with 500 µl of 0.1 M sodium phosphate buffer pH 7.8 containing 1.9 mM methyl [1-^{14}C]-butyrat at 30°C for 30 min. Then 0.5 ml of 0.1 M sodium phosphate buffer and 1 ml of chloroform were added before shaking and centrifugation (1000 g, 5 min). 0.5 ml of the aqueous phase was counted in 10 ml of Instagel scintillation cocktail (Packard Instrument Company).

Statistics

Means and standard error of the mean (SEM) or standard deviation (SD) were calculated for all data. Significance for differences between the mean values were calculated by the Student's t-test for independent and dependent groups.

RESULTS

Characteristics of the inhalation instrument

The range of the toxic vapour concentration for several membranes of different materials fitted in the diffusion cell for broard temperature ranges is shown in Figure 2. It is important to note that different membrane materials may establish gas concentrations within wide concentration ranges and the concentration increase proportionally with the increase in diffusion-cell temperature. In this dynamic inhalation system the airflow was constant during the exposure (3000 ml/min). The concentration of agent in the inhalation atmosphere was measured prior to start of inhalation and at the end of the inhalation experiment. Gaschromatographic analysis showed that a stable air concentration of the toxic vapour was obtained after 30 minutes and could be maintained for several hours after a stable diffusion cell temperature was attained. In independent tests there were no

significant variation in the atmospheric concentration of soman, as shown in Table 1. Test samples were also drawn from the pipeline before the air-gas mixture inlet (A, Figure 1) and at the outlet (B, Figure 1) and there were no significant differences in the soman concentration. Performing experiments at a diffusion cell temperature of 40°C, the soman concentration in the air inlet to the inhalation chamber was 0.73±0.03 ng/l (SEM, n=6) and 0.68±0.03 ng/l (SEM, n=6) in the air passing through the charcoal bed. This confirms no loss of soman in the inhalation chamber with no significant ($p>0.30$) difference in soman concentration. Several independent applications of soman to the diffusion cell did not increase the air concentration of soman above what was measured before the previous application, but the concentration of the toxic vapour was limited by the fitted membrane only (the material in the membrane and membrane temperature). It was, therefore, no structural changes in the polymeric membranes. Leaving the diffusion cell at room-temperature without new applications of soman for 2, 4 or 8 days gave only minor variations (±5%) in the atmospheric concentration of soman when a new experiment was started.

Due to the high flow of air through the inhalation chamber there were no CO_2-accumulation, no change in temperature, no water-vapour accumulation or O_2-depletion, although total animal volume was approximately 20% of the total chamber volume. The changes in pCO_2 were measured continuously with a capnometer, and there were no significant variations in the CO_2-concentration.

Experiments with rats

The sequence in which the gross symptoms of toxification by soman occurred, was the same as if soman was administered by injection. Salivation was regularly an early symptom. The generalized fibrillations were rapidly followed by convulsions. The LC_{50} curve was steep and the LC_{50} value was 400 mg min/m^3 with a 24 hour observation period after completion of the experiment (Figure 3). The lethal dose of

soman by inhalation was 520±60 mg min/m^3 by continuous exposure until death (Table 3). The inhaled LC_{50} dose was 117 µg/kg when assuming a respiratory minute-volume for the rat to be approximately 73 ml (Sanockij, 1970) and the animal weight was on average 250 g. Isolating the rat except nose and mouth in a small plexiglas tube inside the inhalation chamber, in exposures of less than 30 min, had only minor or no effects on the lethal dose. Pretreatment of the rats with the carboxylesterase inhibitor tri-ortho-cresyl-phosphate (TOCP) 24 hours before exposure to soman, reduced the lethal dose by approximately 70% to 172 mg min/m^3 when animals were observed until death (Table 3). In contrast, pretreatment with phenobarbital (100 mg/kg, i.p.) which increases the carboxylesterase activities in liver and serum but not in lung by 70% relative to control (not shown), increased the lethal dose by approximately 30%. This effect of phenobarbital was reduced by TOCP.

