FACTORS AFFECTING THE RELEASE OF TRANSMITTERS

IN THE HEART

A thesis presented for the Degree of Doctor of Philosophy

by

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CONTENTS

PUBLICATIONS

Various aspects of the work presented in this thesis have been published. Reprints of the following articles may be found inside the back cover. The references are:-

BOYLE S. J. & POLLOCK, D. (1987).

   Effects of drugs on field stimulation-induced ^{3}H-noradrenaline

   overflow and responses of rat isolated atria.

   J. Phsyiol. 386: 53P.

BOYLE, S. J. & POLLOCK, D. (1987).

   Effects of clonidine and atropine on field stimulation-induced

   ^{3}H overflow in rat isolated atria.

   Abstract. P193. IUPHAR 10th Int. Congr. of Pharmacology, Sydney.

BOYLE, S. J. & POLLOCK, D. (1988).

   Interactions between adrenergic and cholinergic nerves in the rat

   isolated atria.

   Br. J. Pharmac. 93: 25P.

BOYLE, S. J. & POLLOCK, D. (1988).

   Involvement of guanylate cyclase in overflow of radioactivity in

   rat atria incubated in [^{3}H]-noradrenaline and [^{14}C]-choline.

   Br. J. Pharmac. (In Press).

BOYLE, S. J. & POLLOCK, D. (1988).

   Effects of sodium nitroprusside on responses of the heart rate to

   nerve stimulation in anaesthetised and pithed rats.

   Br. J. Pharmac. (Submitted).

SUMMARY

1. The aim of this study was to investigate the presynaptic
    interactions that exist between the adrenergic and cholinergic
    nerves in the rat heart.

2. The negative chronotropic response of the heart rate, evoked by
    electrical stimulation of the vagus nerve in the pithed rat, was
    enhanced when the sympathetic cardio-accelerator nerves were
    stimulated simultaneously.

3. The negative chronotropic response of the heart rate, evoked by
    electrical stimulation of the vagus nerve in the anaesthetised
    rat, was inhibited by clonidine and potentiated by a combination
    of yohimbine and prazosin.

4. Field stimulation of rat, isolated, spontaneously beating paired
    atria resulted in a complex post-stimulus response. The initial
    negative inotropic component of this response was abolished by
    atropine. The positive inotropic component of this response was
    inhibited by propranolol and guanethidine. Atropine and yohimbine
    both potentiated the positive inotropic component. Clonidine
    inhibited the positive inotropic component only in the presence of
    atropine.

5. Clonidine and acetylcholine inhibited the field stimulation-
    evoked overflow of ^{3}H from atria previously incubated in [^{3}H]-NA.
    Yohimbine and atropine potentiated the overflow of ^{3}H from such
    atria.

6. Clonidine and acetylcholine inhibited the field stimulation-evoked overflow of ^{14}C from atria previously incubated in [^{14}C]-choline. Atropine and yohimbine potentiated the overflow of ^{14}C from such atria. The results suggest that field stimulation of atria causes the simultaneous release of NA and ACh and that these transmitters neuromodulate each others release.

7. Part of the study investigated the role of cyclic nucleotides in governing the field stimulation-evoked release of NA and ACh from rat atria.

8. Isobutylmethylxanthine potentiated the field stimulation-evoked overflow of ^{3}H from atria previously incubated in [^{3}H]-NA.

9. In the absence and in the presence of atropine, 8-Bromo-cAMP potentiated the field stimulation-evoked overflow of ^{3}H from atria previously incubated in [^{3}H]-NA.

10. Sodium nitroprusside and 8-Bromo-cGMP both potentiated the field stimulation-evoked overflow of ^{3}H from atria previously incubated in [^{3}H]-NA. In the presence of atropine, sodium nitroprosside was unable to potentiate ^{3}H overflow.

11. Sodium nitroprusside inhibited the field stimulation-evoked overflow of ^{14}C from atria previously incubated in [^{14}C]-choline in the absence and in the presence of prazosin and yohimbine. The results suggest that sodium nitroprusside indirectly potentiated the overflow of ^{3}H by removing the cholinergic-mediated restraint on NA release.

12. In the pithed rat, sodium nitroprusside inhibited the negative chronotropic response of the heart rate evoked by electrical stimulation of the vagus nerve. In the anaesthetised rat, sodium nitroprusside inhibited the same response but this required the presence of prazosin and yohimbine.

13. Part of the study investigated the effects of chronically treating rats with thyroxine (T_4). The level of T_4 in the serum was increased in rats pretreated with T_4, and this was accompanied by changes in pre- and post-synaptic receptor sensitivity.

14. Pretreatment with T_4 produced a postsynaptic supersensitivity to isoprenaline amd a postsynaptic subsensitivity to phenylephrine. A presynaptic subsensitivity to clonidine, but not to ACh, was produced by T_4 pretreatment. In these pretreated animals the ability of clonidine but not that of ACh to inhibit the field stimulation-evoked overflow of ^{3}H was diminished.

INTRODUCTION

The modern concept of the autonomic nervous system is based on the anatomical and physiological studies of Gaskell and Langley. Gaskell demonstrated the functional connection through the white rami communicates between the central nervous system and the sympathetic chains, and the connections of the grey rami to spinal nerves. Gaskell designated the cranial, sacral and thoracolumbar outflows from the spinal cord together with the sympathetic trunks and the prevertebral and other ganglia as the "involuntary nervous system". Langley first proposed the designation "autonomic nervous system", which included both the sympathetic and parasympathetic divisions (Dale, 1954). Both divisions of the autonomic nervous system are usually tonically active. The sympathetic nerves exert an excitatory influence on the heart and this is opposed by the inhibitory parasympathetic influence. These mutually antagonistic effects are not additive algebraically suggesting that complicated interactions occur between the opposing nerves (Levy, 1971; 1984).

The classical studies of Otto Loewi (Loewi, 1921; Loewi & Navratil, 1926 a, b) provided evidence for the release of an active substance during stimulation of the vagus nerve of the frog heart. Consequently the ground work was laid for the modern concept of neurohormonal transmission. In addition, the studies provided an early indication of the complex nature of neural control of the heart.

Loewi's observations could not be immediately corroborated because of technical difficulties arising from inadvertent stimulation of sympathetic fibres in the mixed vagosympathetic trunk. Stimulation of the trunk produced cardiac inhibition and, at other times, excitation. The intimate anatomical association of sympathetic and parasympathetic

fibres innervating the heart leads to complex interaction between the two divisions of the autonomic nervous system. Such interactions have clouded the issue of neural control of the heart with conflicting and confusing observations.

There are three possible sites of interactions between the two divisions of the autonomic nervous system.

1). In the central nervous system.

2). At the prejunctional level of the terminal nerve fibres.

3). At the postjunctional level of the effector tissue.
Interaction at parasympathetic ganglion cells may arise but this is less likely in the heart.
Much of this study is concerned with the adrenergic-cholinergic interactions arising at the prejunctional level of the terminal nerve fibres in the heart of the rat.

NEUROCHEMICAL TRANSMISSION

At the beginning of this century several reports were published which postulated the existence of "receptive substances" for adrenaline and which demonstrated the similarity between the effects produced by adrenaline and sympathetic nerve stimulation on smooth muscle (Langley, 1901; 1905; Brodie & Dixon, 1904; Elliot, 1905). It was suggested that this parallelism occurred because adrenaline stimulated sympathetic nerve endings (Brodie & Dixon, 1904). However, from studies of denervated tissues it was concluded that adrenaline acted directly on smooth muscle (Elliot, 1905). Elliot (1905) suggested that the effect of adrenaline was mediated by a "myoneuronal junction" whereas Langley referred to this structure as a "receptive-substance" (Langley, 1905).

Elliot proposed that nerve stimulation might also involve a myoneuronal junction and could therefore be mediated by chemical transmission. Such a suggestion was proposed by Elliot in 1905 when he suggested that sympathetic nerves might release adrenaline. Unfortunately, the concept was not well received (see Dale, 1953) and, consequently, was not pursued for several years. Indeed, experimental support was not obtained until it was demonstrated that a chemical mediator, "acceleranstoff" was released from sympathetic nerve terminals innervating the frog heart (Loewi, 1921; Loewi & Navratil, 1926 a,b). This substance was later found to be adrenaline (von Euler, 1946).

Langley (1905) postulated that different receptor subtypes might exist when he stated "different receptive substances are formed, the responsiveness of which to nervous stimuli varies". The nature of these different receptive substances was almost discovered by Henry Dale (Dale, 1906; Barger & Dale, 1910). Moreover, the techniques initiated by Dale were similar to those adopted by Ahlquist almost 40 years later, in his successful classification of the adrenergic receptor (see below).

Initially, Dale (1906) demonstrated that the motor effects evoked by adrenaline or by sympathetic nerve stimulation were reduced or transformed into an inhibitory effect ("adrenaline reversal") in the presence of ergot preparation. Subsequently, Dale found that the relative potencies of and actions produced by a series of amine agonists varied in different smooth muscle preparations (Barger & Dale, 1910). These results prompted him to suggest that the motor and inhibitory effects evoked by adrenaline and nerve stimulation were mediated by different receptor types. In this latter report Barger &

Dale did not study the cardiac effect of these amines, an effect which had previously been shown to be relatively resistant to ergot (Dale, 1906). Although Dale's classification for smooth muscle preparations was correct, the cardiac effects of sympathomimetic agents are now known to be mediated by the normally inhibitory beta-adrenoceptor (Ahlquist, 1948).

Following Loewi's demonstration of neurotransmission in the 1920's, research into the nature of adrenergic transmission was concentrated on determining the identity of this sympathetic mediator. Barger & Dale (1910) concluded that noradrenaline (NA) rather than adrenaline (Elliot, 1905) was more likely to be the chemical mediator of sympathetic nerve transmission. Unhappily, Dale's findings were overlooked and the general opinion continued to be that this mediator was adrenaline (see von Euler, 1971).

In order to explain the apparent disparity in effects evoked by adrenaline and sympathetic nerve stimulation, it was suggested (Cannon & Rosenbleuth, 1933) that there were two types of adrenaline (Sympathin); Sympathin E and Sympathin I. Cannon & Rosenbleuth believed that before Sympathin could activate a muscle cell, it must be bound with another substance in the cell and that the evoked response should be proportional to the amount of combined substance formed. For Sympathin to have such diverse effects then it must be bound and/or modified in different ways by different cells; Sympathin E being formed in cells which it excites and Sympathin I in cells which it inhibits. This sophisticated hypothesis was partially correct insofar as it examined post-receptor events and predicted, albeit unknowingly, the intracellular production of second messengers. Unfortunately, Cannon & Rosenbleuth (1933) proceeded to suggest that

Sympathin E or I could circulate in the blood stream and activate other tissues. Although the Sympathin E and Sympathin I theory received support from Stehle & Ellsworth (1937) it was not generally accepted (Dale, 1954).

The identity of Sympathin was eventually revealed by von Euler (1946) mainly through the multiple biological assay technique pioneered by Gaddum & Kwiatowski (1939). Unfortunately, von Euler employed Cannon's terminology and suggested that Sympathin E was noradrenaline (NA) and that Sympathin I was adrenaline (1946).

Two years later, Ahlquist classified the adrenergic receptors (Ahlquist, 1948). By observing the relative potencies of a series of sympathomimetic amines on different test systems Ahlquist postulated that there were two receptive substances; the alpha- and beta-adrenoceptors. The alpha-adrenoceptor mediated mainly motor effects and was activated more easily by NA than by isoprenaline which could not be antagonised by ergot. The beta-adrenoceptor mediated mainly inhibitory effects (the notable exception being the heart) and was stimulated more easily by isoprenaline than by NA.

A parallel similar to that between the effects of adrenaline and sympathetic nerve stimulation had been observed for the actions of parasympathetic nerve stimulation and the alkaloid muscarine. The effects of exogenous muscarine and parasympathetic nerve stimulation could be blocked by atropine (Dixon, 1907). The concept of chemical transmission was subsequently applied to parasympathetic fibres (Dale, 1914; Le Heux, 1919 and 1921; Dale & Dually, 1929; Loewi 1921; Loewi & Navratil, 1926 a,b). Proof of the hypothesis was provided by the demonstration of the existence of acetylcholine (ACh) in the

perfusion fluid from the mammalian neuromuscular junction following stimulation of the motor nerve (Dale et al, 1936). The effect of curare-like substances in preventing ACh-induced twitch-like responses of skeletal muscle (Brown et al, 1936) lent further support to this view as did the ACh intensifying action and the anti-curare effect of anti-cholinesterase drugs (Brown, et al, 1936; Eccles, 1937).

MORPHOLOGY

A major problem in investigating neurotransmission and the actions of ACh and NA in the heart has been the interactions between the sympathetic and parasympathetic nerves (Levy, 1984). Loewi (1921) encountered this problem when on stimulating the vagal trunk to the heart both acceleration and deceleration of the beating heart was noted.

Sympathetic fibres to the heart emerge from the spinal cord with the upper five or six thoracic white rami, enter the paravertebral sympathetic chains and synapse with the three cervical ganglia and with the upper five thoracic ganglia. The heart, therefore, receives postganglionic sympathetic fibres from a wide area. The post ganglionic sympathetic fibres end around the conducting tissues of the heart and also diffusely in the muscle fibres of both atria and ventricles. Sympathetic nerve stimulation increases pacemaker activity and increases the rate of conduction of impulses through the conducting tissue leading to tachycardia. The parasympathetic supply to the heart is from the vagus, the preganglionic fibres of which synapse with the diffusely arranged ganglion cells situated on the atria. Post-ganglionic fibres arising from these ganglion cells

innervate the conducting tissues of the heart; the sino-atrial node and the atrio-ventricular bundle (Bundle of His) (Day, 1979). Extensive cholinergic innervation of the atria has been detected (Nonindez, 1939) but cholinergic innervation of the ventricles has proven more difficult to establish (Higgins et al, 1973). In various regions of the cat (Brown, 1976) and rat heart (Stanley et al, 1978) the ACh content decreased in the order; right atrium>left atrium>right ventricle>left ventricle. Thus, the potential for greatest interaction between the sympathetic and parasympathetic nerves lies in the atria, in particular at the sino-atrial node (Nilsson & Sporrong, 1970). The terminal cholinergic innervation is composed of long chains of varicose fibres similar to the adrenergic innervations. Varicosities of both fibres exhibit dense accumulations of vesicles and are assumed to release the transmitter en passage, that is, in response to an action potential running from one varicosity to the next. The average density of varicosities (adrenergic and cholinergic) in the frog heart is 1.9 varicosities/100 μm^2 in the sinus venous. Particularly in the atria there appears to be close apposition between adrenergic and cholinergic nerve varicosities and the cardiac tissue (Bojsen-Moller & Tranum-Jensen , 1972).

Postganglionic vagal and sympathetic fibres are often in close apposition in rat atria and, indeed, they may be surrounded by a common Schwann sheath (Ehinger et al, 1970).

Early indications of the presence of cholinergic fibres within postganglionic sympathetic nerve trunks came from experiments demonstrating that muscarinic blocking agents inhibited and anticholinesterases enhanced responses to sympathetic nerve stimulation. In several tissues a release of ACh from organs as a

result of sympathetic stimulation was shown (Campbell, 1970). In addition, there is also an apparent simultaneous activation of adrenergic and cholinergic nerves in the vagosympathetic trunk. Several studies have demonstrated a cardiostimulatory response at the cessation of vagal stimulation (Levy et al 1966; Donald et al, 1967; Misu & Kirpekar, 1968).

The ACh likely to influence sympathetic transmission is probably contained within discrete cholinergic axons. In most cases they represent parasympathetic fibres mixed with the sympathetic nerve supply. However, the existence of separate cholinergic-sympathetic fibres cannot be excluded (Campbell, 1970). The anatomical evidence thus favours an interaction both at a prejunctional and a postjunctional level.

In summary, the mammalian heart receives innervation from the sympathetic and parasympathetic divisions of the autonomic nervous system. These nerves modulate such cardiac activities as sino-atrial and atrio-ventricular automaticity, conduction of the cardiac impulses and the strength of atrial and ventricular contraction.

NON-ADRENERGIC, NON-CHOLINERGIC TRANSMISSION

In the peripheral autonomic nervous system the presence of non-adrenergic, non-cholinergic (NANC) nerves as well as adrenergic and cholinergic nerves has been demonstrated both functionally and morphologically in isolated preparations of the intestine, (Burnstock, 1972; Gershon, 1981) urinary bladder (Ambache & Zar, 1970) and blood vessels (Lee et al, 1978). Immunohistochemical studies have shown the presence of peptide containing nerves, which are distinct from

adrenergic and cholinergic nerves in the heart (Reinecke et al, 1980; Papka et al, 1981; Della et al, 1983). An NANC nerve-mediated response in the guinea-pig and rat right atria has recently been demonstrated (Saito et al, 1986) but has yet to be confirmed. The NANC nerve-mediated response that followed trans-mural stimulation was slow in onset and long lasting and appeared only under certain conditions.

In recent years evidence has accumulated which indicates the existence of neurones containing more than one neurotransmitter —the phenomenon of co-transmission. There are four types of co-transmitter neurones, classification being based on their neurochemical content. The locations of such neurones are widespread and include the central nervous system, adrenal medulla, preganglionic and postganglionic fibres (O'Donohue et al, 1985). Immunohistochemical studies have demonstrated the existence of neuropeptide Y (NPY) containing nerve fibres in atria and to a lesser extent in the ventricles (Allen et al, 1982). NPY is a 36 amino acid peptide (Tatemoto, 1982) and co-exists with NA in many of the sympathetic nerves supplying the cardiovascular bed. NPY produces positive inotropic and chronotropic effects on the isolated spontaneously-beating guinea-pig atria (Lundberg et al, 1984). Other studies however, have shown that NPY is without effect on Lagendorff preparations and in rat isolated atria (Allen, 1986). NPY is reported to be an inhibitor of NA release from adrenergic nerves evoked by electrical stimulation in the vas deferens, femoral artery and portal vein of the rat (Edvinsson et al, 1987). It seems unlikely, therefore, that NA and ACh are the sole neurotransmitters in the heart. However, the physiological roles of NPY and other putative transmitters are still to be elucidated.

POST-SYNAPTIC RECEPTORS IN THE HEART

Adrenergic receptors are classified into isoreceptors according to the relative potencies of various adrenergic agonists and on the susceptibility of the receptor to blockade by specific drugs (Ahlquist, 1948; Powell & Slater, 1958; Lands, 1967; Furchgott, 1972). There are alpha and beta adrenergic receptors (adrenoceptors) on the myocardium. Receptors for ACh are divided into two classes; muscarinic and nicotinic based on their differential sensitivity to the alkaloids muscarine and nicotine (Dale, 1914). Several studies have been carried out regarding muscarinic isoreceptors. There appears to be at least three types of muscarinic receptor classified as M_1, M_2 (Burgen, 1984) and M_3 (Michel & Whiting, 1988). This classification has developed from observations of the distinct pharmacological and biochemical properties of receptors present in various tissues. The M_2 receptor subtype is present on the myocardium (Breitweiser & Szabo, 1985).

The consequences of receptor activation by endogenous neurotransmitters or by exogenous agonists are complex. The parasympathetic neurotransmitter, ACh, regulates atrial rate, nodal conduction and atrial and ventricular contractility through activation of muscarinic receptors (Higgins et al, 1973). The molecular mechanisms that mediate these parasympathetic responses have not been clearly elucidated. ACh hyperpolarizes atrial cells by increasing potassium permeability (Glitsch & Pott, 1978; Fleming et al, 1981) and decreases the slow inward current in some preparations (Hutter, 1961; Giles & Noble, 1976; Pappano et al, 1982). Biochemical responses also accompany cardiac muscarinic receptor activation. These include increased cyclic 3'5'-guanosine monophosphate (cGMP) production via

activation of guanylate cyclase (Mirro et al, 1979; Endoh, 1980) and

stimulation of phosphatidylinositol metabolism (Brown & Brown, 1983,

1984). In addition, ACh activation of the muscarinic cholinoceptor

inhibits beta-adrenoceptor-mediated cyclic 3'5'-adenosine

monophosphate (cAMP) production, but has little effect on basal cAMP

levels (Ingebretsen, 1980).

The consequences of adrenergic receptor stimulation are more readily

understood. It is believed that beta-adrenoceptor activation

increases the rate and force of contraction of the heart. Both these

effects are mediated via activation of adenylate cyclase which

increases cAMP levels which in turn increases the availability of

·intracellular calcium (Loffelholz et al, 1985). The existence of

alpha-adrenoceptors mediating positive inotropic effects in the

mammalian heart is well established (for review see Scholz et al,

1986). Recently, it has been suggested that activation of this

receptor results in the stimulation of phosphatidylinositol metabolism

(Schmitz et al, 1987; Scholz et al, 1987).

There is much discussion in the literature as to how postjunctional

adrenoceptor and cholinoceptor activation interact to control heart

function (Brown, 1979; Brown & Brown, 1984; Linden et al, 1985).

PRESYNAPTIC RECEPTORS

Noradrenergic nerve endings are concerned with the synthesis, storage,

release and inactivation of NA. Recent evidence suggests that

neurotransmitters, hormones and autocoids, such as the prostaglandins,

can all influence the release of neurotransmitters by acting on

presynaptic receptors situated on the nerve terminal. This evidence

was contrary to the original theory of neurochemical transmission which suggested that in the peripheral nervous system the magnitude of the tissue response was proportional to the rate of transmitter release which was in turn controlled by nerve impulse frequency which ultimately was determined centrally (Dale, 1952). This theory was challenged intermittently throughout the years but was generally accepted. In recent years, however, remarkable changes have occurred concerning neurotransmission and it is now generally accepted that nerve terminals are invested with a rich variety of receptors that regulate transmitter release (Stjarne, 1975; Langer, 1977; Starke, 1977, 1981).

The effects of agonists and antagonists on the release of transmitter in response to nerve stimulation has provided evidence for the theory of local regulation. The overflow of the transmitter is that which is recovered in the bathing fluid while transmitter release is deemed to be the amount actually liberated (Gillespie, 1980).

The first observations regarding the effects of alpha-adrenoceptor blockade were derived from experiments in the cat spleen. Phenoxybenzamine (PBA), an alpha-adrenoceptor blocking agent, increased the stimulation-induced overflow of NA in this preparation (Brown & Gillespie, 1957). The increase in transmitter collected in the venous effluent was attributed to a blockade of the post-synaptic alpha-adrenoceptors by PBA, preventing the released NA from combining with the receptors. Overflow of transmitter also increased in relation to the frequency of stimulation; an effect attributed to flooding the receptors with transmitter.

This suggestion was challenged by Paton (1960), who performed similar experiments on the adrenal gland, a tissue devoid of post-synaptic alpha-adrenoceptors. Similar increases in transmitter overflow were observed in response to increasing the frequency of stimulation. Paton suggested that the released amines from both the adrenals and the sympathetic nerves were taken back into the cell from which they were released. Thus, the increase in transmitter overflow at higher frequencies of stimulation was caused by the reduction in time available for uptake of the amine. The existence of a neuronal uptake mechanism (Uptake 1) was soon established (Hertting et al, 1961, a). Uptake 1 was found to be blocked by cocaine (Whitby et al, 1960) and PBA (Hertting et al, 1961, b). Later, a second extraneuronal uptake mechanism (Uptake 2) that could be blocked by PBA but not by cocaine was characterised (Iversen, 1965; Avakian & Gillespie, 1968). Since low concentrations of PBA could inhibit neuronal uptake of NA (Iversen, 1965; Eisenfield et al, 1967), it was suggested that the increase in NA overflow could be related to the blockade of either or both of these sites of loss for the released transmitter.

The suggestion was however unfounded, since complete inhibition of neuronal uptake of NA can be achieved with agents which do not block the alpha-adrenoceptors such as cocaine or desipramine without producing a concomitant increase in transmitter overflow (Blakely et al, 1963; Geffen, 1965). In addition there is evidence that alpha-adrenoceptor antagonists increase transmitter overflow either at concentrations that failed to inhibit neuronal uptake (Starke, 1971; Enero et al, 1972) or even in the presence of potent inhibitors of the neuronal amine pump (Starke, 1977). These observations prompted several independent laboratories to suggest that an increase in the amount of NA released by nerve impulse could occur in the presence of

alpha-adrenoceptor antagonists (Haggendal, 1970; Langer 1970; McCulloch et al, 1972).

Dopamine beta hydroxylase is an enzyme present in adrenergic storage vesicles. It is released with NA by exocytosis during nerve stimulation and is not affected by uptake or degradative enzymes. An increase in overflow of this enzyme reflects an increase in transmitter release. In support of the view that alpha-adrenoceptor antagonists could increase the amount of NA released by nerve impulse it was reported that the release of the enzyme dopamine beta hydroxylase was increased when neurotransmission in the cat perfused spleen was studied in the presence of PBA or phentolamine (De Potter et al, 1971). On the basis of the enhanced exocytotic release of NA observed with alpha-adrenoceptor blocking agents the presence of presynaptic alpha-adrenoceptors involved in the regulation of NA release was postulated (Farnebo & Hamberger, 1971; Kirpekar & Puig, 1971; Langer et al, 1971; Starke, 1971). Activation of these alpha-adrenoceptors is believed to inhibit NA release and it has been suggested that they form part of a negative feedback control mechanism by which the transmitter may regulate its own release (Langer, 1980; Starke, 1977). Several pieces of evidence support this hypothesis.

Alpha-adrenoceptor antagonists enhance the stimulation-evoked release of NA in almost all adrenergically-innervated tissues examined, irrespective of the nature of the postsynaptic receptors on the effector. Enhancement of the evoked-release of NA occurs in the presence of uptake blockers. Antagonists of alpha-adrenoceptors enhance the stimulation-evoked release of the enzyme dopamine beta hydroxylase, an effect which is not seen with amine uptake blockers. A wide range of alpha-adrenoceptor agonists decrease the evoked release

of NA in almost all noradrenergically-innervated tissues examined. In addition, alpha-adrenoceptor agonists abolish the ability of alpha-adrenoceptor antagonists to facilitate the stimulation-evoked release of NA. Antagonists of alpha-adrenoceptors block or reduce the inhibitory effects on neurotransmitter release produced by alpha-adrenoceptor agonists. Finally, inactive enantiomers such as (+)-NA fail to depress the evoked release of NA (Westfall, 1984).

The theory of presynaptic receptors regulating transmitter release is not readily accepted by all workers. Perhaps one observation in particular has caused the voice of dissent to be raised. At high frequencies of stimulation alpha-adrenoceptor antagonists have little effect on transmitter release (Brown & Gillespie, 1957). In much the same way alpha-adrenoceptor agonists also have little effect on transmitter release at high frequencies of stimulation. The latter observation is more readily explained. It is possible that at high frequencies of stimulation the concentration of neurally-released NA is such that the presynaptic alpha-adrenoceptors are fully activated, thus a maximum inhibition is being exerted and additional exogenous agonist is ineffective (Langer, 1977; Starke, 1977). The observation that antagonists have little effect at high frequencies of stimulation cannot be explained in the same way. If at high frequencies of stimulation NA is exerting a maximum inhibition by activating presynaptic alpha-adrenoceptors then alpha-antagonists should be able to further enhance the release of transmitter. In an attempt to explain this anomaly an alternative hypothesis has been postulated (Kalsner, 1984; Kalsner & Quillan, 1984; Kalsner, 1985). The alpha-antagonist-induced increases in overflow should be proportional to the level of ongoing feedback in the absence of antagonists and explicable in terms of the amount of NA in the synapse to activate presynaptic

receptors during stimulation (Forsyth, 1987). However in a variety of tissues from several species this was not found (Kalsner & Quillan, 1984). The alpha-adrenoceptor antagonists proved to be most effective at low frequencies of stimulation when the concentration of transmitter in the synapse was low. The release of transmitter from sympathetic nerves is directly related to the duration of the action potential. If the duration of the action potential is prolonged, the calcium channels stay open longer leading to greater entry of calcium and to an increased release of transmitter (Baker, 1971). Yohimbine and PBA may prolong the duration of depolarisation by indirect modification of the calcium gating mechanism (Kalsner, 1985). This hypothesis is substantiated by the potentiating action of tetraethylammonium (TEA) on transmitter overflow. TEA selectively blocks outward potassium permeability directly, causing prolongation of the action potential and consequently neurosecretion (Kalsner & Quillan, 1984).

Although both the pre- and the post-synaptic alpha-adrenoceptors are stimulated by alpha-adrenoceptor agonists and blocked by alpha-adrenoceptor antagonists, it appears that the post-synaptic alpha-adrenoceptors mediating the responses of the effector organ are not identical with the presynaptic alpha-adrenoceptors that regulate the release of NA during nerve stimulation. Originally, alpha-adrenoceptors were subclassified on the basis of differing anatomical location before complete analysis of their sensitivity to agonists or antagonists could be assessed; $alpha_1$ was the post-junctional receptor whereas $alpha_2$ was the prejunctional receptor (Langer, 1974). The prejunctional or $alpha_2$ receptors were presumed to be located on or near the terminal regions of sympathetic nerves. In accordance with receptor theory, it has been demonstrated that the relative potency of

a series of agonists differed markedly at the pre- and post-junctional site in the rabbit pulmonary artery (Starke et al, 1975). Generally, agonists such as clonidine, alpha-methyl NA and tramazoline were more potent at the prejunctional site whereas phenylephrine and methoxamine were more potent postjunctionally.

The demonstration that $alpha_2$ adrenoceptors were also located in non-neuronal tissues eg platelets, invalidated Langer's (1974) original anatomical classification and receptors are now designated purely on the basis of drug selectivity (Starke & Langer, 1979). Recent studies, based on drug selectivity, have demonstrated the presence of presynaptic $alpha_1$-adrenoceptors in the pithed rat heart and vas deferens (Docherty, 1984). Activation of these receptors inhibits NA release as reflected by a decreased postjunctional response to nerve stimulation.

In addition to the presynaptic alpha-adrenoceptors regulating NA release it appears that the adrenergic nerve ending is endowed with a variety of receptors that serve the same purpose. For example, facilitation of the stimulation-evoked release of NA through presynaptic beta-adrenoceptors has been proposed (Langer et al, 1974). Further support for the presence and role of presynaptic beta-adrenoceptors was obtained in experiments in which exposure to low concentrations of isoprenaline facilitated release of NA during low frequency nerve stimulation (Adler-Graschinsky & Langer, 1975; Pelayo et al, 1978).

Experimental evidence obtained with specific agonists suggest that the presynaptic facilitatory beta-adrenoceptors might be of the $beta_2$ subtype (Gillespie,1980). However in skeletal muscle of the cat the

presynaptic beta-adrenoceptors are blocked by metoprolol a $beta_1$-adrenoceptor antagonist suggesting that in this case they are of the $beta_1$ subtype. This suggests that the nature of the presynaptic beta-adrenoceptor may differ between the somatic and the autonomic nerves.

It is possible that presynaptic facilitatory beta-adrenoceptors are mainly activated by circulating adrenaline to enhance noradrenergic neurotransmission. This view is supported by the observation that when adrenergic nerves are labelled with adrenaline (instead of NA) propranolol, the beta-adrenoceptor antagonist, becomes more effective in reducing transmitter release elicited by sympathetic nerve stimulation (Rand et al, 1979).

In addition, there is evidence for the existence of inhibitory dopamine receptors situated on peripheral noradrenergic nerve endings. These presynaptic dopamine receptors designated to be of the DA_2 subtype appear to be responsible for the hypotension and bradycardia produced by certain dopamine agonists (Lokhandwala & Jandyala, 1979).

Electrically-stimulated release of NA can be regulated by morphine acting via presynaptic opiate receptors (Dubocovich & Langer, 1980; Forsyth, 1987) and inhibited by prostaglandins of the E series (Hedqvist, 1970), by adenosine and adenine nucleotides (Hedqvist & Fredholm, 1976) and by serotonin acting on presynaptic serotonin receptors on the noradrenergic nerve terminals of vascular tissues (McGrath, 1977).

In addition to the presynaptic receptors mentioned above there exists an inhibitory presynaptic, muscarinic receptor. Activation of muscarinic receptors on adrenergic nerve endings results in inhibition

of NA release. These receptors are not only activated by drugs but also by the endogenous transmitter released from cholinergic nerves. The studies that ultimately led to the concept of muscarinic inhibition of NA release were initially designed to investigate the effects of nicotinic drugs on the peripheral post-ganglionic adrenergic nerve fibre. The rabbit perfused heart preparation was used for this work as it permitted pre-ganglionic stimulation of the vagus nerve. In these experiments the nicotinic actions of ACh were compared with those of dimethylpiperazinium (DMPP). Whereas ACh has both muscarinic and nicotinic effects, DMPP is a purely nicotinic agonist. As often occurs, discoveries such as these depend on serendipity and indeed, it was noted that after accidental omission of atropine (normally present in the perfusion fluid) that the transmitter output into the perfusion fluid was greatly inhibited by ACh but not by DMPP. It was soon established that muscarinic receptor activation or inhibition did not interfere with the neuronal uptake or output of NA from the heart. Atropine did not enhance the amine liberating effect of ACh (via nicotinic receptors) by delaying its metabolism. The post synaptic effects of cholinomimetic drugs on heart rate, myocardial tension development and coronary flow could not explain the facilitatory action of atropine on NA overflow evoked by ACh (Lindmar et al, 1968).

These findings led to the suggestion that the peripheral adrenergic nerve fibre contains inhibitory muscarinic receptors in addition to the excitatory nicotinic receptors mediating NA release (Lindmar et al, 1968). It was subsequently shown that exogenous application of ACh could also inhibit electrically-evoked NA output in the rabbit perfused heart (Loffelholz & Muscholl, 1969). This effect was blocked by atropine but not by hexamethonium or DMPP (Loffelholz and

Muscholl, 1969), thus confirming the clear distinction between the excitatory nicotinic effect and inhibitory muscarinic effect on transmitter release (Muscholl, 1980).

PRESYNAPTIC ADRENERGIC-CHOLINERGIC INTERACTIONS IN THE HEART

The possibility of cholinergic-adrenergic interactions has been the subject of much experimentation and several review articles (Burn & Rand, 1962; Campbell, 1970; Kosterlitz & Lees, 1972; Fozard, 1979; Levy, 1971 and 1984). Since the discovery of the autonomic grand plexus in which terminal cholinergic and adrenergic axons run side by side without intervention of insulating Schwann cell processes (Hillarp, 1959), many such fibres have been found in a variety of systems. These include the iris (Ehinger et al, 1970), vas deferens (Thoenen & Tranzer, 1968), myenteric plexus of the gut (Manber & Gershon, 1979) and atria (Thoenen & Tranzer, 1968; Ehinger et al, 1970).

There is now a large body of evidence which suggests that simultaneous stimulation of adrenergic and cholinergic nerves results in endogenous ACh-mediated inhibition of release of NA by activation of presynaptic muscarinic receptors situated on adrenergic nerve terminals. Transmitter release studies have revealed such a mechanism in hearts from cat (Haeusler et al, 1968), dog (Levy & Blattberg, 1976), and rat (Fuder et al, 1982;) but not in the guinea-pig (Story et al, 1975) or chicken (Engel & Loffelholz, 1976). The case for endogenous cholinergic regulation of NA release is therefore well documented.

Recent evidence suggests that endogenous ACh and NA regulate ACh release from cholinergic nerves, particularly in the gut. For example, exogenous ACh inhibits the field-stimulated induced release of [³H] from guinea-pig ileum previously incubated in [³H]-choline. This inhibition could be blocked by atropine (Fosbraey & Johnson, 1980). A reciprocal adrenergic-cholinergic inhibitory interaction has been proposed following experiments carried out on the rabbit jejunum. This study involved simultaneous stimulation of the perivascular and cholinergic nerves. Under such circumstances the release of [³H] was inhibited. Sympathetic denervation by 6-hydroxydopamine (6-OHDA) pre-treatment prevented this inhibition, suggesting an adrenergic involvement in the inhibition of ACh release. Since atropine enhanced the release of [³H]-NA during simultaneous nerve stimulation it appears that ACh inhibits the release of NA in the rabbit jejunum (Manber & Gershon, 1979). Morphological evidence also supports the view that adrenergic nerves synapse with cholinergic nerves in the myenteric plexus suggesting that a reciprocal adrenergic-cholinergic inhibitory interaction is not unreasonable. There is considerable pharmacological evidence for the existence of presynaptic alpha-adrenoceptors on the parasympathetic nerves in the gut (Knoll & Vizi, 1970; Wickberg, 1978; Davey, 1980) in addition to the presynaptic muscarinic receptors proposed by Fosbraey & Johnson (1980).

There exists, therefore, a substantial body of evidence for a reciprocal adrenergic-cholinergic inhibition in the gut. Such a mechanism would require the release of NA from sympathetic nerves to be inhibited by the activation of prejunctional muscarinic receptors by ACh, released from the adjacent parasympathetic nerves and similarly, the release of ACh to be inhibited by activation of

prejunctional alpha-adrenoceptors by endogenous NA. The evidence is much less substantial for such a system occurring in cardiac tissue.

However,such an inhibitory cholinergic-adrenergic interaction has been proposed for the heart (Rand et al, 1975 and 1980), although little evidence has emerged to verify its existence. Differing experimental techniques and species differences have prevented verification of such an interaction. Thus, the guinea-pig atria has produced conflicting and confusing results over the last few years. For example, no evidence was found for inhibitory presynaptic receptors on the cholinergic nerve terminal in this preparation (Lew & Angus, 1983). Furthermore, no evidence was found for the inhibition of cholinergic transmission by NA released from sympathetic nerves. In contrast to this, by examining the field-stimulated induced release of [^{3}H]-ACh from atria previously incubated in [^{3}H]-choline it can be shown that exogenous NA inhibits the release of ACh from the guinea-pig atria (Loiacono & Story, 1986). The inhibitory effect of exogenous NA was abolished by idazoxan and phentolamine but not by prazosin suggesting the inhibition was mediated by activation of presynaptic alpha$_2$-adrenoceptors. However, since clonidine could not inhibit [^{3}H]-ACh release, there remains some doubt as to the nature of the receptor. In addition to this anomaly, NA was ineffective in blocking the negative chronotropic responses to extrinsic nerve stimulation in atrial preparations with a branch of the vagus intact. Since neither phentolamine, idazoxan nor prazosin had any effect on the stimulation induced release of [^{3}H]-ACh, no evidence for an endogenous NA-mediated attenuation of ACh release has been found in the guinea-pig atria. Similarly, in the chick heart no evidence was found for presynaptic alpha-adrenoceptors on cholinergic nerve terminals or for an

inhibitory action of endogenous NA on ACh release (Loffelholz et al, 1984).

Studies on the rat atria have, however, proved fruitful. Potassium-induced [^{3}H]-overflow from rat atria previously incubated in [^{3}H]-choline can be inhibited by ACh and potentiated by atropine (Wetzel et al, 1985). This suggests that a mechanism for muscarinic feedback inhibition exists that is analogous to the autoregulation of NA release by alpha-adrenoceptors on adrenergic nerve endings (Wetzel & Brown, 1985). In addition alpha-adrenoceptor agonists inhibit the potassium-evoked release of [^{3}H]-ACh via a presynaptic alpha$_1$-adrenoceptor situated on the cholinergic nerve terminal (Wetzel et al, 1985).

The rat atria provides a good opportunity to examine possible cholinergic-adrenergic presynaptic interactions. This thesis will seek to investigate this possibility and to examine the effects of such an interaction on the field-stimulated release of NA and ACh.

UNDERLYING MECHANISMS OF TRANSMITTER RELEASE

The mechanisms by which activation of presynaptic beta-adrenoceptors facilitate release of transmitter and by which activation of presynaptic muscarinic cholinoceptors and alpha-adrenoceptors inhibit the release of NA has not yet been elucidated. With more recent evidence suggesting the presence of presynaptic inhibitory muscarinic cholinoceptors and alpha-adrenoceptors on the cholinergic nerve terminals (see above), there exists perhaps a new opportunity to examine events which regulate transmitter release. Transmitter release appears to be promoted by increasing cytosolic free calcium

concentration (Blaustein, 1979). A nerve action potential opens calcium voltage-operated channels in the varicosities thus increasing the cytosolic free calcium concentration. This increases calcium binding to the protein calmodulin. The calcium-calmodulin complex activates kinases which trigger the release of transmitter (Illes, 1986). The mechanism by which presynaptic receptor activation perturbs this system is unknown. One possible hypothesis is that the adrenoceptor and cholinoceptor effects are due to alterations in adenylate cyclase activity and consequently cyclic AMP formation (Jakobs, 1985; Illes, 1986). It is suggested that the $alpha_2$-adrenoceptor and possibly the muscarinic cholinoceptor are coupled to adenylate cyclase via the inhibitory G protein G_i. When activated by their respective agonists the receptors reduce cAMP production and consequently protein kinase A (PKA) dependent phosphorylation. It is thought that PKA and the calcium-calmodulin complex feed into the same phosphorylation pathway. PKA increases the calcium sensitivity of the release process. Therefore, agents that reduce cAMP production reduce calcium sensitivity and transmitter release. Conversely, facilitatory presynaptic beta-adrenoceptors are thought to be coupled to adenylate cyclase via the stimulatory G protein G_s. Activation of beta-adrenoceptors enhances adenylate cyclase activity, increases cAMP production and promotes transmitter release. Additionally, PKA causes phosphorylation of voltage-operated calcium channels causing an increase in intracellular calcium (Rosenthal & Schulz, 1987). Therefore, presynaptic receptor activation can affect transmitter release in two ways, first by affecting the calcium sensitivity of the release process via PKA and secondly, by affecting calcium influx also via PKA.

It has been suggested that the presynaptic muscarinic cholinoceptor and the presynaptic $alpha_2$-adrenoceptor on the adrenergic nerve terminal in the rat atria are linked to a common pathway, since the effect of ACh on transmitter release was enhanced in the presence of the $alpha_2$-adrenoceptor blocking agent phentolamine (Loiacono et al, 1985). A possible candidate for such a pathway is the phosphorylation pathway described above.

Further support for the involvement of cyclic nucleotides in transmitter release is provided by the observation that stimulation-induced release of NA is enhanced by cell permeable analogues of cAMP (dibutyryl-cAMP, 8-bromo cAMP) as well as phosphodiesterase inhibitors that inhibit the breakdown of cAMP (Wooten et al, 1973; Stjarne et al, 1979; Hentrich et al, 1985) and the adenylate cyclase activator forskolin (Hovevei-Sion et al, 1983; Alberts et al, 1985).

Recent studies in mouse atria (Johnston et al, 1987) have shown that prejunctional beta-adrenoceptor effects were mediated through adenylate cyclase while the inhibitory alpha-adrenoceptor effects were not. In addition, it was found that the cGMP analogue, 8-bromo cGMP facilitated the stimulated-induced release of NA. This effect, also reported by Cubeddu (Cubeddu et al, 1975), was assigned to inhibition of cAMP phosphodiesterase activity. The selective cGMP phosphodiesterase inhibitor M & B 22948 $(9_\nu M)$ failed to enhance the stimulation-induced overflow of NA. However at $(90_\nu M)$ an enhancement was observed.

The enhancement of NA release by permeant cGMP analogues is not a common observation, indeed increases in neuronal cGMP levels are usually associated with inhibition of NA release (Pelayo et al, 1978).

The presence of a calcium-dependent presynaptic mechanism for the generation of cGMP has been described (O'Dea & Zatz, 1976). This cGMP generating system appeared to be linked to an alpha-adrenoceptor because the increased cGMP levels obtained by either NA or potassium were blocked by alpha-adrenoceptor blocking agents. Exposure to dibutyryl cGMP reduced the potassium-evoked release of $[^3H]$-NA from the rat pineal. In addition, a decrease in the stimulation-evoked release of $[^3H]$-NA was observed during exposure to an inhibitor of a cGMP phosphodiesterase (Pelayo et al, 1978). These results suggest that, in the rat pineal gland, cGMP may be a link in the chain of events following activation of presynaptic alpha-adrenoceptors and may lead to a decrease in the release of NA. In contrast, in the cat perfused spleen and in the rat vas deferens analogues of cGMP did not affect the release of NA during nerve stimulation (Langer, 1980). However, an important effect of cGMP was observed in cortical slices of rat brain. In this preparation cGMP derivatives reduced the stimulation evoked release of ACh through a presynaptic muscarinic receptor (Yonehara et al, 1980). This observation has serious implications when examining the role of cyclic nucleotides in the atria, due to the possible influence that the adrenergic and cholinergic systems may exert over one another.

Therefore, in view of the close apposition of adrenergic and cholinergic nerves in preparations such as the atria (Ehinger et al, 1970) and the possible interactions between the two divisions of the autonomic nervous system (Rand et al, 1975 ; 1980) it is perhaps imprudent to assign drug induced effects to one aspect of the system without considering that the drugs may also be affecting the other aspect. Consequently, the apparent direct effects of drugs on the sympathetic division of the autonomic nervous system may be

indirectly due to an action on cholinergic nerves or to a combination of both effects. Due to interaction between the two sets of nerves a misinterpretation of results may occur. This thesis will examine the role of cyclic nucleotides in transmitter release in the rat atria and will attempt to interpret the results achieved against a background of possible adrenergic cholinergic interaction.

SENSITIVITY CHANGES IN AUTONOMIC EFFECTORS

The survival of the organism is often governed by its ability to adapt to its own environment. Such adaptations, as prompted by the environment, range from evolutionary changes taking many years to develop, to the rapid adaptions required to protect the animal from hostility (Gibson, 1981). Rapid responses often depend on the release of chemical mediators which provide a physiological means of adapting tissue sensitivity to meet the demands of the animal (Guyton, 1971). The continued existence of an organism is maintained by two systems of prime importance; the autonomic nervous system and the endocrine glands (Bernard, 1878). Both of these systems are involved in influencing the sensitivity of cardiac muscle. Under certain conditions skeletal, cardiac or smooth muscle can be rendered either supersensitive or subsensitive to various stimuli. Supersensitivity is believed to have occurred when the amount of stimulant required to produce a given response is less than normal. The converse is true when subsensitivity has occurred; that is a certain amount of agonist produces a smaller response.

Supersensitivity

Supersensitivity (Cannon & Rosenbleuth, 1949; Trendelenburg, 1972; Trendelenburg and Graefe, 1975; Fleming et al, 1973; Thesleff, 1974; Westfall, 1981) generally occurs as a result of the removal of a tonic stimulus. Surgical denervation provided an early opportunity to examine the phenomenon of supersensitivity (Budge, 1855). Denervation of the cat nictitating membrane results in two types of supersensitivity (Langer & Trendelenburg, 1966; Trendelenburg, 1966), one of which was specific for NA, the other being non-specific. The specific supersensitivity produced to NA was due to the destruction of a major site of loss for exogenously administered NA, that is, the adrenergic neurone. As a consequence of the removal of the site of loss, the concentration of agonist that reaches the receptors of the effector organ increases, resulting in an enhanced response.