The activities of carboxylesterases, acetylcholinesterase and cholinesterases in different tissues showed a concentration dependent inhibition by soman (Table 2). In the bronchi and lung, the organs first exposed to the toxic vapour, there was a marked decrease in all enzyme activities. It was a larger inhibition of the carboxylesterase activities in the bronchi than in lung tissue. The liver carboxylesterase and cholinesterase activities were not inhibited. The brain, diaphragm and duodenum cholinesterase activities were on the other hand strongly inhibited by inhalation of soman, but the carboxylesterase activities in the diaphragm and duodenum were not significantly reduced.

DISCUSSION

Inhalation instrument

The results show that the inhalation instrument is suitable for experiments with highly toxic gases, such as organophosphorus compounds. The advantages of the system developed are mainly that experi-

ments can be run for several hours and days at constant homogenous concentrations of toxic vapour with only a small amount of toxic liquid. The highly toxic gases are completely isolated from the atmosphere in a closed dynamic inhalation system. Since there was no variation in the concentration of soman at inlet and outlet of the chamber one can conclude that there was no wall loss in this inhalation system.

In addition to the complete mixing that occured before the two airstreams entered the inhalation-chamber, there was also a mixing effect both by the diffuser and by the constant animal movement. Therefore, there is strong reason to believe that a homongenous gas concentration was established in the inhalation chamber.

There was only a minor decrease in gas concentration from respiration or from absorption, although the animal to chamber volume is rather high (20%). This is due to the relatively high flow of gas-air mixture (3000 ml/min) through the inhalation chamber of 1100 ml. Licking of the fur and extremities could interfere and increase the total absorbed dose when performing experiments of long duration (weeks), and nose exposure systems would therefore in some instances be more suitable. The gas concentration in the inhalation chamber was kept at a constant level, and could be varied within wide concentration limits depending only on the membrane, membrane material and the diffusion cell temperature. Therefore it is possible to perform both acute and chronic exposure studies. When performing chronic studies, the advantage with the present system is the simple method for production of a constant concentration of a toxic vapour. A disadvantage when doing chronic experiments is the low number of animals exposed. On the other hand with some reconstructions more animals than one might be exposed in the system.

The CO_2 produced by the rats was continuously flushed out of the chamber as measured with the capnometer. There was no increase in CO_2 concentration and therefore no stimulation of the respiration and increase in the respiratory minute-volume. Consequently there were not any increase in the absorbed dose of soman due to a stimulation of the respiration.

Experiments with rats

Since only very few experiments on inhalation of the organophosphorus agents have been published there are very few comparable data in the literature. Crook et al.(1969) and Oberst (1961) have compared the toxicity of methylphosphonic difluoride vapour with vapour of sarin (a closely related organophosphorus compound to soman) and the results indicate a large variation in the mortality data. The LC_{50} of sarin vapour from 24 hr mortality data in rat was 320 mg min/m^3. This was lower than for mouse (515 mg min/m^3) but somewhat higher than for dog (135 mg min/m^3) and monkey (83 mg min/m^3). Fredrikson et al.(1960) studied the effects of sarin in anaesthetized and unanaesthetized dogs following inhalation and determined a mean lethal dose of 300 mg min/m^3 in unanaesthetized dogs and 400 mg min/m^3 in anaesthetized animals. Although there are considerably variations in the toxicity data on inhalation exposure to sarin, the data for sarin are in the same range as we have found.

There was a steep LC_{50} curve (Figure 3), which indicate that the rats received only a small dose of soman in excess when kept until death in the inhalation chamber, compared to animals taken out of the chamber prior to death. The acute LD_{50} of soman determined by subcutaneous injection in rat was 80 µg/kg (Sterri et al., 1980), by intramuscular injection 96 µg/kg (Elsmore, 1981) and 110 µg/kg (Petras, 1981) or by intraperitoneal injection 200 µg/kg (Sterri et al., 1980). This is in the same range as what was estimated in rat by inhalation (117 µg/kg) by using 73 ml as the respiratory minute volume in rat (Sanockij, 1970).