Non-specific supersensitivity has been attributed to an increase in receptor number (Langer & Trendelenburg, 1968; Bito & Danson, 1970). Surgical denervation does not produce an immediate change in sensitivity. The phenomenon is slow in onset occurring several days after surgery and the sensitivity changes occur at several receptors. It is unlikely that the supersensitivity arises solely at the site of agonist receptor interaction. It is possible that the system is affected beyond the plasma membrane for example, at sites where the excitation of several receptors converge on a common biochemical pathway (Hudgins & Fleming, 1968; Fleming, 1968).

A leftward displacement of the dose response for one or more agonists reflects supersensitivity of a tissue to a receptor ligand (Cannon & Rosenbleuth, 1949). In such circumstances the maximum response of the tissue remains unchanged while responses to submaximal doses of the agonist are increased. However in some situations the maximum response of the tissue is increased and this can also be interpreted as a type of supersensitivity (Pollock et al, 1972; Gibson & Pollock, 1975).

Subsensitivity

Desensitisation of a tissue to a receptor ligand, or subsensitivity, is reflected in a rightward displacement of the dose response curve (Rang & Ritter, 1969). As with supersensitivity, a decrease in the maximum response of the tissue, coupled with, or in the absence of , a rightward displacement of the dose response curve can be interpreted as subsensitivity (Waud, 1975). Two types of desensitisation are recognised, namely homologous and heterologous.

The term heterologous desensitisation describes the non-specific reduction in sensitivity that occurs when cells are incubated with one agonist and subsequently found to be subsensitive not only to that agonist, but to several others (Su et al, 1970). Heterologous desensitisation involves functional uncoupling of receptors in the absence of sequestration or down-regulation (Harden, 1983).

Homologous desensitisation is the term used to described the specific desensitisation that occurs when cells are re-challenged with the same agonist used in the initial exposure. In this situation agonists other than that used in the initial exposure produce a normal response

of the tissue (Su et al, 1976). In the case of beta-adrenoceptors,
this type of desensitisation is thought to involve the sequestration
of the receptors away from the plasma membrane. The sequestered
receptors may then undergo degradation (Harden, 1983; Perkins &
Hertel, 1987).

Changes in the sensitivity of a tissue to a specific receptor ligand
are usually examined by one of two techniques. First, by using direct
receptor labelling techniques, radio-ligand binding, and secondly by
more traditional pharmacological methods using isolated tissues or in
situ experiments. Each technique has both advantages and
disadvantages. Radio-ligand binding minimises the problem of drug-
receptor equilibirum and eliminates the effects of drugs distal to the
receptor (Minneman & Molinoff, 1980 ; Nahorski, 1981). This
technique, which examines the specific binding of radiolabelled high
affinity agonists or antagonists, has been successful in demonstrating
that alterations in receptor number may contribute to at least some
forms of drug induced desensitisation (Creese & Sibley, 1981; Harden,
1983). In these experiments the lack of a biological response, often
due to the fact that highly specific antagonists with little intrinsic
activity are employed, is a disadvantage. This can be overcome by
employing traditional pharmacological techniques that allow the
biological response to be measured. There is perhaps an advantage in
being able to detect alterations in the mechanisms linking receptor
activation to a change in cellular response.

Circulating hormones released from the endocrine glands are known to
exert a regulatory influence on distant tissues. For example,
corticosteroids enhance the response of smooth muscle to
catecholamines (Gibson & Pollock, 1975). The thyroid hormones,

triiodothyronine (T_3) and thyroxine (T_4) are released from the thyroid gland which is under the control of a pituitary hormone, thyrotrophin. The thyroid hormones have wide ranging effects throughout the body on growth and metabolism. They also exert a strong influence on the sensitivity of sympathetically-innervated tissues to agonists (Gibson, 1981).

The similarity between the symptoms of hyperthyroidism and increased activity of the sympathetic nervous system is long established (Reith, 1865). It appears that neuro-effector response to catecholamines are enhanced in hyperthyroidism and diminished in hypothyroidism (Waldstein, 1966). In some circumstances, however, increased sympathetic activity is not observed in conditions of hyperthyroidism (MacMillan & Rand, 1962) and therefore the role of thyroidal influence governing the sympathetic nervous system is far from being elucidated.

Most studies on the effects of thyroid hormones on autonomic effector sensitivity have involved the use of cardiac tissue. Hyperthyroidism increases the sensitivity of the heart to beta-adrenoceptor agonists, and decreases the sensitivity of the heart to alpha-adrenoceptor agonists. Conversely, hypothyroidism enhances the response to alpha-adrenoceptor agonists and diminishes that of beta-adrenoceptor agonists (McNeill, 1987; Kunos, 1977). These changes in cardiac sensitivity are supported by ligand binding studies, which indicate that hyperthyroidism increases the number of beta-adrenoceptors and decreases the number of alpha-adrenoceptors, whilst hypothyroidism exerts the opposite effect (Kunos, 1977). In addition, thyroid hormones can apparently regulate the number of muscarinic receptors in the heart; hyperthyroidism decreases and hypothyroidism increases the number of muscarinic receptors (Sharma & Banerjee, 1977). Indeed, the

actions of the thyroid hormones are complex and may exert intracellular effects in addition to those at the level of the receptor (Tse et al, 1980; McNeill, 1987).

The apparent parallel changes in adrenergic receptor sensitivity and number led to the suggestion that there might be a single adrenoceptor that can exist as either the alpha- or the beta-adrenoceptor and which can interconvert to one or other according to the circulatory levels of T_4 (Kunos, 1977). This theory, unfortunately, fails to take into account the existence of prejunctional alpha- and beta-adrenoceptors known to exist in the heart and the effect that thyroid hormones may exert on the sensitivity and number of these receptors. Generally, ligand binding studies fail to take into account the anatomical site of non-neuronal and neuronal receptors. However, one study attempted to calculate the number of presynaptic alpha-adrenoceptors by comparing receptor binding in control and chemically sympathectomised atria. It was found that 33% of alpha-adrenoceptors appeared to be localised on the prejunctional sites (Sharma & Banerjee, 1978). Some doubt must be cast on this study however as the chemical sympathectomy will also affect the number and sensitivity of postjunctional receptors (Chiu, 1978; Hawthorn & Broadley, 1984).

It is difficult to accept a theory that proposes an allosteric change in a single receptor when the hypothesis fails to take into account that the proposed changes in receptor number and sensitivity occur at different anatomical sites.

Surprisingly little attention has been focussed on the effects of thyroid hormones on presynaptic receptors. This is despite the important role of these receptors in regulating transmitter release

at the neuroeffector junction (Gillespie, 1980). Part of this study,
therefore, investigated the effects of excess T_4 levels on the
sensitivity of both pre- and post-synaptic receptors in the rat atria.

The heart possesses a dense adrenergic and cholinergic innervation.
Since the earliest studies this has proved to be both advantageous and
disadvantageous to pharmacological examination. With this in mind the
study set out to determine:

(1)   The extent of presynaptic adrenergic-cholinergic interaction in
      the rat heart

(2)   To what extent does such an interaction influence the effect of
      drugs on the release of transmitter in the heart.

(3)   Are cyclic nucleotides involved in the release of
      neurotransmitters in the heart.

(4)   What effect does altering the levels of circulating T_4 have on
      the sensitivity of pre- and post-synaptic receptors.

MATERIALS AND METHODS

MECHANICAL RESPONSES OF THE RAT ATRIA

i) Preparation of Tissues

Adult, male, Wistar rats (250-320g) were killed by stunning and exsanguination. The thorax was opened and the heart was rapidly removed. The left and right atria were separated from the ventricles and placed in a beaker containing Krebs bicarbonate solution of the following composition (mM): NaCl (118.1), KCl (4.7), $MgSO_4$ (1.0), KH_2PO_4 (1.2), $CaCl_2$ (2.5), $NaHCO_3$ (25.0) and glucose (11.1).

The atria were then inserted into silver ring electrodes, the left atria being attached with thread to a hook at the bottom end of the electrode. The atria were then transferred to a 25ml double-jacketed organ bath containing Krebs solution at $35^{o}C$ and gassed with 95% O_2/5% CO_2. The paired atria were attached by thread from the right atria to a Statham force displacement transducer and placed under a resting tension of 1.0g. Changes in tension of the isolated atria were recorded isometrically on a Grass Polygraph (model 79D). Under these conditions the atria beat spontaneously at a mean rate of 251 ± 4 beats min^{-1},(n=38) and a mean force of 0.35 ± 0.01g. tension.

In some experiments the left atrium alone was inserted into silver ring electrodes and subsequently transferred to an organ bath whereupon it was placed under 1.0g tension. This preparation did not beat spontaneously but could be paced electrically at a constant frequency produced by electrical stimulation.

ii) Spontaneously Beating Paired Atria

The responses of isolated spontaneously beating paired atria to the beta$_1$-adrenoceptor agonist, isoprenaline, and the muscarinic cholinoceptor agonist, carbachol were examined. Increasing concentrations of the respective agonists were added to the organ bath to produce a cumulative dose-response. The effect of various concentrations of the agonists on the rate of atrial beating was examined.

The responses were examined in 3 ways. First, as absolute changes in the rate of beating caused by addition of the agonist. Secondly, as the incremental change caused by the specific concentration of agonist; this allows the maximum effect of the agonist to be seen. Finally, the results were expressed as a percentage of the maximum response of the tissue.

The effects of pretreatment with thyroxine (T$_4$) on the sensitivity of the atria to these agonists were examined. Thus, responses to these agonists from control animals and animals pretreated with T$_4$ were compared.

Spontaneously-beating paired atria were stimulated electrically by field stimulation through silver ring electrodes with a Palmer Square Wave stimulator or a Grass S88 stimulator. Field stimulation of atria caused the release of transmitters from nerve endings and produced a complex mechanical response immediately after cessation of stimulation (Fig 1). During stimulation the atria was driven electrically if the frequency of stimulation was above the inherent rate of beating. Immediately following stimulation the atria produced a complex
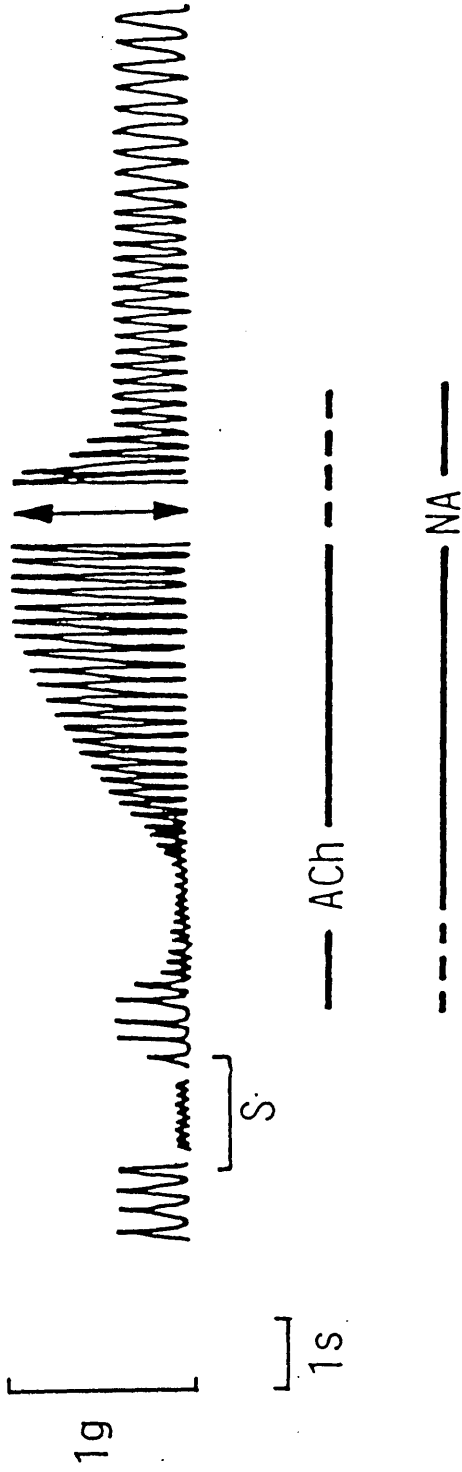
Post Stimulus Response

FIGURE 1. Schematic diagram of the response of the spontaneously beating paired atria that arises after field stimulation (S). The diagram illustrates the 3 components of the post-stimulus response. These are the negative inotropic component, the positive inotropic component and the positive chronotropic component.

response. First, a negative inotropic response developed which was transient and which was rapidly (2-3sec.) superseded by positive inotropic and chronotropic responses. The positive component of the response was also transient and the tissue returned to pre-stimulation control levels. The technique of stimulating spontaneously-beating paired atria has been employed previously (Idowu & Zar, 1977) but was not fully exploited. These workers confined their efforts to an examination of the chronotropic response of the atria. In the present study the three components of the post-stimulus response were examined and appeared to reflect the release of transmitters during the stimulation period. The preparation allowed examination of the effects of drugs which act to inhibit or promote the release of the transmitters or which inhibit the post-synaptic effects of the released transmitters.

Field stimulation-induced inotropic and chronotropic responses were measured isometrically following stimulation. The inotropic and chronotropic post-stimulus responses occurred at different frequencies of stimulation (20 pulses, pulse width 1 m s , 0.2-20Hz supramaximal voltage). The atria were stimulated 2 minutes after the tissue had returned to the pre-stimulation control values of rate and force.

The post-stimulus responses were expressed as percentage change above or below basal control levels immediately preceding stimulation. Comparisons were made between frequency-response curves obtained in the absence and presence of drugs.

In addition to the above experiments atria from animals pretreated with T_4 and reserpine were field stimulated. Comparisons were made between responses from such atria and atria from control animals.

iii) <u>Paced Atria</u>

The post-synaptic inotropic effect of the $alpha_1$-adrenoceptor agonist phenylephrine was examined on the paced left atria of the rat in the absence and presence of the $alpha_1$-adrenoceptor antagonist prazosin. The left atria was stimulated through silver ring electrodes by a Palmer Square Wave stimulator at a frequency of 1Hz., 5m s pulse width and 1.5 x supramaximal voltage. The experiment was carried out in the presence of $5x10^{-7}$ M tetrodotoxin (TTX), which prevented Na^+ conductance and the production of action potentials in the nerves and consequently the release of transmitter and 10^{-6} M propranolol which blocked the myocardial beta-adrenoceptor. This ensured that any response produced was solely due to phenylephrine acting on a post-synaptic $alpha_1$-adrenoceptor. A cumulative dose-response curve was obtained for phenylephrine. The positive inotropic response to each dose was allowed to reach a maximum level prior to addition of the next dose. Results were expressed as a percentage increase in response above control levels. Comparisons were made between the effects of phenylephrine on control atria and atria from animals pretreated with T_4.

THE FIELD STIMULATION-INDUCED RELEASE OF RADIOACTIVITY FROM ATRIA INCUBATED IN [^{3}H]-NORADRENALINE AND [^{14}C]-CHOLINE

Adult, male, Wistar rats (250-320g) were stunned and killed by exsanguination. The hearts were dissected and the paired atria separated from the ventricles. The atria were then transferred to 0.5ml of Krebs bicarbonate buffer and gassed with a mixture of O_2, 95%/CO_2, 5% containing 10μCi of [^{3}H]-NA (500nM 43 Ci mmol^{-1}). Atria were incubated in this radioactive solution (30 min., 37°C) and inserted into silver ring electrodes, transferred to organ baths (2ml capacity) and attached to Statham force displacement transducers (resting tension 1.0g) to record motor responses isometrically (Fig. 2). These atria were continuously superfused with Krebs solution (37°C, 4 ml/min, 1.5hr) to remove loosely bound [^{3}H]-NA not taken up into the nerves. At the end of this preliminary washing period the superfusion was stopped. Atria were field stimulated with a Palmer Square Wave stimulator (60 pulses, pulse width 1m s , 2Hz, supramaximal voltage) every 10 minutes. At 2 minute intervals before the first stimulation and between each subsequent stimulation the contents of the organ bath were removed and collected in liquid scintillation vials, each containing 10ml of Ecoscint (National Diagnostics). The organ baths were then filled with fresh Krebs solution. Drugs were added to the organ bath at 2 minutes or 10 minutes prior to stimulation and were dissolved in Krebs solution unless otherwise stated. The radioactivity in each scintillation vial was counted in a Packard Tri-Carb liquid scintillation counter. Each pair of atria was digested in KOH (1ml, 0.5M, 24 hr, 60°C) so that the residual radioactivity in the tissue could be determined. All radioactive counts were corrected for quenching enabling the counted radioactivity to be expressed as disintegrations per minute. The field

Apparatus for Overflow Experiment

Oxygenated Krebs

Pump

Heating
Coil

to Transducer

to Water
Heater

Electrode
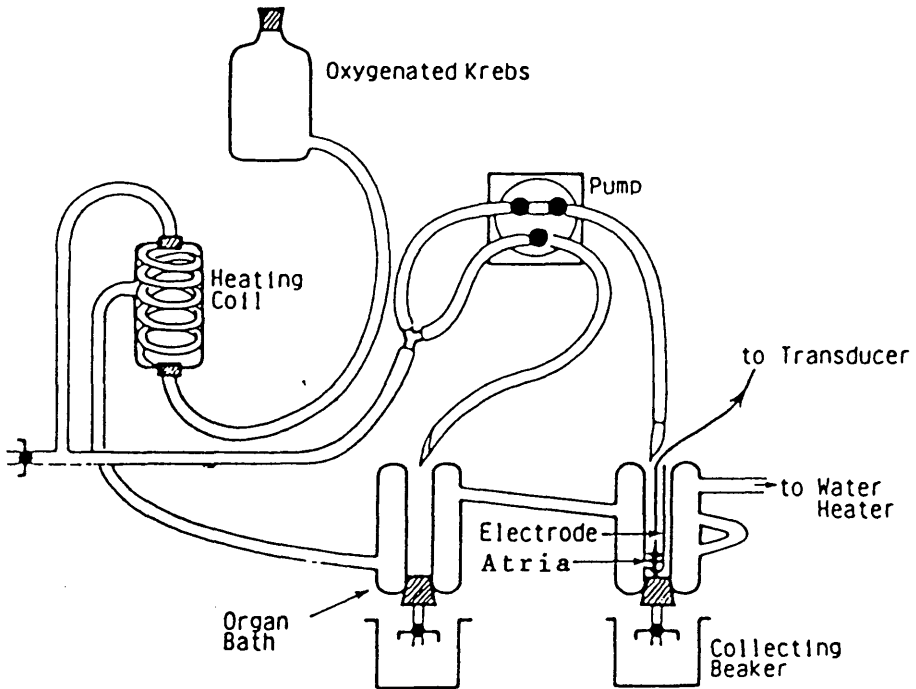Atria

Organ
Bath

Collecting
Beaker

FIGURE 2.   Diagram of apparatus used for overflow experiments.

stimulation-induced release of radioactivity was expressed as fractional release. The fractional release value was obtained by calculating the d.p.m. produced by field stimulation above that of basal release immediately prior to stimulation. This figure was subsequently expressed as a fraction of the total amount of radioactivity released coupled with that obtained from the digested tissue.

The [^{3}H]-NA released into the organ baths during stimulation was separated from its metabolites by column chromatography.

In some experiments paired atria were dissected free of the ventricles and transferred to 0.5ml of Krebs bicarbonate buffer containing 10 μCi of [^{14}C]-choline (500nM 43 Ci mmol^{-1}) and gassed with a mixture of O_2, 95%/CO_2, 5%. Atria were incubated in this radioactive solution (60 mins, 37°C), mounted in silver ring electrodes and placed in organ baths as described previously. Incubation of atria with choline results in the synthesis of a pool of ACh, contained in nerves, which can be released by potassium depolarisation (Wetzel & Brown, 1983, 1985). This study examined the effects of field stimulation on release of [^{14}C]-choline. Occasionally, atria were simultaneously incubated in [^{3}H]-NA and [^{14}C]-choline and the effect of field stimulation and drugs on the release of both [^{3}H] and [^{14}C] were examined.

Once the atria had been incubated in [^{14}C]-choline they were transferred to 2ml organ baths. As before the atria were superfused with Krebs solution (37°C, 4ml/min 2 hrs). This Krebs solution included physostigmine (10^{-6}M) and hemicholinium — 3.(10^{-5}M). Physostigmine and hemicholinium-3. prevented respectively, enzymatic

degradation of ACh and re-uptake of choline (Fosbraey & Johnson, 1980).

At the end of this preliminary washing period the superfusion was stopped. Atria were field stimulated with a Palmer Square Wave stimulator (525 pulses, pulse width 1 m s , 5Hz, supramaximal voltage) every 10 minutes. At two minute intervals before the first stimulation and between each subsequent stimulation the contents of the organ bath were collected in scintillation vials containing 10ml Ecoscint. Drugs were added to the organ baths and were present throughout the stimulation period. None of the drugs employed affected the basal release of either [^{3}H] or [^{14}C]. As before, the amount of radioactivity contained in each sample was determined as was the residual radioactivity remaining in the tissue. This enabled the fractional release obtained with each stimulation to be calculated.

METABOLISM STUDIES

A metabolite assay (Fig.3) was carried out in order to determine the components of the [^{3}H] overflow produced from field stimulated atria.

Alumina was treated according to the method of Crout (1961) and washed with 0.2M sodium acetate before drying. Dowex 50W x 4 (200-400 mesh) was washed several times with 2M NaOH (containing 1% Na-EDTA) at 50°C until the supernatant was clear, then washed with H$_2$O, 2M HCl, H$_2$O and, finally, equilibrated with 0.01M HCl. Columns used for chromatography were 50mm in diameter and stoppered with cotton wool. Acid-activated alumina (200mg) was washed before use with 0.2M sodium
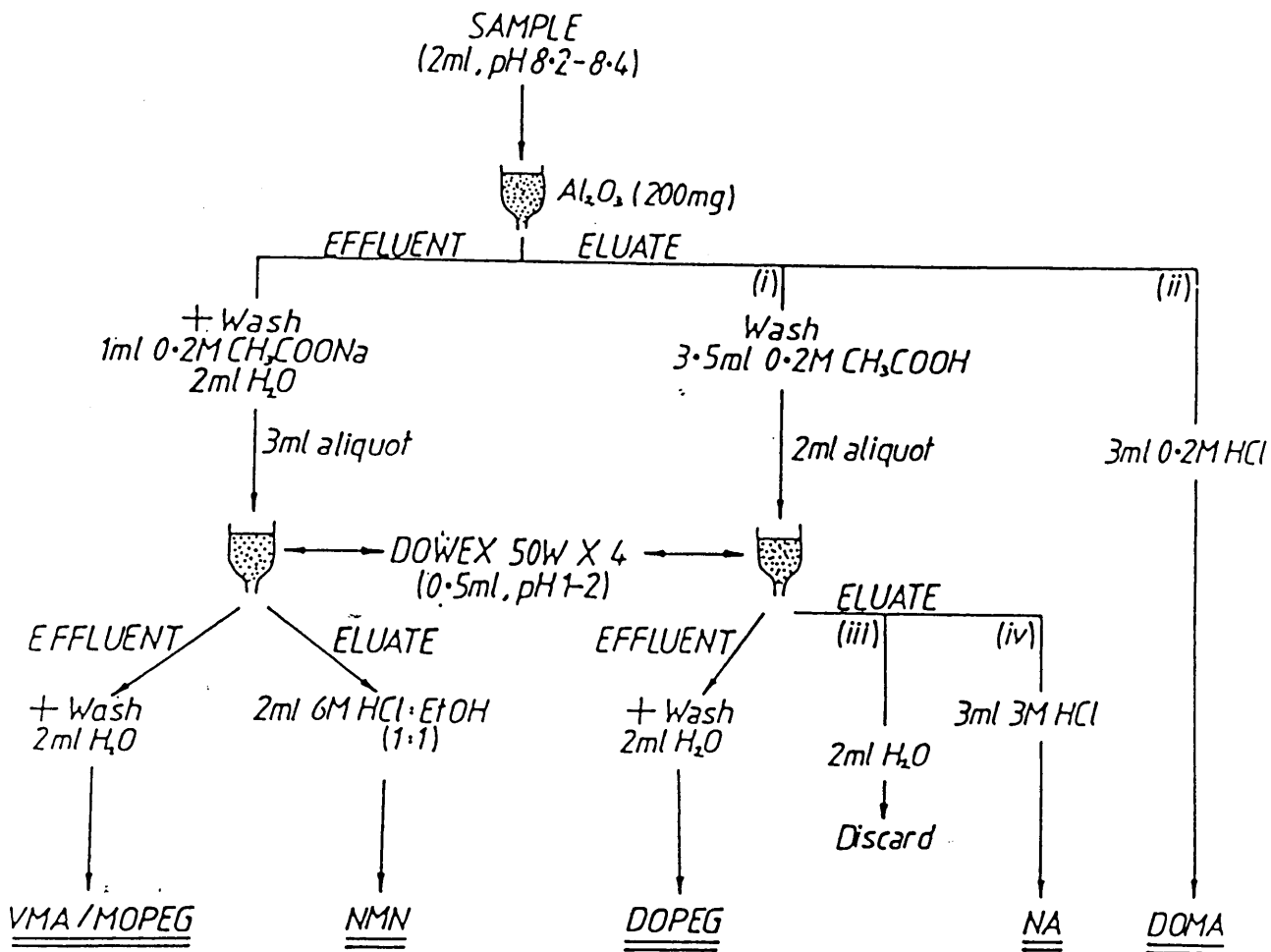
FIGURE 3. Flow diagram of the chromatographic separation of (^3H)-NA from its metabolites. 2 ml samples were applied to the alumina columns. The effluent and washings contained COMT metabolites. The acetic acid eluate contained the NA and DOPEG. The HCl eluate from the alumina contained DOMA. The COMT metabolites were separated by a DOWEX-50W x 4 column into VMA/MOPEG and NMN. The NA-DOPEG fraction was also separated by a DOWEX-50W x 4 column into DOPEG and NA.

acetate (pH 8.2). Dowex columns were filled to a height of 1.5cm (0.5ml) with resin and washed with 2 ml H_2O.

10 μg of unlabelled NA and of each NA metabolite were added as carrier to each sample. The pH of the samples was adjusted to 8.2-8.4 with (0.5m) Tris buffer (pH8.2). The samples were then immediately placed in the columns. After addition, the column was washed through with a further 1ml of 0.2M sodium acetate and 2 ml H_2O. The effluent and washing contained the O-methylated metabolites 3-methoxy-4-hydroxyphenylglycol (MOPEG), 3-methoxy-4-hydroxymandelic acid (VMA) and normetanephrine (NMN).

The [^{3}H]-NA and [^{3}H]- 3,4-dihydroxygphenylglycol (DOPEG) fraction was then eluted with 3.5ml 0.2M acetic acid. After washing the column with 4ml 0.5M acetic acid, which elutes approximately 5% of both NA and DOPEG, the deaminated acid, 3,4 dihydroxymandelic acid (DOMA) was eluted with 3ml 0.2M HCl.

[^{3}H]-NA was separated from the [^{3}H]-DOPEG by means of the strong cation exchange resin Dowex 50W x 4. 2ml of the [^{3}H]-NA/[^{3}H]-DOPEG fraction was poured onto the Dowex column and washed through with 2ml H_2O. The effluent and washings contained DOPEG. After washing with a further 2ml H_2O, which was discarded, the NA was eluted with 3ml 3M HCl.

The O-methylated fraction was separated using Dowex-50W x 4 columns. The O-methylated metabolites (VMA and MOPEG) passed through the column and were collected in the effluent. NMN was retained by the Dowex and subsequently eluted with 2ml 6M HCl/ethanol (1:1 v/v).

1ml of each separated fraction was added to 10ml of Ecoscint in a counting vial and radioactivity was measured in a liquid scintillation counter.

The method of separation was essentially that of Graefe et al (1973) and Nicol (1975) with the following modifications. Volumes of eluates were increased so that aliquots could be taken for counting before column separation. This allowed corrections for recovery to be made in each sample. The volume of 0.2m acetic acid used to elute the NA/DOPEG fraction from the alumina column was increased from 2ml to 3.5ml and the 0.2M HCl used for elution of [^{3}H]-DOMA was increased from 2.5ml to 3ml. The concentration of HCl required to elute [3H]-NA was increased from 2M to 3M and the volume from 2ml to 3ml. These alterations were made to ensure complete recovery of [^{3}H]-NA.

Calculation of Metabolism Results

The amount of radioactivity appearing in each of the metabolite samples was compared to that in the sample before separation to give an indication of the recovery and efficiency of each column. The recovery for the alumina column was 85.3$\pm$ 2.3% (n=8) and for the Dowex columns 95.3$\pm$ 3.9% (n=8) and 93.7$\pm$ 4.7% (n=8) respectively.

The amount of [^{3}H]-NA and [^{3}H]-metabolites present in the pre-stimulation sample were subtracted from the amount in each sample obtained after field stimulation.[^{3}H]-NA and its metabolites were expressed as a percentage of the total [^{3}H] present in the samples. Results were expressed as mean $\pm$ standard error of the mean. Significance was assessed by student's t-test.

IN VIVO EXPERIMENTS

i) Anaesthetised Rats

Male Wistar rats (250-300g) were anaesthetised with pentobarbitone (60mg/kg, i. p.). In each animal the trachea was cannulated to allow respiration to be maintained artificially with a Palmer pump. The left carotid artery was cannulated and blood pressure (B.P.) recorded using a Statham pressure transducer. In addition, the arterial pulse was used to trigger a Devices Instantaneous Ratemeter for measurement of heart rate (H.R.). Both variables were recorded on a Devices M2 physiological recorder. The left femoral vein was cannulated to allow administration of drugs. The right branch of the vagus nerve was carefully separated from the carotid artery and was placed over bipolar silver electrodes which were connected to a Palmer Square Wave stimulator. The vagus nerve was stimulated at various frequencies at a pulse width of 0.1ms and at supramaximal voltage. Such stimulation caused a depressor effect on both the H.R. and B.P. The effect of various drugs on the chronotropic response to vagal stimulation was examined.

ii) Pithed Rats

Animals were pithed according to the method of Gillespie et al, 1970. Male, Wistar rats were anaesthetised with pentobarbitone (60mg/kg i.p.) and respired artificially through a tracheal cannula. Rats were pithed by inserting a short steel tube through the orbit and foramen magnum into the spinal canal. Through this trocar was passed a fine steel wire contained in a teflon tube and this was pushed down to the sacral end of the spinal canal. A separate steel rod, inserted behind the skull and pushed down between the vertebral column and the skin acted as an indifferent electrode. The steel rod extruding from the

spinal canal and the indifferent electrode were connected to a Palmer Square Wave stimulator. The temperature of the pithed animal was maintained at approximately $37^{\circ}C$ by radiant heat. As with the anaesthetised rats the left carotid artery was cannulated in order to record B.P. and H.R. and the left femoral vein cannulated to permit administration of drugs. Again, as before, the right branch of the vagus was exposed and placed over bipolar silver electrodes connected to a Palmer Square Wave stimulator. The pithed animal was left for 60 minutes to allow H.R. and B.P. to stabilise. After this period tubocurarine was administered (1mg/kg, i.v.) to prevent electrical stimulation-induced contractions of voluntary muscle interfering with the recordings of B.P. and H.R.

The length of the stimulating electrode extruding from the shielding teflon tube could be varied but was maintained at 1cm. This 1cm length was positioned at C_7-T_1 in the spinal canal, the position of the sympathetic cardioaccelerator nerves. Stimulation via the spinal electrode at this level at various frequencies, at 0.03ms. pulse-width and at supramaximal voltage produced increases in H.R. with very small changes in B.P. Stimulation of the exposed vagus nerve at various frequencies, 0.1ms pulse width and at supramaximal voltage produced a fall in H.R. at low frequencies (5Hz) of stimulation. At high frequencies of stimulation (10Hz) there was a more complex, biphasic response, which consisted of a fall followed by a rise in H.R. This preparation allowed independent or simultaneous stimulation of the cardioaccelerator and vagus nerves. The effect of various drugs on the chronotropic responses to stimulation of these nerves was examined. Drug concentrations were administered and are expressed as $\mu g\ Kg^{-1}$.

RADIOIMMUNE ASSAY OF FREE SERUM THYROXINE LEVELS

Blood samples were collected from control and T_4 pretreated rats. Radioimmune assay of serum T_4 allowed the effectiveness of the pretreatment to be monitored. An Amersham Amerlex T_4 RIA kit (IM3050) was used for the immune assay of free T_4 in the serum obtained. This method is dependent upon the competition between $[^{125}I]-T_4$ and free T_4 in the serum for a limited number of binding sites on a T_4-specific antibody. The proportion of the $[^{125}I]-T_4$ bound to the antibody is inversely related to the concentration of free T_4 present in the serum. Standards (containing known amounts of T_4) and the serum samples were mixed with both $[^{125}I]-T_4$ and T_4-specific antibody suspension and then allowed to stand for 60 min at 37^0C. Centrifugation (2000 x g, 5min, room temperature) produced a pellet of antibody containing bound $[^{125}I]-T_4$ and T_4 beneath a liquid supernatant. The supernatant was discarded and the pellet was placed in a gamma counter to record the level of radiation. A standard curve was constructed showing the concentration of free T_4 versus $[^{125}I]$ counts. From this standard curve it was possible to calculate the amount of free T_4 in the serum from control and treated rats.

STATISTICS

Each response is expressed as a mean with a standard error of the mean ($\bar{x} \pm$ S. E. mean). To compare results, the student's t-test, or where appropriate, the paired t-test was used. Levels of significance: $*0.05 > p > 0.01$; $** 0.01 > p > 0.001$; $*** p < 0.001$.

DRUGS USED

The drugs used during the project were acetylcholine chloride (Sigma), L-ascorbic acid (Sigma), atropine sulphate (Sigma), 8-bromo-cyclic 3',5'-adenosine monophosphate (Sigma), 8-bromo-cyclic 3',5'-guanosine monophosphate (Sigma), carbamylcholine chloride (Sigma), [methyl-^{14}C]-choline (Amersham), clonidine hydrochloride (Sigma), desipramine hydrochloride (Sigma), dL-3,4-dihydroxymandelic acid (Sigma), dL-3,4-dihydroxyphenylglycol (Sigma), dimethylsulphoxide (Sigma), forskolin (Sigma), guanethidine sulphate (Ciba), dL-4-hydroxy-3-methoxymandelic acid (Sigma), bis-4-hydroxy-3-methyoxyphenylglycol (Sigma), 3-isobutyl-1-methylxanthine (Sigma), isoprenaline sulphate (Sigma), L-[7,8-^{3}H] noradrenaline (Amersham), L-noradrenaline bitartrate (Koch-Light), dl-normetanephrine hydrochloride (Sigma), 17 beta-oestradiol (Sigma), pentobarbitone sodium (May and Baker), phenoxybenzamine hydrochloride (Smith Kline and French), phentolamine mesylate (Ciba), trans-2-phenylcyclopropylamine hydrochloride (Sigma), phenylephrine hydrochloride (Sigma), prazosin hydrochloride (Pfizer), propranolol hydrochloride (ICI), reserpine (Sigma), sodium nitroprusside (Analar), tetrodotoxin (Boehringer), L-thyroxine (Sigma), d-tubocurarine (Wellcome) and yohimbine hydrochloride (Sigma).

The drugs were prepared in Krebs bicarbonate buffer solution unless otherwise stated.

L-[7,8-^{3}H]-noradrenaline (10-48 Ci mmol^{-1}) supplied in 0.02M acetic acid: ethanol (9:1 v/v), was resuspended in distilled water containing ascorbic acid (5.7 x 10^{-3}M) to prevent breakdown of the amine.

Forskolin was dissolved in dimethylsulphoxide (DMSO). DMSO was never added in a concentration greater than 1%.

Reserpine was dissolved in glacial acetic acid (0.3ml, 17.5M) and diluted with distilled water.

Phenoxybenzamine and 17, beta-oestradiol were made up in absolute alcohol but were never added in volumes greater than 0.25 ul/ml of Krebs solution.

Pertussis toxin was dissolved in 50mM Tris-IM NaCL pH8 buffer.

ANIMAL PRETREATMENTS

(i) Pertussis Toxin

Pertussis toxin was administered to rats intravenously in a dose of 10 µg/kg body weight contained in 0.1-0.4ml of vehicle. Control rats were injected with a similar volume of vehicle. Experiments were carried out 72 hours after injection of the vehicle or pertussis toxin.(i.v. tail vein).

(ii) Reserpine

Reserpine was administered to rats intraperitoneally in a dose of 1mg/kg body weight contained in 0.3-0.6ml of vehicle. Control rats were injected with a similar volume of vehicle. Animals were treated with reserpine or vehicle for 3 consecutive days and on the fourth day were killed.

(iii) <u>L-Thyroxine</u>

Thyroxine was administered orally in the drinking water. Preliminary studies indicated that a 250g. rat drinks 50ml of water per day. Thyroxine was added to the water to give a solution of 25mg/litre. This gave each rat a dose of 5mg/kg/day. The rats were treated for 14 days before being used.

RESULTS

PART I

RESPONSES OF THE HEART RATE IN ANAESTHETISED AND PITHED RATS TO VAGAL STIMULATION AND CARDIO-ACCELERATOR NERVE STIMULATION, AND THE EFFECTS OF DRUG ADMINISTRATION ON THESE RESPONSES

Electrical stimulation of the vagus nerve produced a frequency-dependent decrease in heart rate in both pithed and anaesthetised rats. The negative chronotropic response to vagal stimulation was greater in the anaesthetised rat than in the pithed rat (Fig. 4).

Electrical stimulation of the cardio-accelerator nerves in the spinal column at C7-T1 resulted in a frequency-dependent increase in heart rate (Fig. 5).

Intravenous (i.v) administration of propranolol (50 µg Kg^{-1}) abolished the positive chronotropic response of the heart evoked by cardio-accelerator nerve stimulation or by administration of NA (0.1 µg Kg^{-1}). Atropine (50 µg Kg^{-1}) abolished the negative chronotropic response of the heart evoked by vagal stimulation or by administration of acetylcholine (0.6 µg Kg^{-1}) (Table 1).

When the vagus nerve was stimulated during stimulation of the cardio-accelerator nerves the negative chronotropic response to vagal stimulation was enhanced (Figs. 6, 7).

In the pithed rat, administration of NA (0.1 µg Kg^{-1}) resulted in an increase in heart rate. Stimulation of the vagus nerve in the presence of NA resulted in an enhanced bradycardia (Figs. 8, 9).
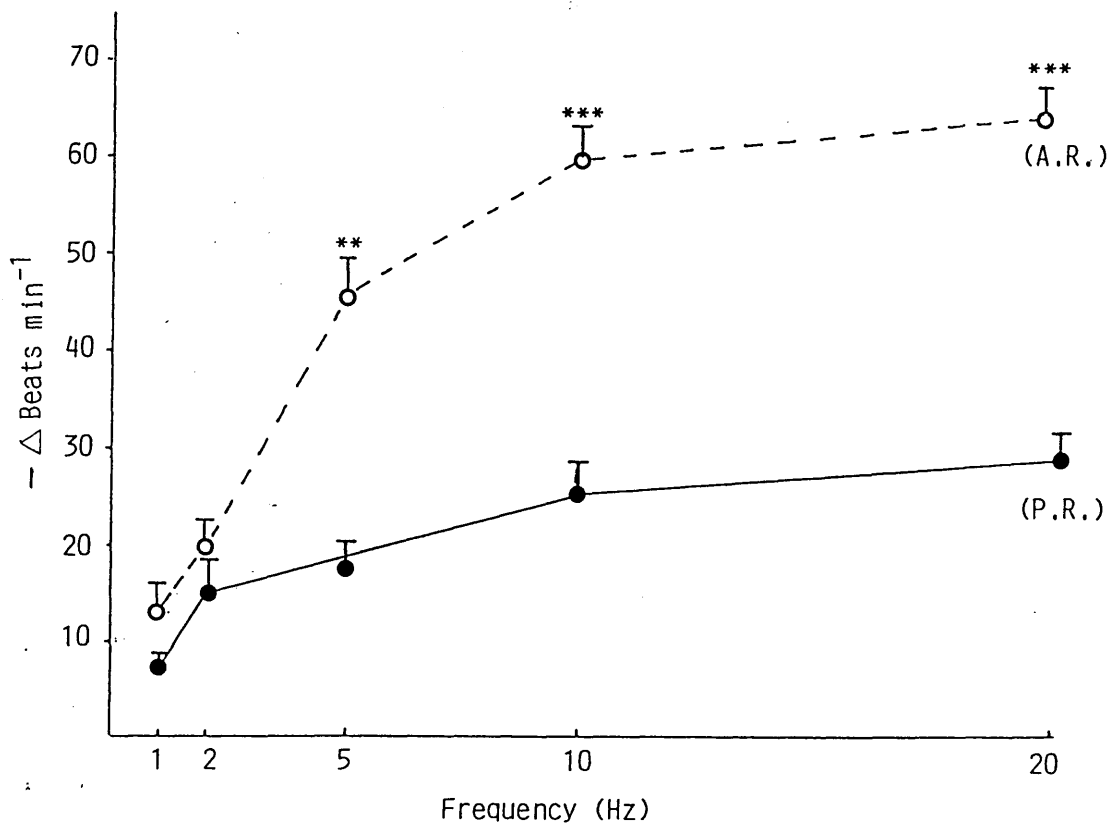
FIGURE 4. Frequency response curve showing the effects of stimulation of the vagus nerve on heart rate (Beats min^{-1}) in situ in anaesthetised (O- - -O) and pithed (●——●) rats. Each point is the mean ($\pm$ S.E. mean) of 4 observations. Stimulation results in a decrease in heart rate.

** $0.01 > P > 0.001$, ***$P < 0.001$.

P.R.: Resting heart rate: 200 beats min^{-1}.
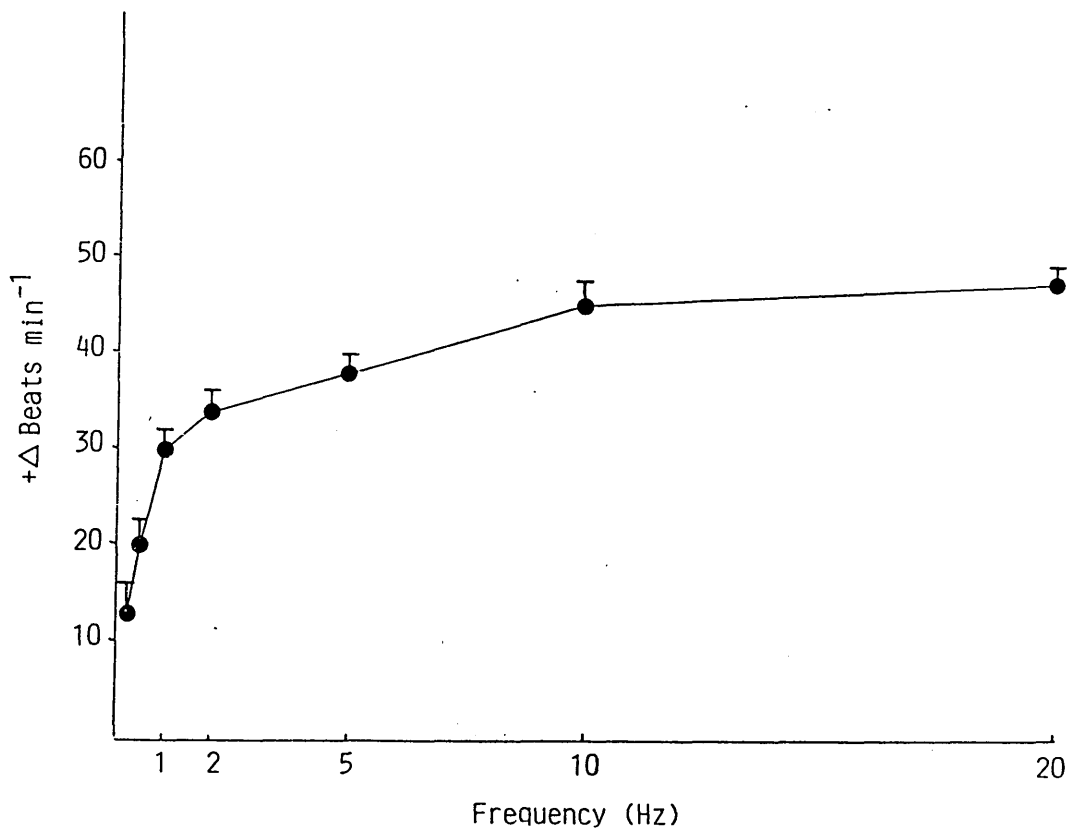A.R.: Resting heart rate: 350 beats min^{-1}.

FIGURE 5. Frequency response curve showing the effects of stimulation of the cardio-accelarator nerves _via_ a spinal electrode on heart rate in the pithed rat.

Stimulation results in an increase in heart rate.
Each point is the mean ($\pm$ S.E. mean) of 6 observations.

Table 1. Effect of atropine and propranolol on the chronotropic responses of the heart to nerve stimulation, ACh and NA.

Treatment	Changes in heart rate $\pm\triangle$BPM ($\pm$ S.E. mean)		
	Control	50µgKg^{-1} Atropine	50µgKg^{-1} Propranolol
a) Pithed rat			
Cardio-accelerator nerve stimulation (2Hz x 30s)	+34.8$\pm$4.2	–	0.0$\pm$0***
0.1 µgKg^{-1} Noradrenaline	+23.5$\pm$0.8	–	0.0$\pm$0***
b) Anaesthetised rat			
Vagal nerve stimulation (5Hz x 10s)	-64$\pm$7.2	0.0$\pm$0***	–
0.6 µgKg^{-1} ACh	-274$\pm$25	0.0$\pm$0***	–

Each value is the mean of ≮ 6 observations.