Since the respiratory tree is the primary route of entry for nerve agents or organophosphorus insectisides, this is also the organ first exposed to these toxic chemicals. The bronchial smooth muscle contracts on application of soman (Fonnum et al., 1984) in addition to the large secretion of mucus which is induced in the airways during

inhalation of soman. Soman penetrates rapidly from the respiratory tree to the bloodstream, indicated by the low activities of the plasma cholinesterases and erythrocyte acetylcholinesterease. The enzymes were inhibited to the same degree as the cholinesterases in lung and bronchi (Table 2). On the other hand the carboxylesterase activities in the respiratory tissues were higher than in plasma. The enzyme activities in the accessory muscles of respiration such as the diaphragm are also strongly inhibited as shown by the low levels of cholinesterase and acetylcholinesterase activities in this tissue. But the carboxylesterase activities were only marginally inhibited in this muscle, as was also shown in the liver and in the duodenum. In liver this is probably due to the presence of a high phosphorylphosphatase activity (Sterri et al., 1983).

Injection of TOCP, a treatment which inhibits the carboxylesterase activities (Myers, 1959; Sterri et al., 1981), reduced the lethal dose inhaled by the rats by approximately 70%. This illustrates the important function of these enzymes in binding soman as a part of the detoxifying mechanism. The importance of the carboxylesterases was also shown by the relatively high activities these enzymes exhibited in both lung and bronchi, which indicate that the lung is a primary tissue in the detoxification of vapours, such as vapours of soman. The carboxylesterases and cholinesterases were inhibited to nearly 100% in the bronchi, which show that the bronchial smooth muscle is strongly affected by soman and therefore contracts during a soman intoxication (Fonnum et al., 1984). This is due to the excess of acetylcholine in the synaptic cleft and stimulation of postsynaptic muscarinic receptors in the smooth muscle. This may be one of the primary causes of death in addition to weakness of the accessory muscles of respiration.

In conclusion, the inhalation instrument has proved to be a relevant instrument in the study of toxicity by inhalation of highly toxic liquids such as soman and several advantages of the instrument has been pointed out. In our opinion knowledge of the influence of organophosphorus agents on the respiratory system and the cholinergic part of its nervous system may be of great value in the prophylactic treatment of organophosphorus intoxication.

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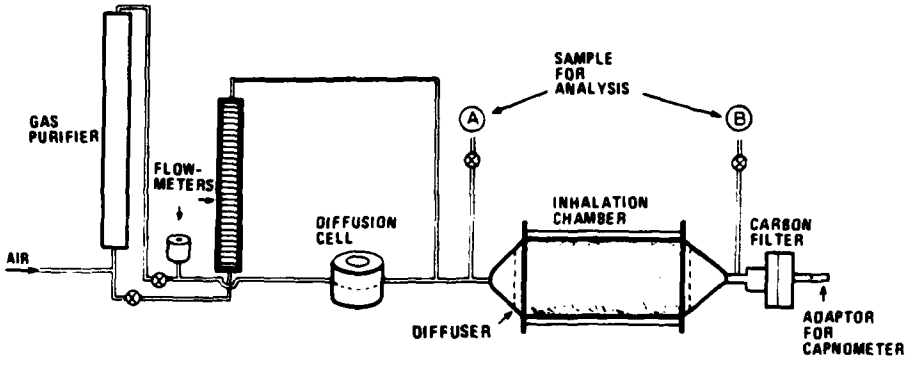
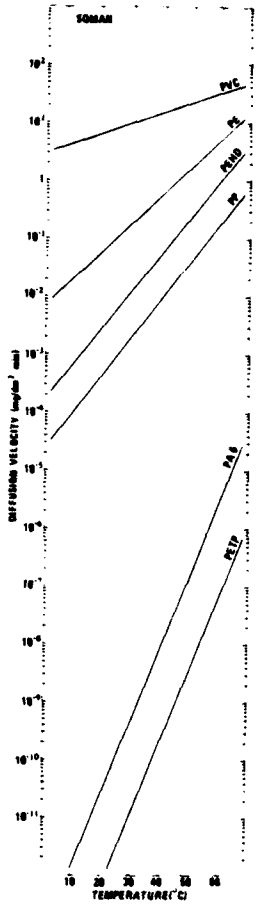


Figure 1. Schematic diagram of the inhalation instrument

Figure 2. The diffusion velocity (mg/dm^2× min) of soman through different diffusion barriers (membranes) as a function of temperature (°C).



PVC = Polyvinylchloride (50 µm)
PE = Polythylene (15 µm)
PEHD = Polyethylene-high density (70 µm)
PP = Polypropylene (23 µm)
PA = Polyamide (20 µm)
PETP = Polyethyleneterepthalat (12 µm)

Employing the equation:

$$Log_{10}(D) = A_i + B \times T \quad ,$$

the diffusion velocity (D) may be calculated by inserting values for temperature (°C) and the respective constants (A_i and B) from the table below.

D = Diffusion velocity (mg/dm^2× min)
A_i = Intercept y-axis
T = Temperature (°C)
B = Slope

Membrane material	A_i ± SD	B ± SD	n
PVC	-0.219 ± 0.045	0.0176 ± 0.0011	6
PE	-2.348 ± 0.087	0.0484 ± 0.0019	6
PEHD	-4.326 ± 0.106	0.0570 ± 0.0018	7
PP	-4.568 ± 0.164	0.0570 ± 0.0025	8
PA	-11.228 ± 0.805	0.0935 ± 0.0083	7
PETP	-12.272 ± 0.212	0.0914 ± 0.0020	5

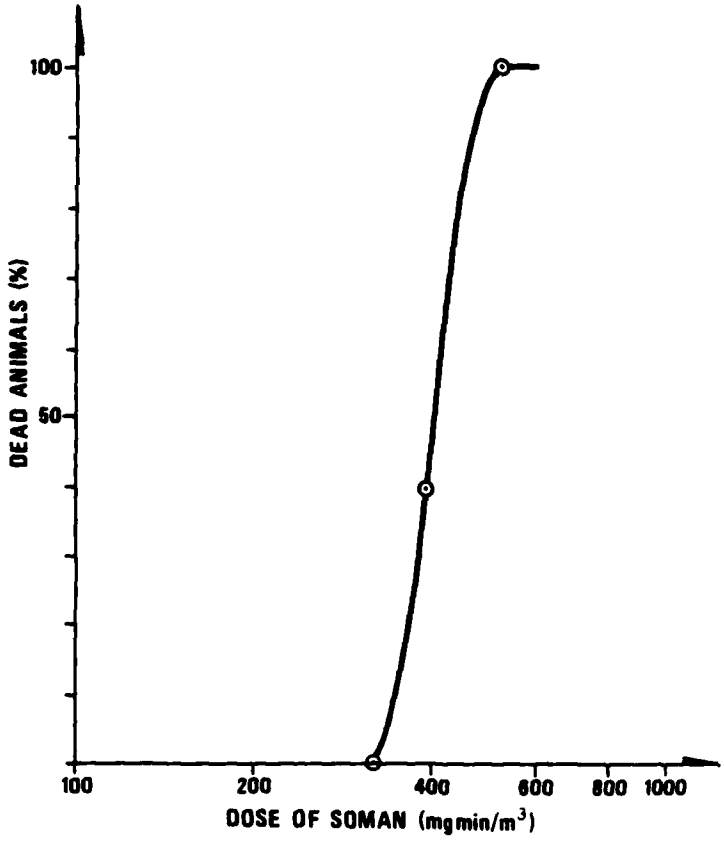


Figure 3. Mortality of rats following inhalation of soman. Each point represents 6 animals.

TABLE 1 Concentrations of soman in the inhalation atmosphere

Diffusion-cell temperature (^{o}C)	Concentrations of soman (µg/l)	
	t_o	t_1
70	21.1±1.2	21.4±1.2

The concentrations (µg/l) of soman ±SEM (n=10) before start of experiment (t_o) and after completion of inhalation experiment, approximately 8 hours later (t_1).