***P<0.001 for comparison with control prior to drug addition.
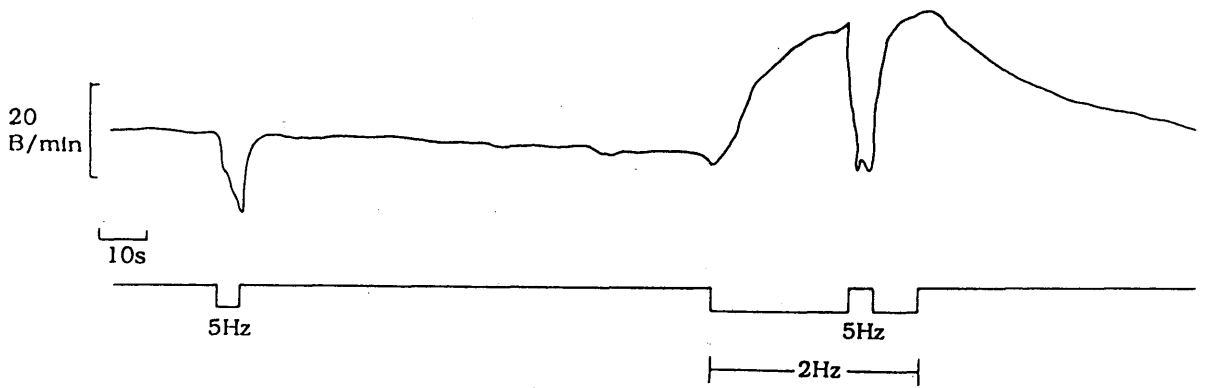
Figure 6. Recording of the response of the heart rate in a
pithed rat to vagal stimulation (5Hz x 5s) alone and against
a background of increased heart rate produced by stimulation
of the cardio-accelerator nerves (2Hz x 30s) in the spinal
column. The response to vagal stimulation was enhanced by
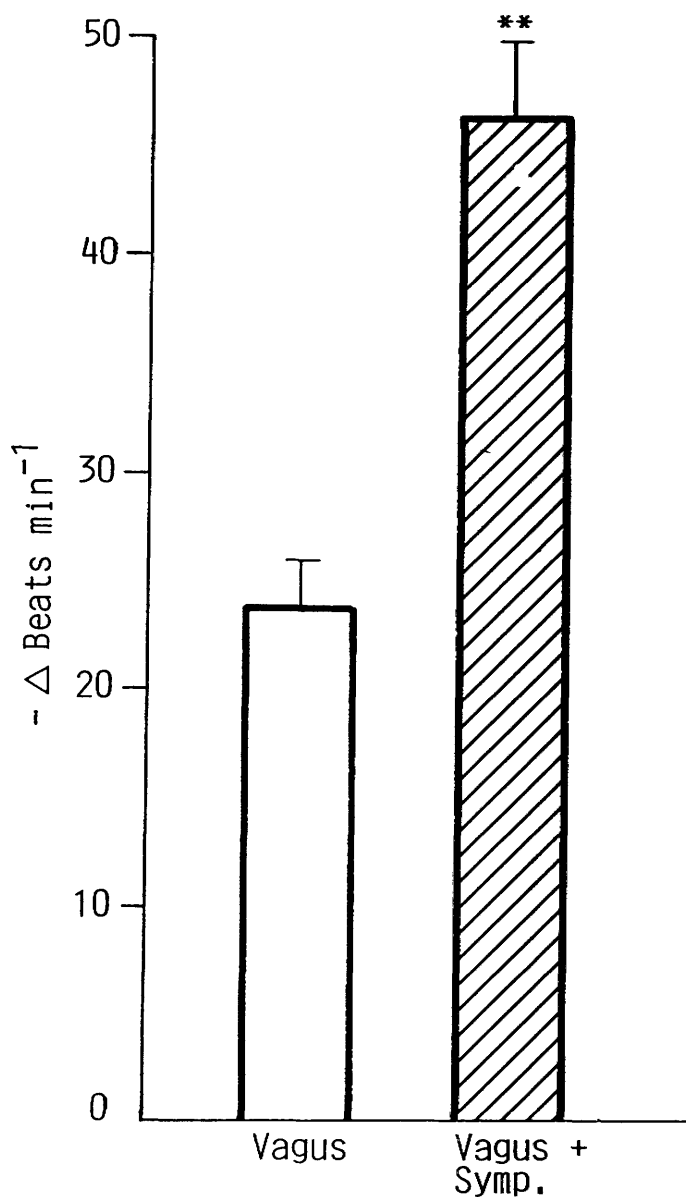the concommitant stimulation of the cardio-accelerator
nerves.

FIGURE 7. Effect on heart rate produced by stimulating the
vagus nerve (5Hz x 5s), in the pithed rat, alone and against
a background of cardio-accelerator nerve stimulation
(2Hz x 30s). The open histogram shows the effect on heart
rate produced by vagal stimulation alone and the diagonal
striped histogram shows the effect of vagal stimulation
against a background of cardio-accelerator nerve
stimulation. Each column represents the mean ($\pm$ S.E. mean)
of 5 observations. ** 0.01>P>0.001.
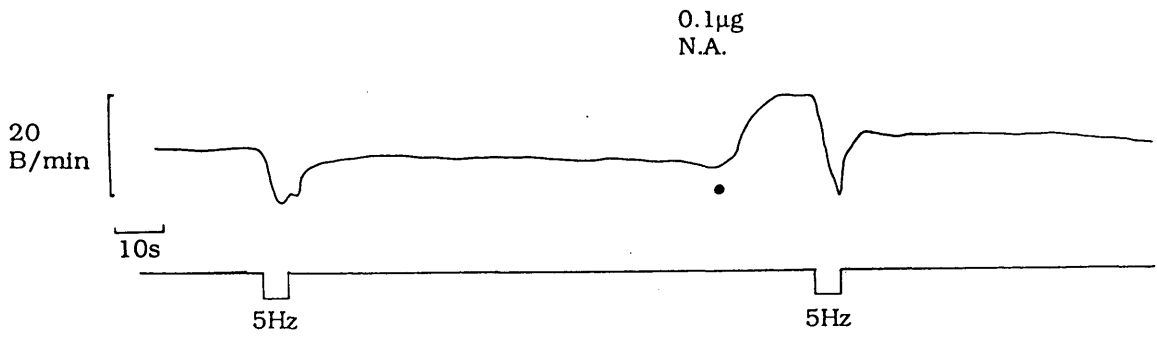
FIGURE 8. Recording of the response of the heart rate in a pithed rat to vagal stimulation (5Hz x 5s) alone and against a background of increased heart rate produced by intravenous (i.v.) administration of exogenous NA. The response to vagal stimulation was enhanced in the presence of NA.
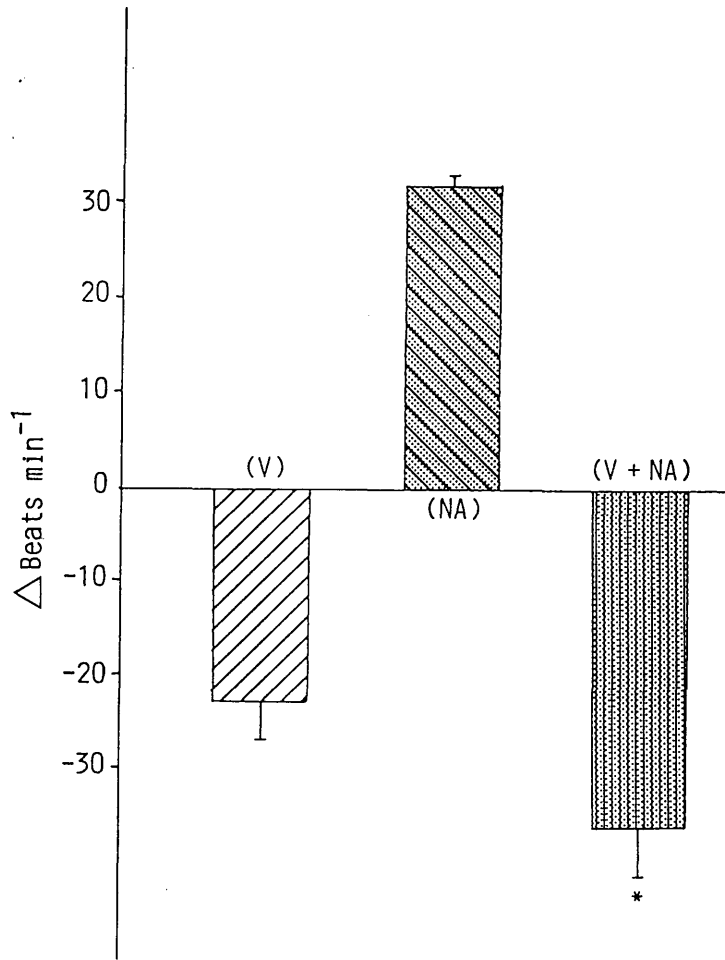
FIGURE 9. Effects of vagal stimulation and exogenous NA
alone and combined on heart rate in the pithed rat. The
upper stippled diagonal striped histogram shows the effec
of NA on heart rate. The lower diagonal striped histogram
shows the inhibitory effect of vagal stimulation on heart
rate. The lower stippled vertical striped histogram shows
the effect of vagal stimulation against the raised heart
rate produced by exogenous NA. Each column represents the
mean ($\pm$ S.E. mean) of 4 observations. * 0.05>p>0.01.

Clonidine (5 µg Kg^{-1}) inhibited the response evoked by electrical stimulation of the cardio-accelerator nerves in the spinal cord. Yohimbine (100 µg Kg^{-1}) reversed the inhibitory effect of clonidine (Fig. 10). In contrast, neither clonidine (5 µg Kg^{-1}) nor yohimbine (100 µg Kg^{-1}) affected the increase in heart rate obtained with exogenous NA (0.1 µg Kg^{-1}) (Fig. 11).

In the pithed rat, clonidine (5 µg Kg^{-1}) attenuated the inhibitory effect on heart rate obtained by electrical stimulation of the vagus. Yohimbine (100 µg Kg^{-1}) did not completely reverse the effect of clonidine (Figs. 12, 13).

In the anaesthetised rat, clonidine (5 µg Kg^{-1}) inhibited the negative chronotropic response obtained by electrical stimulation of the vagus. Yohimbine (100 µg Kg^{-1}) partially reversed the attenuating effect of clonidine (Fig. 14).

In the anaesthetised rat, phenylephrine (1 µg Kg^{-1}) inhibited the negative chronotropic response obtained by electrical stimulation of the vagus. Prazosin (100 µg Kg^{-1}) reversed the attenuating effect of phenylephrine (Fig 15).
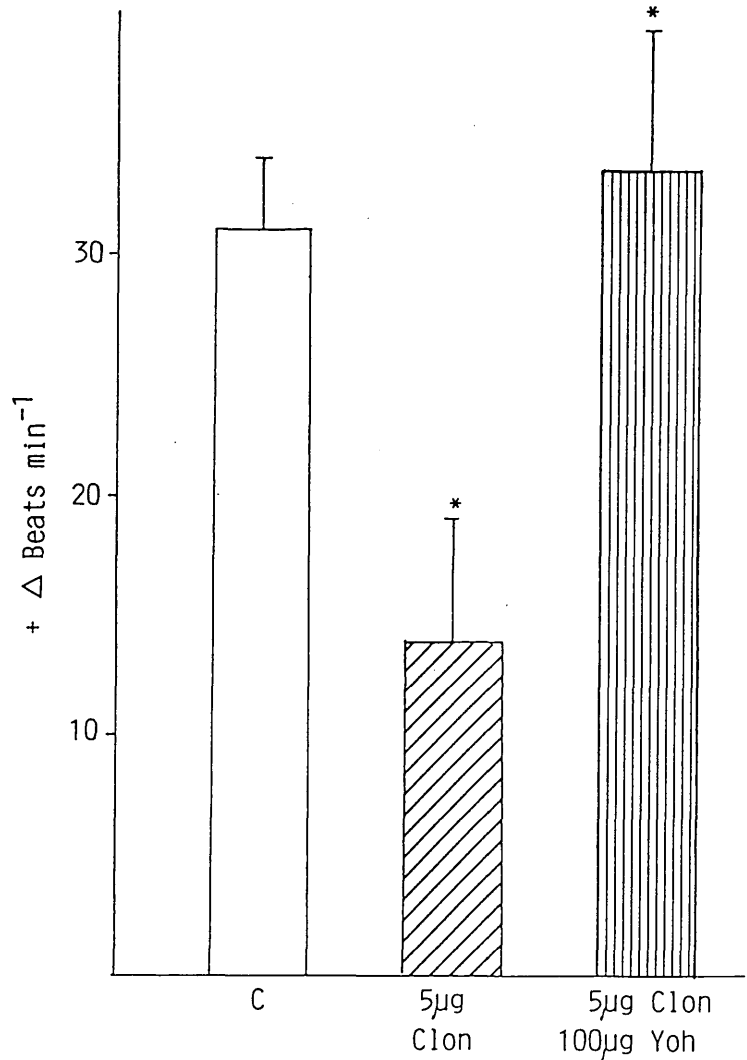
FIGURE 10. Inhibitory effect of clonidine and reversal by yohimbine on the response of the heart rate to cardioaccelerator nerve stimulation (2Hz x 30s) in the pithed rat. The open histogram shows that stimulation of the cardioaccelarator nerves results in an increase in heart rate. The diagonal striped histogram shows the inhibitory effect of clonidine on this response. The vertical striped histogram shows that yohimbine can reverse the inhibitory effects of clonidine. Each column represents the mean ($\pm$ S.E. mean) of 4 observations. * 0.05>P>0.01.
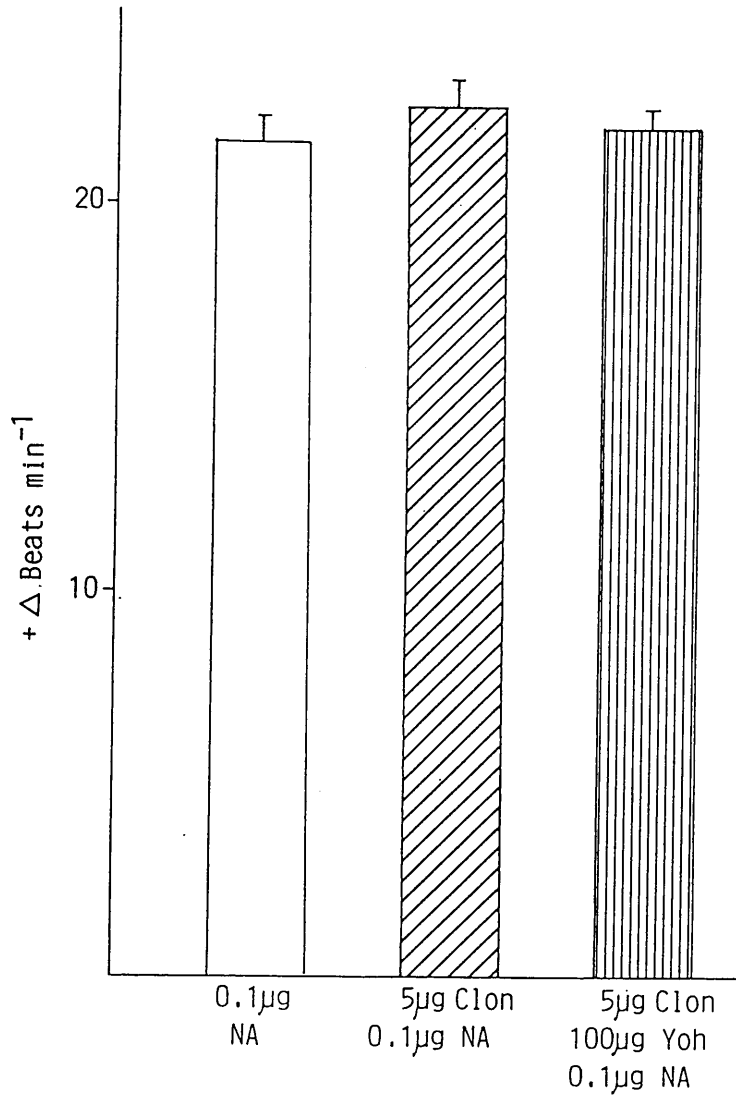
FIGURE 11. Effect of clonidine and yohimbine on the response

of the heart rate to exogenous NA in the pithed rat. The

open histogram shows the control response produced by NA.

The diagonal striped histogram shows the lack of effect of

clonidine on this response. The vertical striped histogram

shows the lack of effect of yohimbine on the response to NA

in the presence of clonidine. Each column represents the
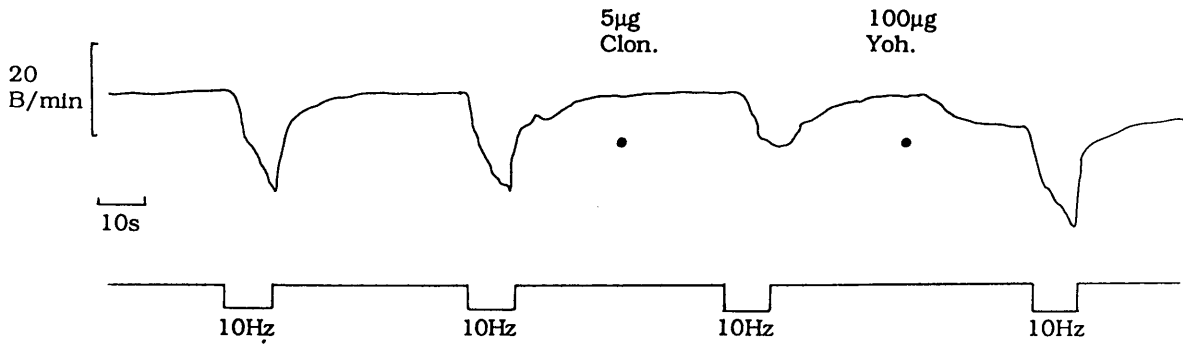
mean ($\pm$ S.E. mean) of 4 observations.

FIGURE 12. Recording of the effects of vagal stimulation
(10Hz x 10s) on heart rate in the pithed rat and the effects
of clonidine and yohimbine on this response. Stimulation of
the vagus resulted in a decrease in heart rate.
Administration of clonidine attenuated this response and
further administration of yohimbine reversed the inhibitory
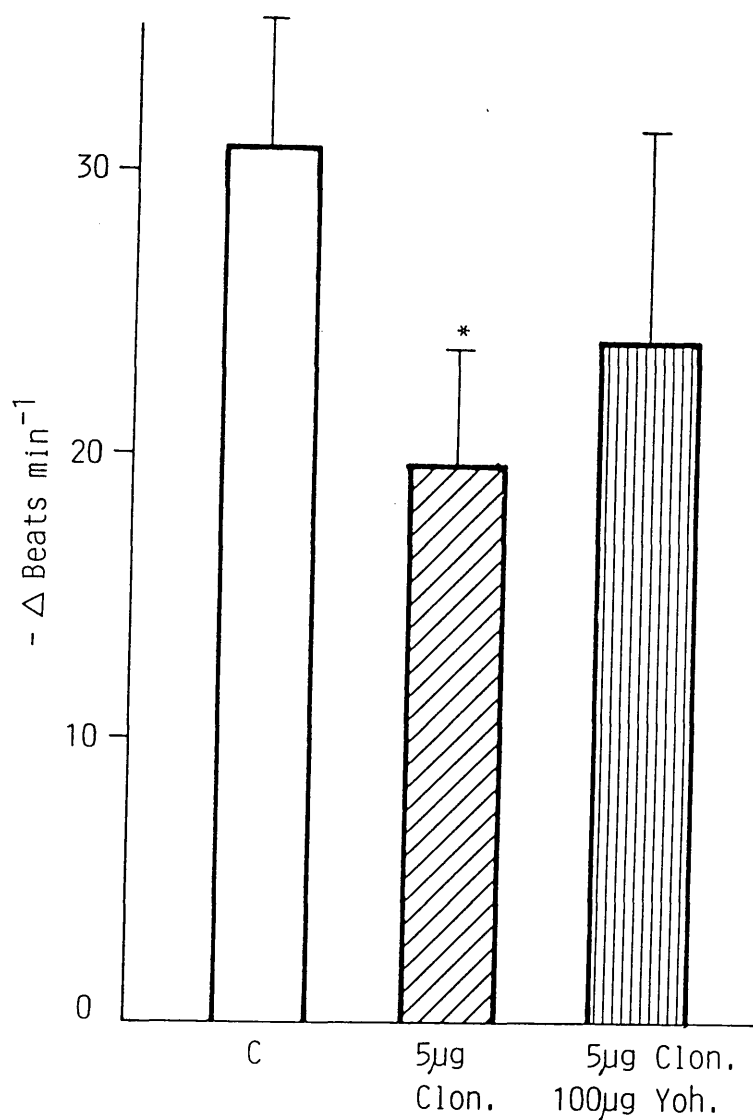effect of clonidine.

FIGURE 13. Inhibitory effect of clonidine on the response of
the heart rate to vagal stimulation (10Hz x 10s) in the
pithed rat. The open histogram shows that stimulation of the
vagus nerve results in a decrease in heart rate. The
diagonal striped histogram shows the inhibitory effect of
clonidine on this response. The vertical striped histogram
shows that yohimbine did not significantly reverse the
inhibitory effect of clonidine. Each column represents the
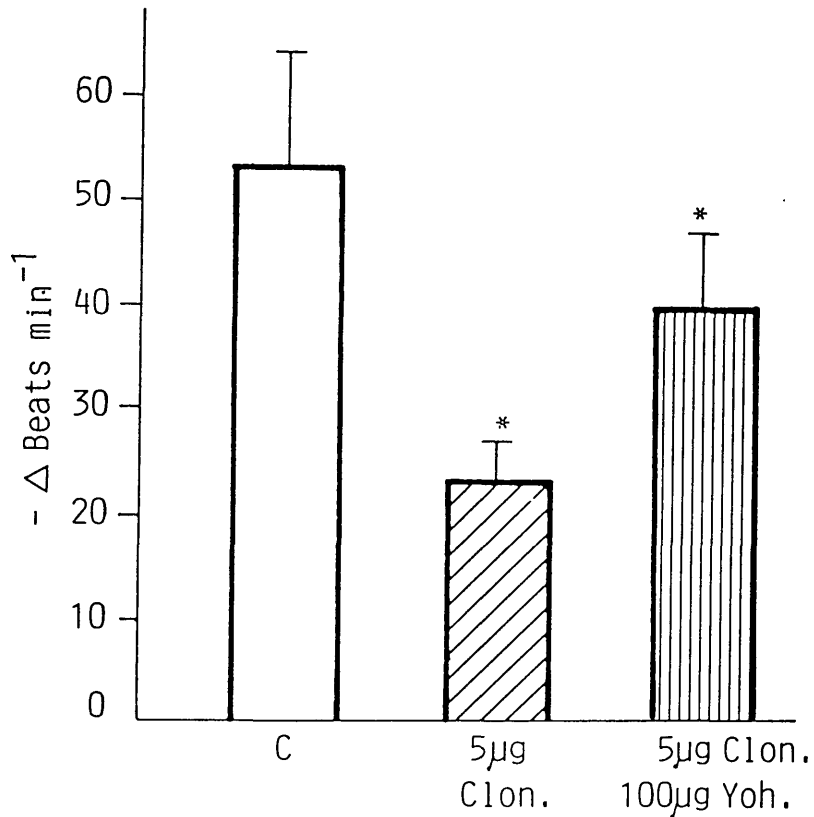mean ($\pm$ S.E. mean) of 4 observations.   * 0.05>P>0.01.

FIGURE 14. Inhibitory effect of clonidine and reversal by
yohimbine on the response of the heart rate to vagal
stimulation (5Hz x 10s) in the anaesthetised rat. The open
histogram shows that stimulation of the vagus nerve results
in a decrease in heart rate. The diagonal striped histogram
shows the inhibitory effect of clonidine on this response.
The vertical striped histogram shows that yohimbine can
reverse the inhibitory effect of clonidine. Each column
represents the mean ($\pm$ S.E. mean) of 4 observations.
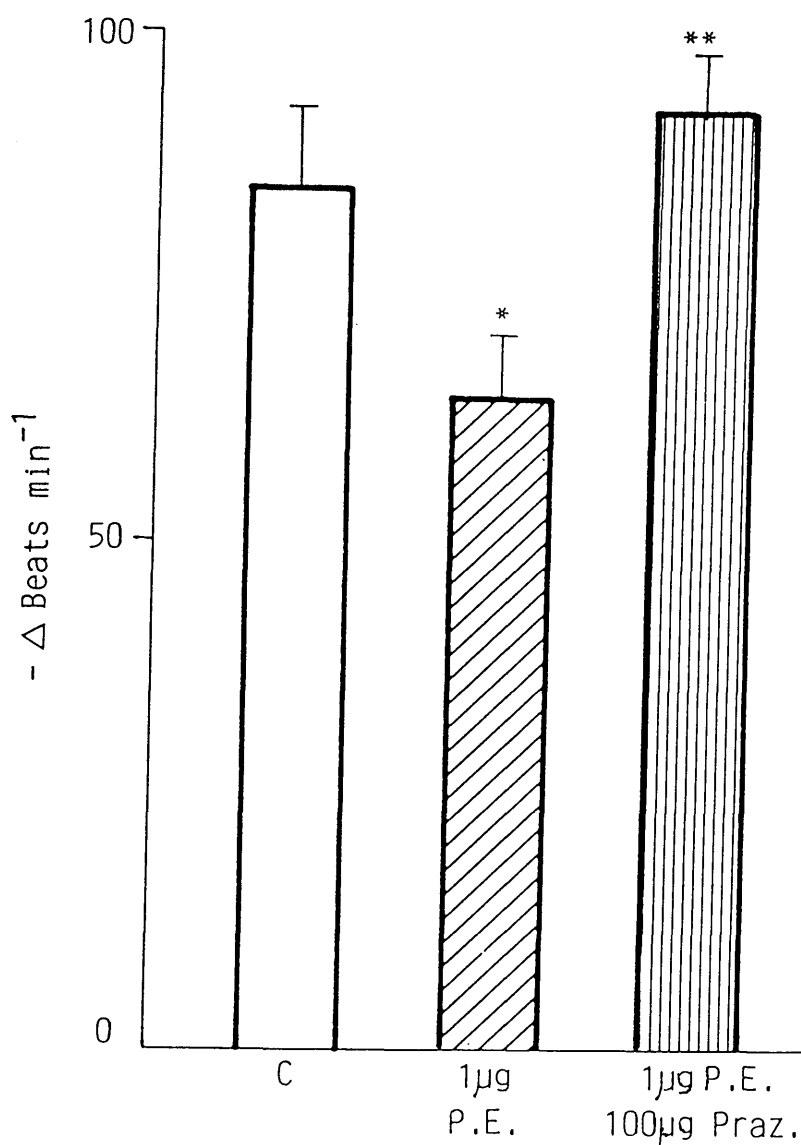* 0.05>P>0.01.

FIGURE 15. Inhibitory effect of phenylephrine and reversal by prazosin on the response of the heart rate to vagal stimulation (5Hz x 10s) in the anaesthetised rat. The open histogram shows that stimulation of the vagus nerve results in a decrease in heart rate. The diagonal striped histogram shows the inhibitory effect of phenylephrine on this response. The vertical striped histogram shows that prazosin can reverse the inhibitory effect of phenylephrine. Each column represents the mean ($\pm$ S.E. mean) of 4 observations. * 0.05>P>0.01, ** 0.01>P>0.001.

PART II

THE EFFECT OF DRUGS ON THE POST-STIMULUS RESPONSE OF THE SPONTANEOUSLY BEATING PAIRED ATRIA OF THE RAT

Field stimulation of isolated spontaneously beating paired atria resulted in a post-stimulus response that consisted of a negative inotropic component, a positive chronotropic component and a positive inotropic component (Fig. 1).

Field stimulation at increasing frequencies resulted in a frequency-dependent increase in the force of contraction of the atria. Production of several frequency response curves in the same preparation did not affect the reproducibility of the response (Fig. 16).

Field stimulation also resulted in a negative inotropic response. This response reached a maximum at 6-8Hz and was unaffected by propranolol (10^{-6} M) (Fig. 17). The response was, however, abolished by atropine.

A small positive chronotropic response was obtained by field stimulating isolated spontaneously beating paired atria. This component was unaffected by atropine (10^{-6} M) (Fig, 18). This response was, however, abolished by propranolol (10^{-7} M) amd guanethidine (10^{-6} M).

FIGURE 16. Frequency response relationship at increasing frequencies of stimulation of the positive inotropic component of the post-stimulus response of the spontaneously beating paired atria of the rat. The frequency response curve was produced several times to ensure repeated stimulation did not affect the reproducibility. Frequency response curve 1, (0———0), frequency response curve 2, (▲———▲), frequency response curve 3, (●———●). Repeated production of frequency response curves did not affect the reproducibility of the response. Each point is the mean ($\pm$ S.E. mean) of k 4 observations.
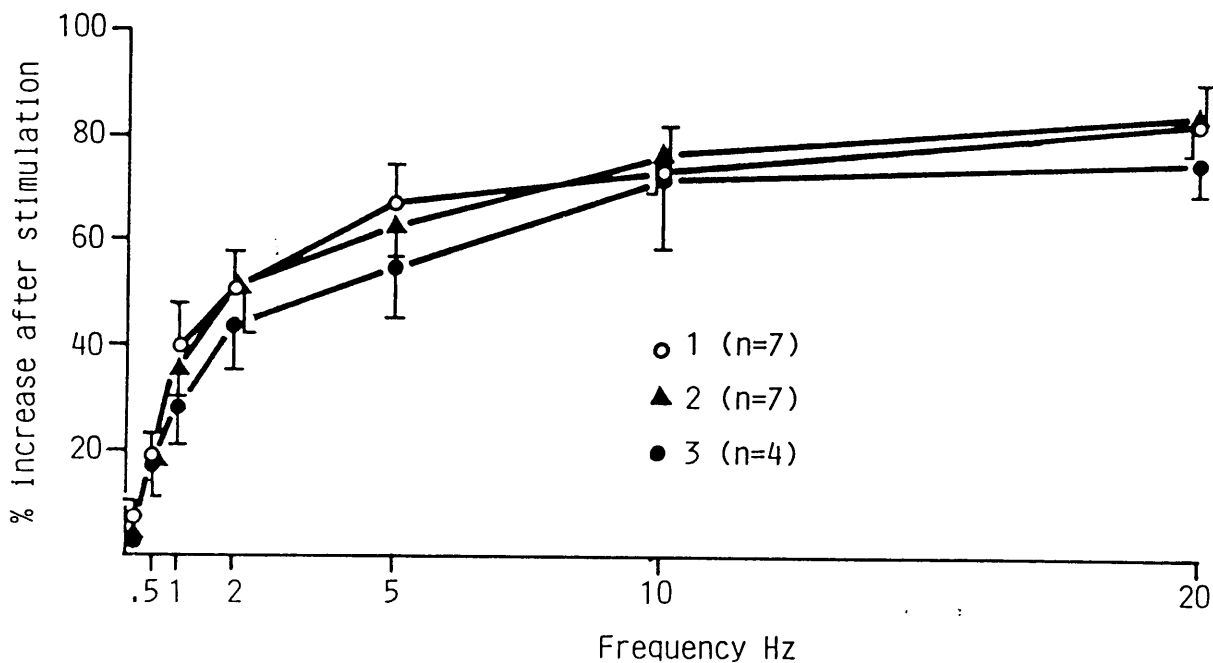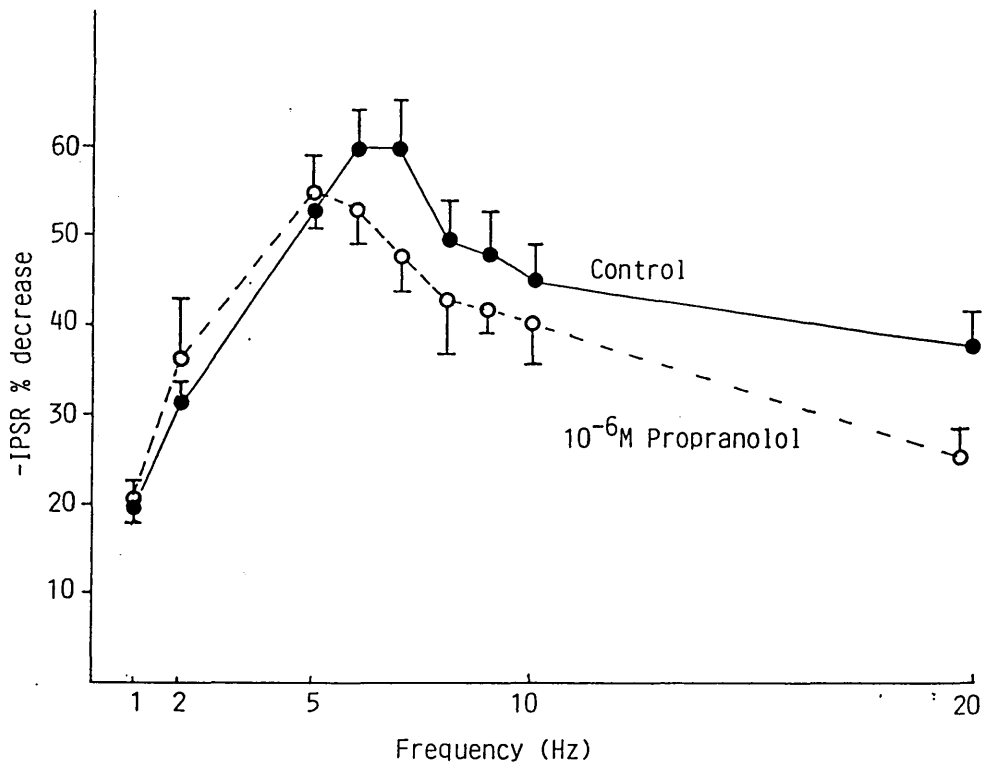
FIGURE 17. Frequency response relationship at
increasing frequencies of stimulation of the negative
inotropic component of the post-stimulus response of
the spontaneously beating paired atria of the rat.
Frequency response curves were produced in the absence
($\bullet$————$\bullet$) and in the presence of propranolol (10^{-6} M)
(O- - - -O). Propranolol did not affect this response of
the atria to field stimulation. Each point represents
the mean ($\pm$ S.E. mean) of 6 observations.

Figure 18. Frequency response relationship at increasing frequencies of stimulation of the chronotropic component of the post-stimulus response of the spontaneously beating paired atria of the rat. Frequency response curves were produced in the absence (o——o) and presence of atropine (10^{-6} M) (■———■). Atropine did not affect this response of the atria to field stimulation. Each point represents the mean (± S.E. mean) of ⟨ 6 observations.
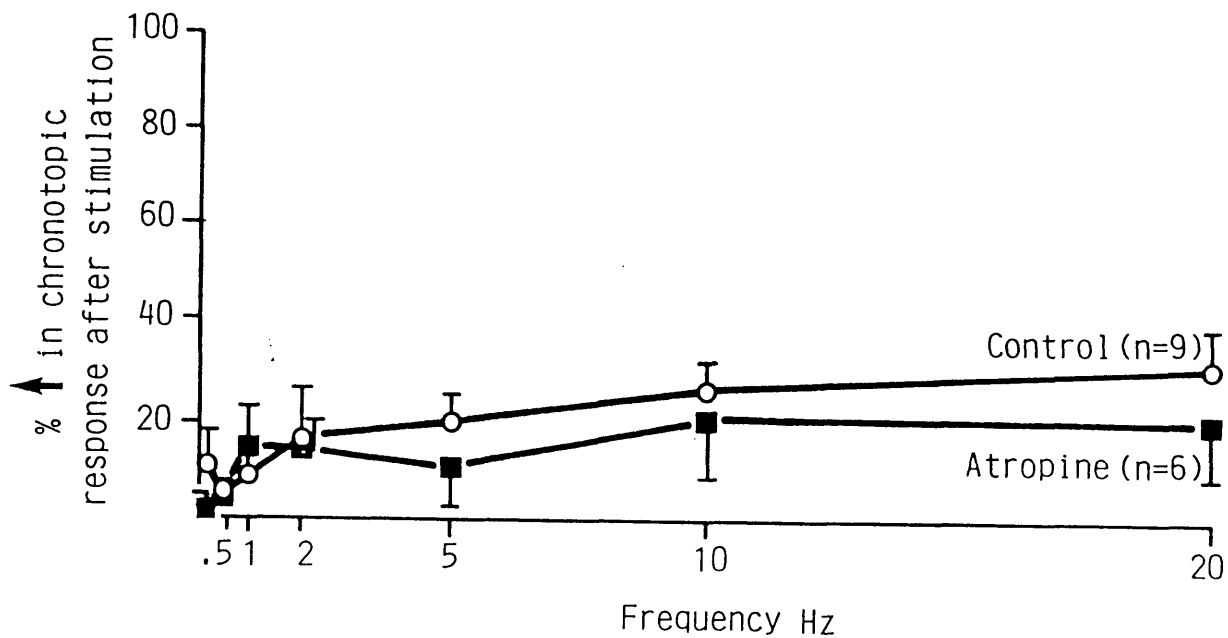
Atropine (10^{-6} M) potentiated the positive inotropic component of the post-stimulus response of the atria (Fig. 19).

The positive inotropic post-stimulus response was inhibited by propranolol (10^{-6} M) and guanethidine (10^{-6} M) (Fig. 20).

Phenoxybenzamine (10^{-6} M) potentiated the positive component of the post-stimulus response of the atria (Fig. 21).

Reserpine abolished the positive components of the post-stimulus response of the atria.

FIGURE 19. Effect of atropine on the positive inotropic component of the post-stimulus response of the atria. Frequency response curves were produced in the absence (0————0) and presence (●————●) of atropine (10^{-6} M). Atropine potentiated this response of the atria following field stimulation. Each point represents the mean of ($\pm$ S.E. mean) of ξ 6 observations.

* 0.05>P>0.01,   ** 0.01>P>0.001,   *** P<0.001.

FIGURE 20. Effect of propranolol and guanethidine on the positive inotropic component of the post-stimulus response of the atria. Frequency response curves were produced in the absence (0———0) and presence of propranolol (10^{-7} M) (☐----☐) and in the presence of guanethidine (10^{-6} M) (●———●). Both propranolol and guanethidine inhibited this response of the atria following field stimulation. Each point is the mean ($\pm$ S.E. mean) of ≮ 7 observations. *** P<0.001.
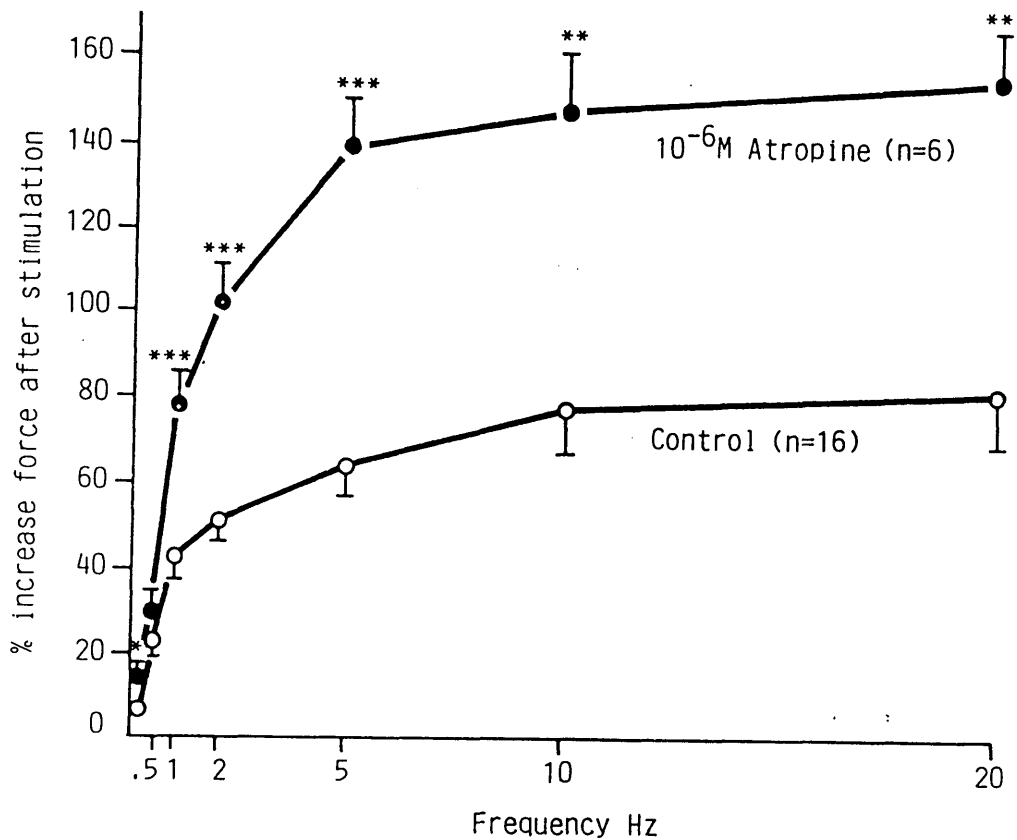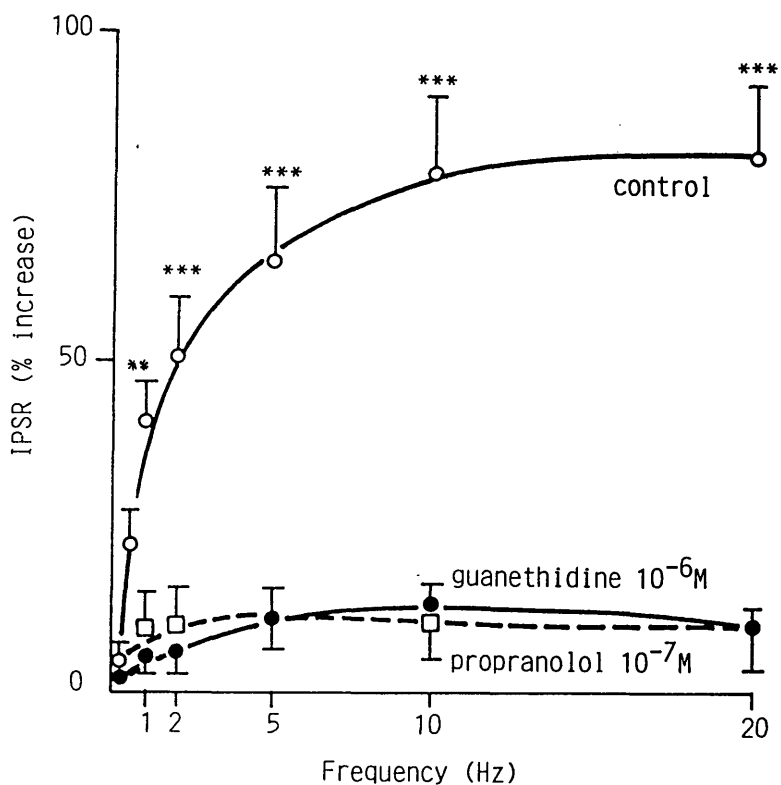
Figure 21. Effect of phenoxybenzamine on the positive
inotropic component of the post-stimulus response of
the atria. Frequency response curves were produced in
the absence (0————0) and presence (▲————▲) of
phenoxybenzamine (10^{-6} M). Phenoxybenzamine
potentiated this response of the atria following
field stimulation. Each point is the mean
($\pm$ S.E. mean) of ≮ 6 obsevations.
** 0.01>P>0.001, *** P< 0.001.

Clonidine (10^{-7} M) did not affect the positive inotropic component of the post-stimulus response of the atria. Yohimbine (10^{-7} M), however, potentiated this response in the presence of clonidine (Fig. 22).

In the presence of atropine (10^{-6} M), clonidine (10^{-6} M) did inhibit the positive inotropic component of the post-stimulus response of the atria (Fig. 23).
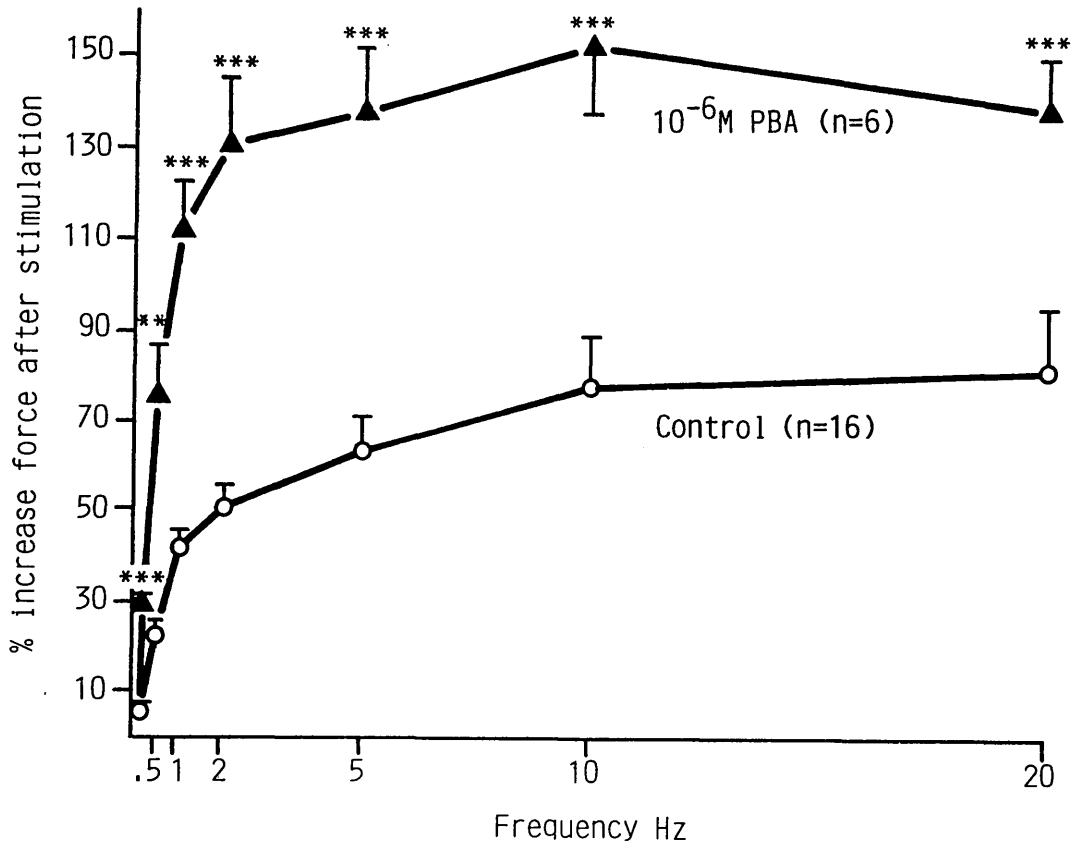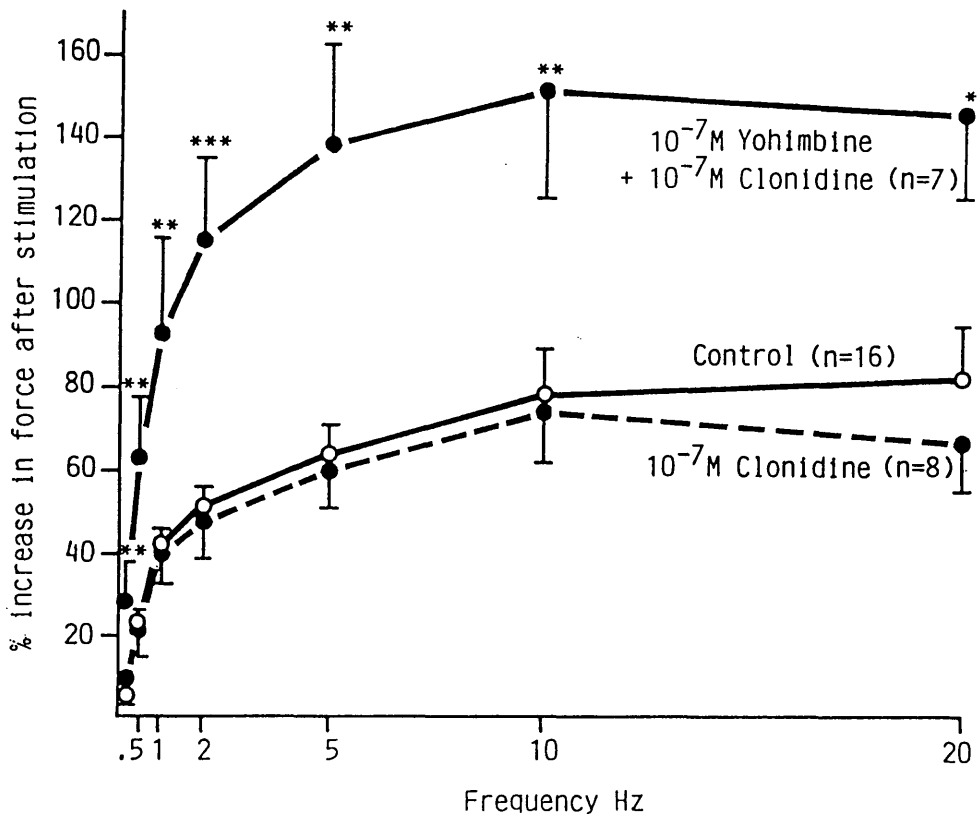
Figure 22. Effect of clonidine and yohimbine on the
positive inotropic component of the post-stimulus
response of the atria. Frequency response curves were
produced in the absence (O————O) and presence of
clonidine (10^{-7} M) (●– – –●) and in the presence of
yohimbine (10^{-7} M) (●————●). Clonidine did not affect
this response of the atria. Yohimbine, however,
potentiated the response in the presence of
clonidine. Each point is the mean ($\pm$ S.E. mean) of
$\textit{k}$ 7 obsevations.