TABLE 2 THE EFFECT OF SOMAN ON ALIESTERASE, ACETYLCHOLINESTERASE AND TOTAL CHOLINESTERASE ENZYME ACTIVITIES IN RAT BY INHALATION

A CONTROL	ENZYME ACTIVITIES IN CONTROL ANIMALS (nmol hr^{-1} mg tissue^{-1}) ± SEM (n = 12)																							
	BRONCHI			LUNG			PLASMA			ERYTHROCYTE			BRAIN			DIAPHRAGM			LIVER			DUODENUM		
	CarbE	ChE	AchE	CarbE	ChE	AchE	CarbE	ChE	AchE	CarbE	ChE	AchE	CarbE	ChE	AchE	CarbE	ChE	AchE	CarbE	ChE	AchE	CarbE	ChE	AchE
	1213 · 82	50 · 3	21 · 1	3305 · 488	28 · 4	8 · 1	0 · 1	23 · 2		-		49 · 5	-	404 · 30	512 · 21	200 · 20	66 · 6	86 · 7	11124 · 700	33 ± 2	-	1054 · 121	204 ± 17	104 · 17
B SOMAN DOSE (mg min/m^3)	ENZYME ACTIVITY (% OF CONTROL) · SEM (n = 6)																							
	CarbE	ChE	AchE	CarbE	ChE	AchE	CarbE	ChE	AchE	CarbE	ChE	AchE	CarbE	ChE	AchE	CarbE	ChE	AchE	CarbE	ChE	AchE	CarbE	ChE	AchE
520 00 (LETHAL)	23 · 6	3 · 0 4	11 · 1	53 · 3	3 · 0 6	9 · 1	13 · 2	6 · 1	-	-	-	4 ± 0.3	-	3 ± 0.7	1 ± 0.3	77 · 14	5 · 0 4	5 · 0 6	89 · 6	88 · 7	-	101 · 18	55 · 6	20 · 3
	**	***	***	***	***	**	***	***				*		***	**	*	***	***	*	*		*	**	***
317 · 9	55 · 10	15 · 1	25 · 3	101 · 16	10 · 0 9	32 · 11	43 · 8	14 · 2	-	-	-	7 · 1		30 · 9	46 · 13	105 · 25	52 · 6	52 · 7	102 · 11	78 · 7	-	123 · 19	85 · 6	62 · 3
	*	*	*	*	**	**	***	**				***		***	*	*	***	**	*	*		*	*	*
207 · 3	81 · 9	30 · 13	40 · 6	72 · 7	27 · 6	43 · 4	98 · 12	43 · 15				10 ± 3		87 · 3	75 · 2	91 · 8	84 · 7	80 · 6	116 ± 12	72 ± 3	-	108 ± 21	88 · 9	72 · 10

CARBOXYLESTERASE (CarbE), ACETYLCHOLINESTERASE (AchE) AND TOTAL CHOLINESTERASE (ChE) ACTIVITIES (% OF CONTROL) IN DIFFERENT TISSUES FROM RAT AFTER TREATMENT WITH SOMAN BY INHALATION (D) (ATMOSPHERIC CONCENTRATION OF SOMAN WAS 21 $\mu g/l$). THE RESULTS WERE COMPARED WITH UN TREATED RATS AS *CONTROLS (Al. (-. INDICATE LOW ACTIVITY)*

(* p 0.05 · **: ** p 0.05 *** p 0.01)

TABLE 3 Lethal dose of soman in rat by inhalation

Treatment of rats	Lethal dose in rat	
	mg min/m^3	µg/kg
Untreated	520±60	142±16
TOCP-treated	172± 7	57± 4

The lethal dose ±SEM(n=6) of soman in rat by inhalation in untreated rats and rats pretreated with TOCP (tri-ortho-cresyl-phosphate) (100 mg/kg, S.C.) 1x24 hrs before inhalation experiment. The lethal dose (µg/kg) was estimated on the basis of a respiratory minute volume of 73 ml. The air concentration of soman was 21 µg/l.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