* 0.05>P>0.01,   ** 0.01>P>0.001,   *** P<0.001.
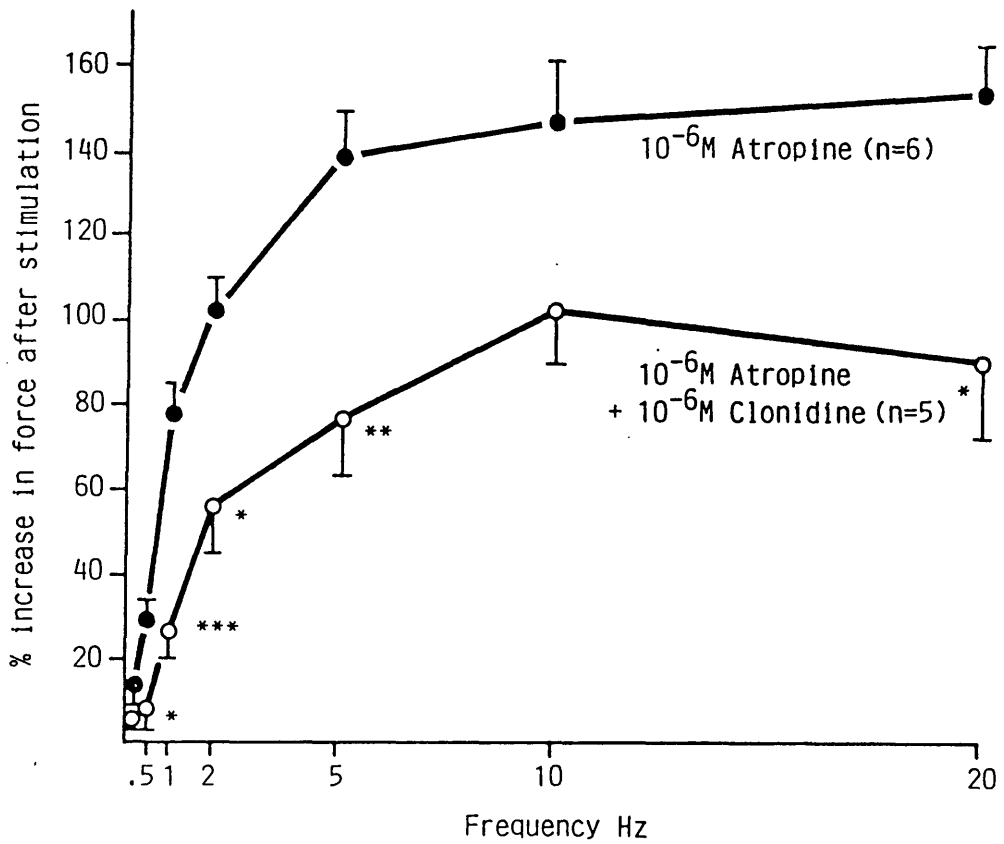
FIGURE 23. Effect of clonidine in the presence of atropine on the positive component of the post-stimulus response of the atria. Frequency response curves were produced in the presence of atropine (10^{-6} M) (●———●) and in the presence of atropine and clonidine (10^{-6} M) (O———O). In the presence of atropine, clonidine inhibited this response of the atria. Each point is the mean ($\pm$ S.E. mean) of $\nmid$ 5 observations.

* 0.05>P>0.01,   ** 0.01>P>0.001,   *** p<0.001.

PART III

EFFECTS OF FIELD STIMULATION ON THE OVERFLOW OF ^{3}H FROM ATRIA PREVIOUSLY INCUBATED IN [^{3}H]-NA, AND THE EFFECTS OF DRUGS ON THIS OVERFLOW

Electrical stimulation of atria increased the amount of radioactivity released into the Krebs solution in the organ bath. Collections of Krebs solution between stimulations contained only low levels of radioactivity. Consecutive stimulations at regular intervals (10 min) resulted in reproducible increases in the amount of radioactivity evoked by field stimulation (Fig. 24).

Chromatographic separation of [^{3}H]-NA from its metabolites showed that field stimulation increased the overflow of [^{3}H]-NA. Whereas only 13% of the spontaneous ^{3}H overflow was recovered as [^{3}H]-NA, almost 80% of the ^{3}H radioactivity released into the bathing solution during field stimulation was [^{3}H]-NA (Table 2).

Tetrodotoxin (5×10^{-9} M – 5×10^{-6} M) inhibited the overflow of radioactivity from atria in a dose-dependent manner (Fig. 25).
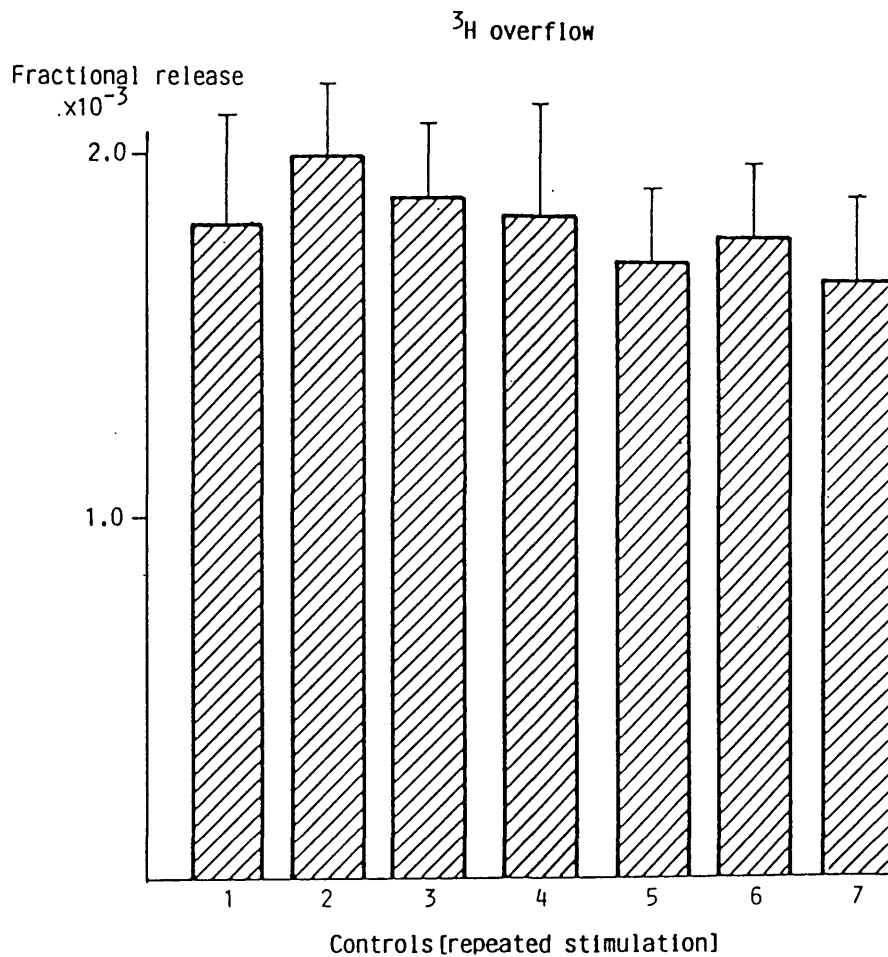
FIGURE 24. Effect of 7 consecutive stimulations at a frequency of 2Hz for a period of 30 sec on the release of ^{3}H from atria previously incubated in [^{3}H]-NA. Each column represents the mean ($\pm$ S.E. mean) of 7 observations.

Table 2. Percentage of (^{3}H)-NA and its (^{3}H)-metabolites in Krebs solution bathing isolated, field stimulated atria

	Compound	Mean Percentage ($\pm$ S.E. Mean) of ^{3}H Released
Before stimulation	NA	13.2 $\pm$ 0.8
	DOPEG	25.3 $\pm$ 6.3
	OMDA	28.0 $\pm$ 2.5
	NMN	12.9 $\pm$ 2.1
	DOMA	20.6 $\pm$ 2.6
After stimulation	NA	78.2 $\pm$ 3.1 ***
	DOPEG	3.0 $\pm$ 0.2 **
	OMDA	3.0 $\pm$ 1.2 **
	NMN	9.2 $\pm$ 3.1 NS
	DOMA	6.6 $\pm$ 2.7 **

NA   noradrenaline

DOPEG   3,4-dihydroxyphenyl glycol

OMDA   O-methylated deaminated metabolites

NMN   normetanephrine

DOMA   3,4-dihydroxymandelic acid

Each value is the mean of $\neq$ 5 observations.

** $0.01 > P > 0.001$, *** $P < 0.001$.

For comparison between corresponding values measured before and after stimulation.
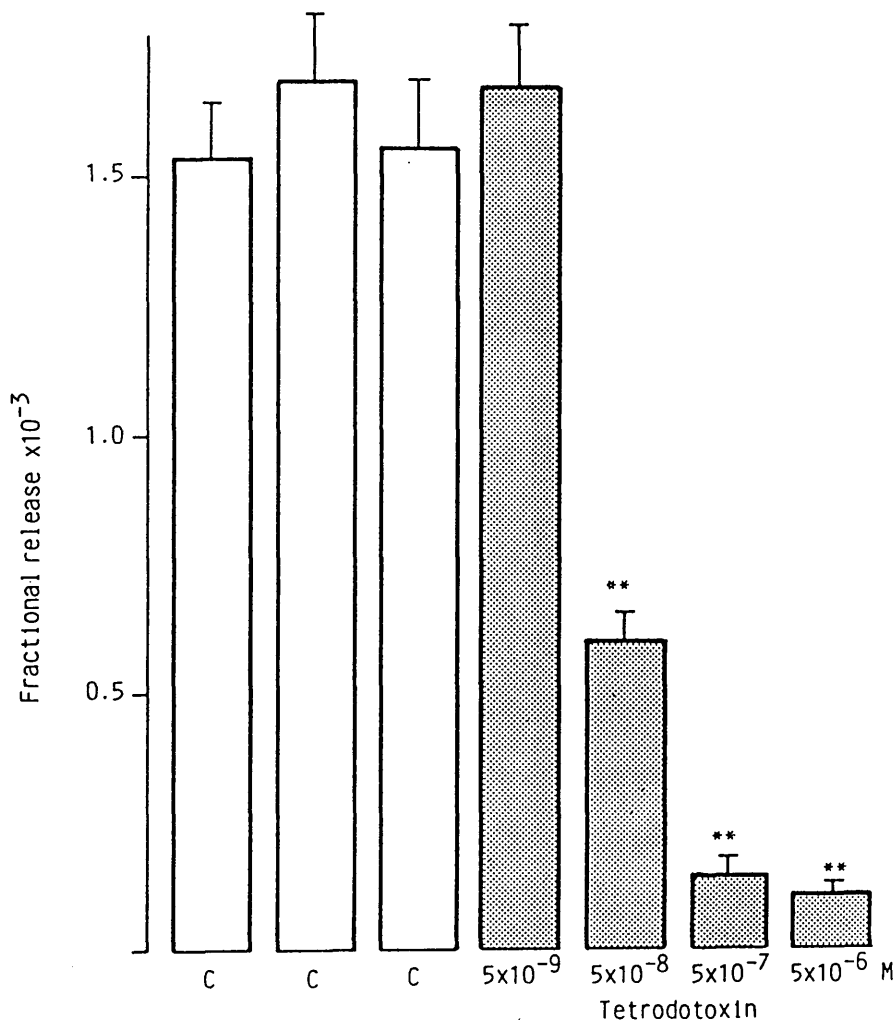
FIGURE 25. Effect of tetrodotoxin on field stimulation-
evoked overflow of ^{3}H in atria. The open histograms show
successive control responses prior to drug addition. The
succeeding 4 shaded histograms show the inhibitory effect
of tetrodotoxin added in increasing concentrations
(5×10^{-9} M - 5×10^{-6} M) in the same experiments. Each
column represents the mean ($\pm$ S.E. mean) of 4
observations.
** 0.01>P>0.001 for comparison with control prior to drug
addition.

Atropine and yohimbine (10^{-8} M- 10^{-5} M) potentiated the overflow of ^{3}H from atria. The combined effects of atropine and yohimbine at 10^{-5} M were additive (Fig. 26). The ability of yohimbine (10^{-8} M – 10^{-5} M) to potentiate ^{3}H overflow was enhanced in the presence of 10^{-6} M atropine (Fig 27).

Clonidine (10^{-8} M – 10^{-5} M) inhibited the stimulation-evoked overflow of ^{3}H from atria in a dose-dependent manner (Fig. 28).

Acetylcholine (10^{-8} M) potentiated the overflow of radioactivity from atria. In the same experiments acetylcholine (10^{-7} M – 10^{-5} M) inhibited the overflow of radioactivity in a dose-dependent manner (Fig. 29).

^{3}H overflow was potentiated by treatment with a combination of tranylcypromine (10^{-5} M), desmethylimipramine (10^{-5} M) and 17,β - oestradiol (10^{-5} M), which block respectively monoamine oxidase and the neuronal and extraneuronal uptake of NA (Figs. 30, 31). The ability of clonidine (10^{-5} M) to inhibit ^{3}H overflow was retained in the presence of these blocking drugs (Fig. 30). The ability of yohimbine (10^{-5} M) to potentiate ^{3}H overflow was also retained in the presence of the blocking drugs (Fig. 31).
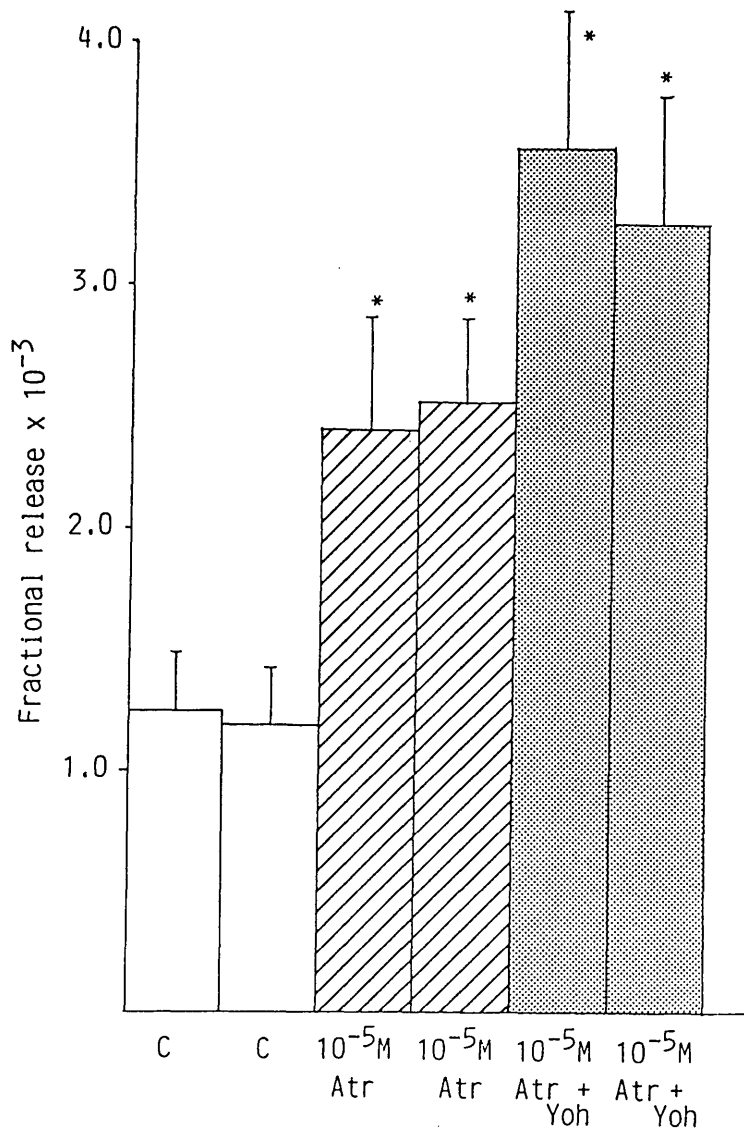
FIGURE 26. Effect of atropine and yohimbine in the
presence of atropine on the stimulation-evoked release
of ^{3}H in atria. The open histograms show successive
control responses prior to drug addition. The succeeding
2 diagonal striped histograms show the potentiating
effect of atropine (10^{-5} M) and the final 2 shaded
histograms show the further potentiating effect of
yohimbine (10^{-5} M) in the presence of
atropine (10^{-5} M). Each column represents the mean ($\pm$
S.E. mean) of 4 observations.

* 0.05>P>0.01 for comparison with control prior to
atropine addition and for comparison with atropine prior
to yohimbine addition.
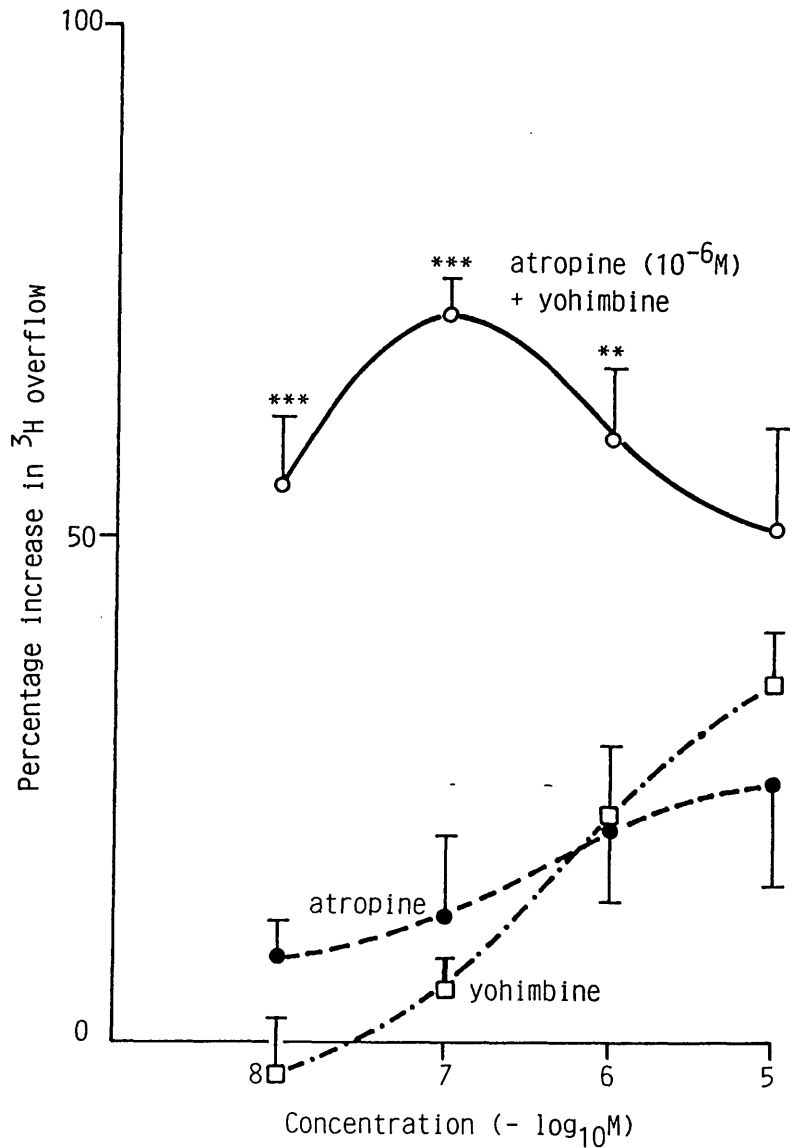
FIGURE 27. Dose %-response curves showing the effects of
atropine (●---●), yohimbine (□·····□) and yohimbine in the
presence of atropine (10^{-6} M) (○———○) on the
stimulation-evoked overflow of ^3H from atria. Asterisks
indicate a significant difference between yohimbine and
yohimbine in the presence of atropine. Each point is the
mean (± S.E. mean) of ≮ 5 observations.
** $0.01 > P > 0.001$, ***$P < 0.001$.

^{3}H overflow

Fractional release
x10^{-3}

Clonidine

2 —

1 —

Controls   10^{-8} 10^{-7} 10^{-6} 10^{-5}M
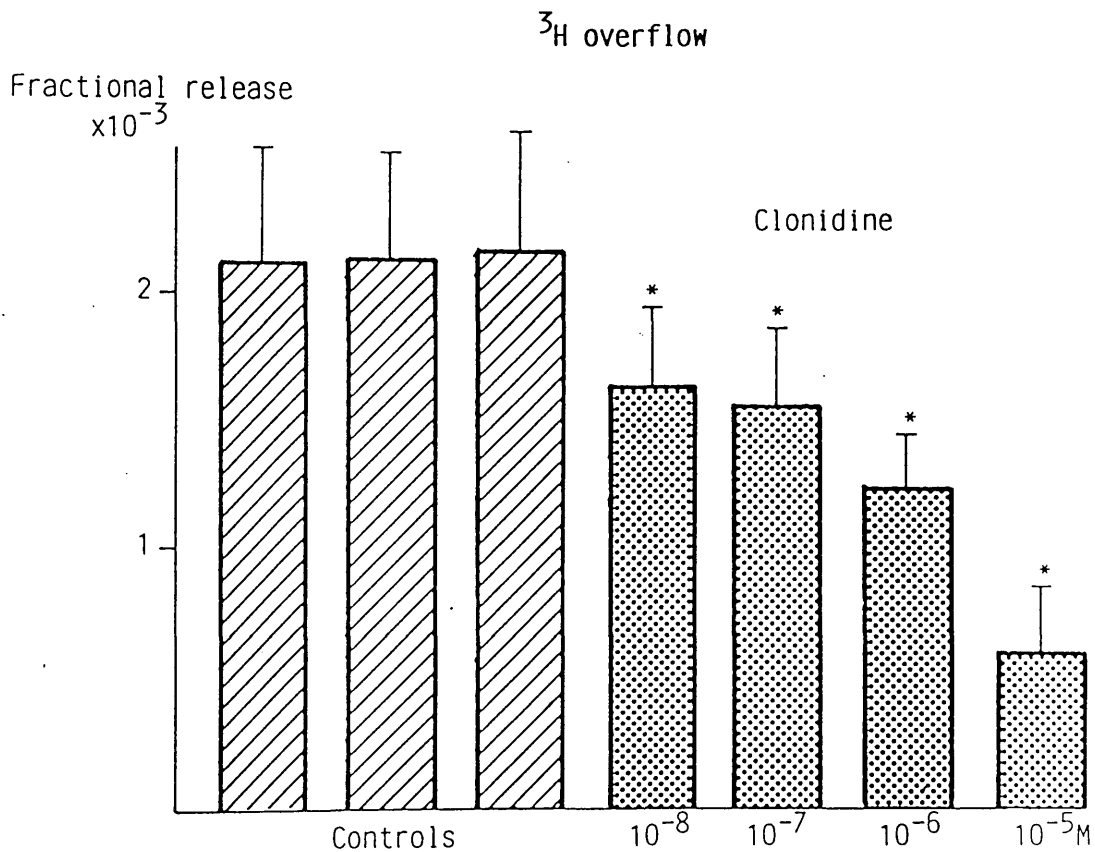
Figure 28. Effect of clonidine on field stimulation-
evoked overflow of ^{3}H in atria. The diagonal striped
histograms show successive control responses prior to
drug addition. The succeeding 4 stippled histograms show
the inhibitory effect of clonidine added in increasing
concentrations (10^{-8} M – 10^{-5} M) in the same
experiments. Each column represents the mean
($\pm$ S.E. mean) of ≮ 4 observations.   * $0.05 > P > 0.01$.

FIGURE 29. Effects of ACh on field stimulation-evoked overflow of ^{3}H in atria. The open histograms show successive control responses prior to drug addition. The succeeding 4 diagonal striped histograms show the potentiating effect (10^{-8} M) and inhibitory effect (10^{-7} M - 10^{-5} M) of ACh in the same experiments. Each column represents the mean ($\pm$ S.E. mean) of 4 observations.

* 0.05>P>0.01, ** 0.01>P>0.001 for comparison with control prior to drug addition.
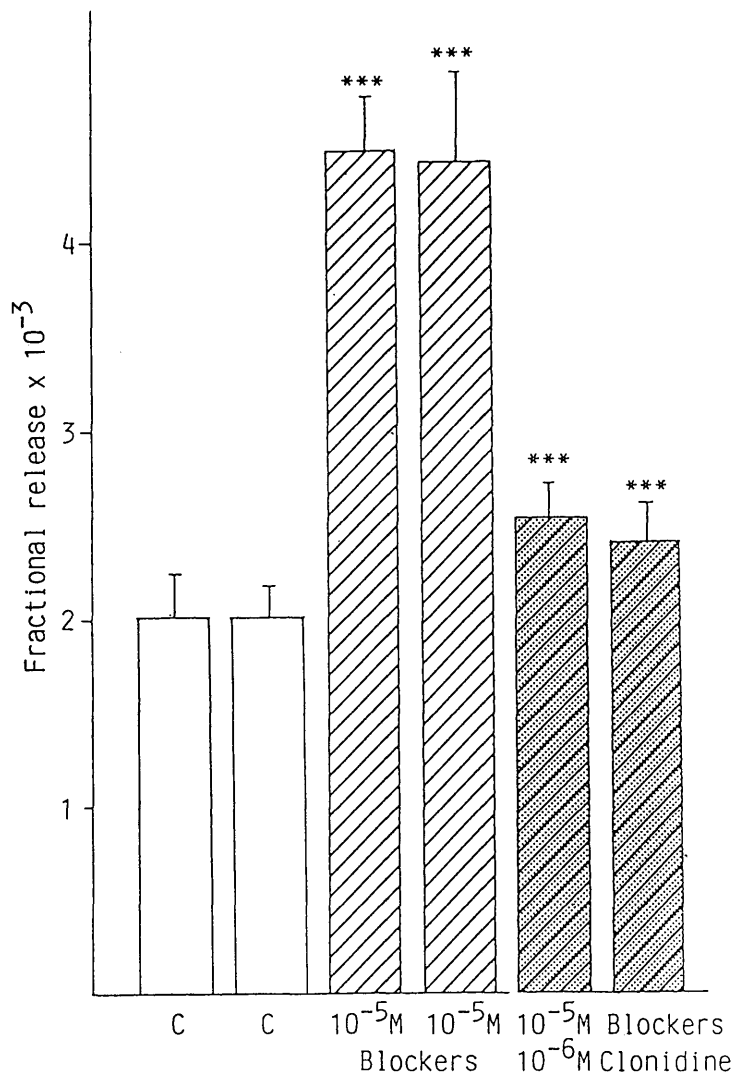
FIGURE 30. Effect of clonidine (10^{-6} M) in the presence
of 10^{-5} M tranylcypromine, desmethylimipramine and $17,\beta-$
oestradiol on field stimulation-evoked overflow of ^{3}H
in atria. The open histograms show successive control
responses prior to drug addition. The 2 diagonal
striped histograms show the significant potentiating
effect of 10^{-5} M tranylcypromine, desmethylimipramine
and $17,\beta-$oestradiol on this response. The 2 stippled
diagonal striped histograms show the significant
inhibitory effect of clonidine in the presence of
tranylcypromine, desmethylimipramine and $17,\beta-$
oestradiol. Each column represents the mean ($\pm$ S.E.
mean) of $\{$ 4 observations. *** P<0.001.
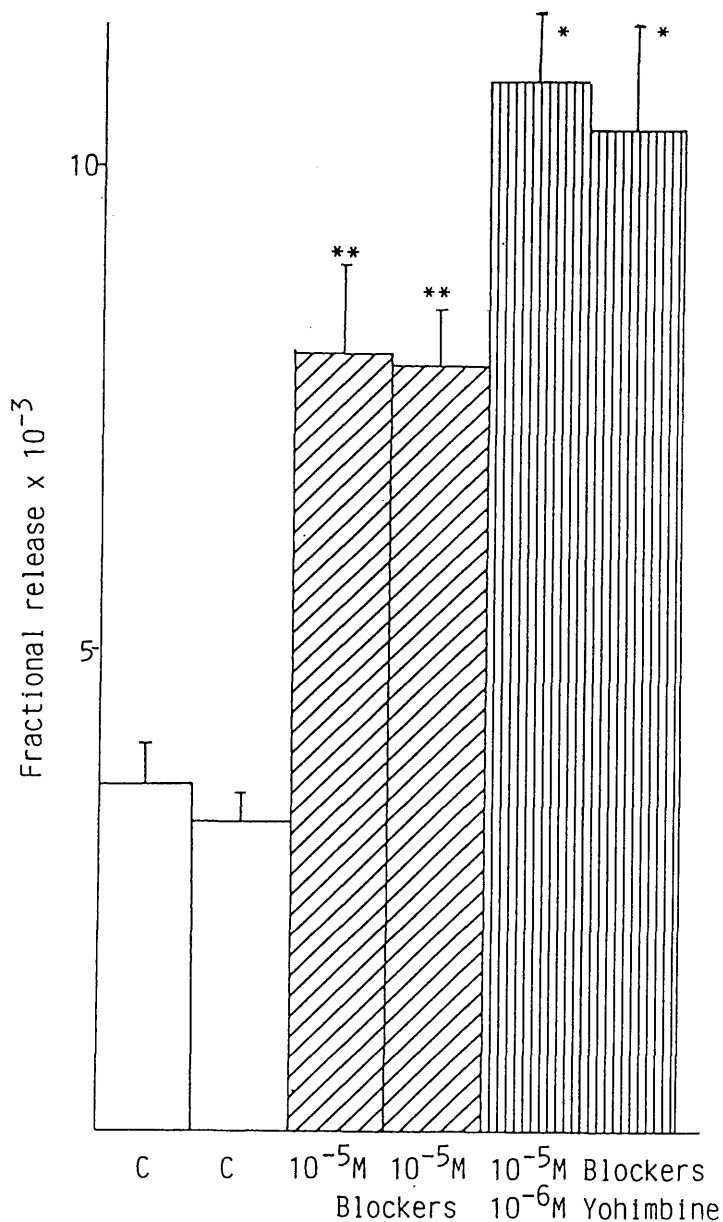
FIGURE 31. Effect of yohimbine (10^{-6} M) in the presence of
10^{-5}M tranylcypromine, desmethylimipramine and 17, β—
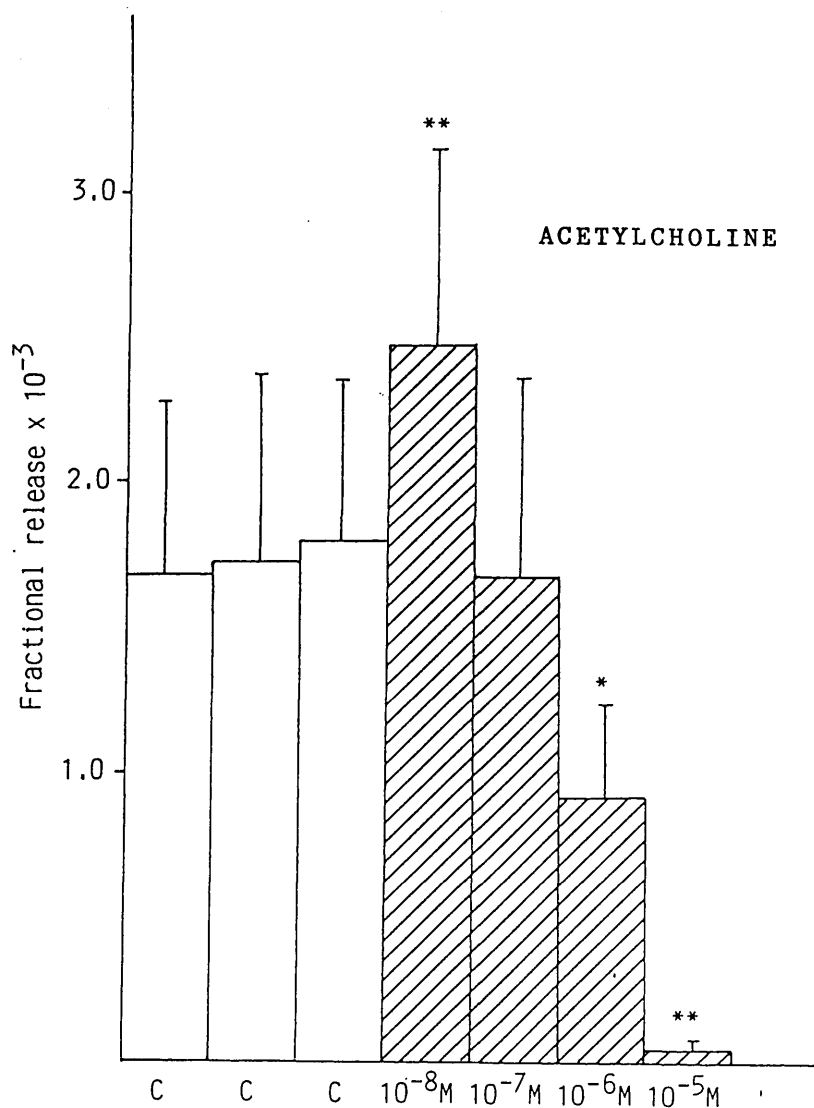oestradiol on field stimulation-evoked overflow of ^{3}H in
atria. The open histograms show successive control
responses prior to drug addition. The 2 diagonal striped
histograms show the potentiating effect of
tranylcypromine, desmethylimipramine and 17,β—oestradiol.
The 2 vertical striped histograms show the potentiating
effect of yohimbine in the presence of tranylcypromine,
desmethylimipramine and 17,β—oestradiol. Each column
represents the mean ($\pm$ S.E. mean) of 4 observations.
* $0.05 > P > 0.01$, ** $0.01 > P > 0.001$.

PART IV

EFFECT OF FIELD STIMULATION ON THE OVERFLOW OF ^{14}C AND ^{3}H FROM ATRIA PREVIOUSLY INCUBATED IN [^{14}C]-CHOLINE AND [^{3}H]-NA, AND THE EFFECTS OF DRUGS ON THIS OVERFLOW

Electrical stimulation of atria increased the amount of ^{14}C and ^{3}H released into the Krebs solution in the organ bath. Collections of the bathing solution between stimulations produced only low levels of ^{14}C and ^{3}H. Consecutive stimulations at regular intervals (10 min) resulted in a decreasing level of ^{14}C and ^{3}H overflow evoked by field stimulation (Fig. 32).

Tetrodotoxin (5×10^{-6} M) inhibited the overflow of ^{14}C from atria previously incubated in [^{14}C]-choline (Fig. 33).

Clonidine (10^{-5} M) and acetylcholine (10^{-5} M) inhibited the simultaneous stimulation-evoked release of ^{14}C and ^{3}H (Fig. 34).

Yohimbine (10^{-5} M) and atropine (10^{-6} M) potentiated the simultaneous stimulation-evoked release of ^{14}C and ^{3}H (Fig. 35).

Prazosin (10^{-5} M) potentiated the stimulation-evoked release of ^{3}H (Fig. 36) but not that of ^{14}C (Fig. 37) in the same experiments.
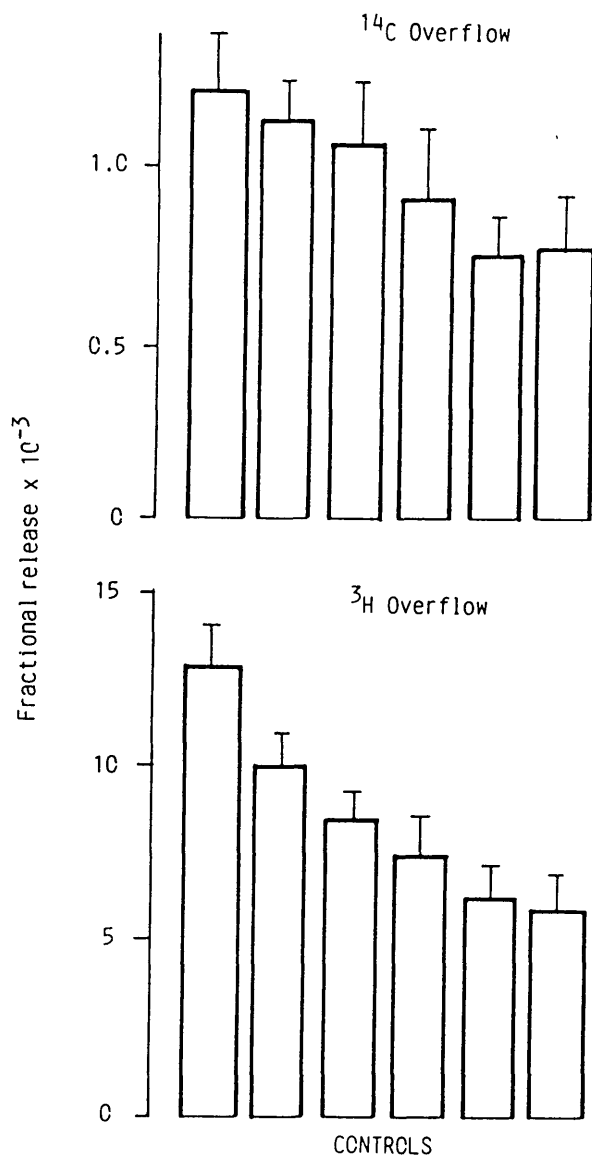
FIGURE 32. The top panel shows the effect of 6 consecutive stimulations at a frequency of 5Hz for 105 sec on the release of ^{14}C from atria previously incubated in [^{14}C]-choline. Each column represents the mean ($\pm$ S.E. mean) of 6 observations. The lower panel shows the effect of the same stimulations on the simultaneous release of ^{3}H from the same atria also incubated in [^{3}H]-NA. Each column represents the mean ($\pm$ S.E. mean) of 6 observations.
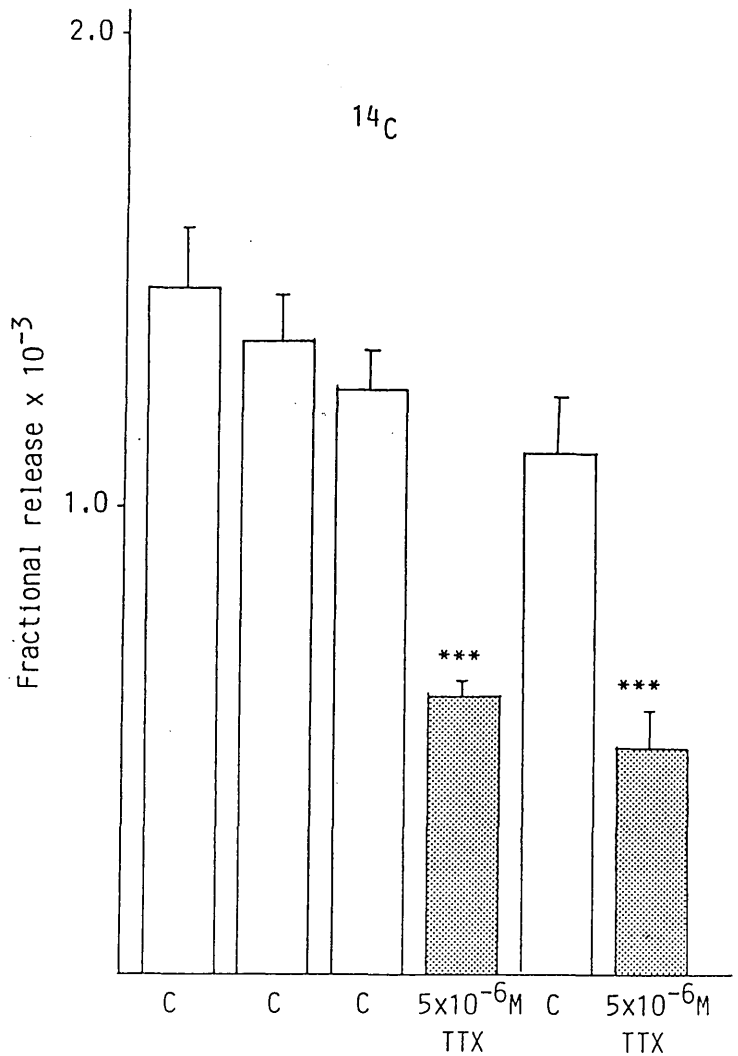
FIGURE 33. Effect of tetrodotoxin on the field
stimulation-induced overflow of ^{14}C in atria. The first
three open histograms show control responses prior to
drug addition. The first shaded histogram shows the
inhibitory effects of tetrodotoxin (5 x 10^{-6} M) on this
response. The next open histogram is a control response
and the inhibitory effect of tetrodotoxin is reversed.
The final shaded histogram shows again the inhibitory
effect of tetrodotoxin (5 x 10^{-6} M). Each column
represents the mean ($\pm$ S.E. mean) of 6 observations.
*** P<0.001 for comparison with control prior to drug
addition.
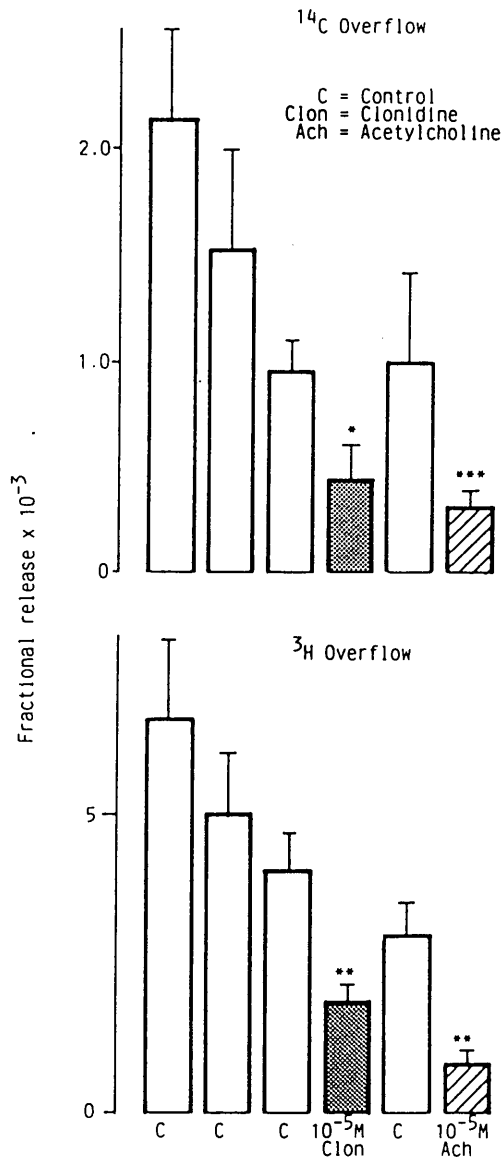
FIGURE 34. Effect of clonidine (10^{-5} M) and ACh (10^{-5} M) on the field stimulation-induced overflow of ^{14}C (top panel) and ^{3}H (lower panel) in atria. The first three open histograms show control responses prior to drug addition. The first shaded histogram shows the inhibitory effect of clonidine on the overflow of both ^{14}C and ^{3}H. The next open histogram is a control response and the inhibitory effect of clonidine is reversed. The final diagonal striped histogram shows the inhibitory effect of ACh on the overflow of both ^{14}C and ^{3}H. Each column represents the mean ($\pm$ S.E. mean) of 6 observations. * $0.05 > P > 0.01$, ** $0.01 > P > 0.001$, *** $P < 0.001$ for comparison with control prior to drug addition.

^{14}C Overflow

2.0

C = Control
Yoh. = Yohimbine
Atr. = Atropine

**

**

1.5

1.0

0.5

0

Fractional release x 10^{-3}

^3H Overflow

10

*

*

5

0

C   C   C $10^{-5}M$ C $10^{-6}M$
                  Yoh.   Atr.

FIGURE 35. Effect of yohimbine (10^{-5} M) and atropine (10^{-6} M) on the field stimulation-induced overflow of ^{14}C (top panel) and ^3H (lower panel) in atria. The first three open histograms show control responses prior to drug addition. The first shaded histogram shows the potentiating effect of yohimbine on the overflow of both ^{14}C and ^3H. The next open histogram is a control response and the potentiating effect of yohimbine is reversed. The final diagonal striped histogram shows the potentiating effect of atropine on the overflow of both ^{14}C and ^3H. Each column represents the mean ($\pm$ S.E. mean) of 4 observations.

* $.05 > P > 0.01$,   ** $0.01 > P > 0.001$ for comparison with control prior to drug addition.
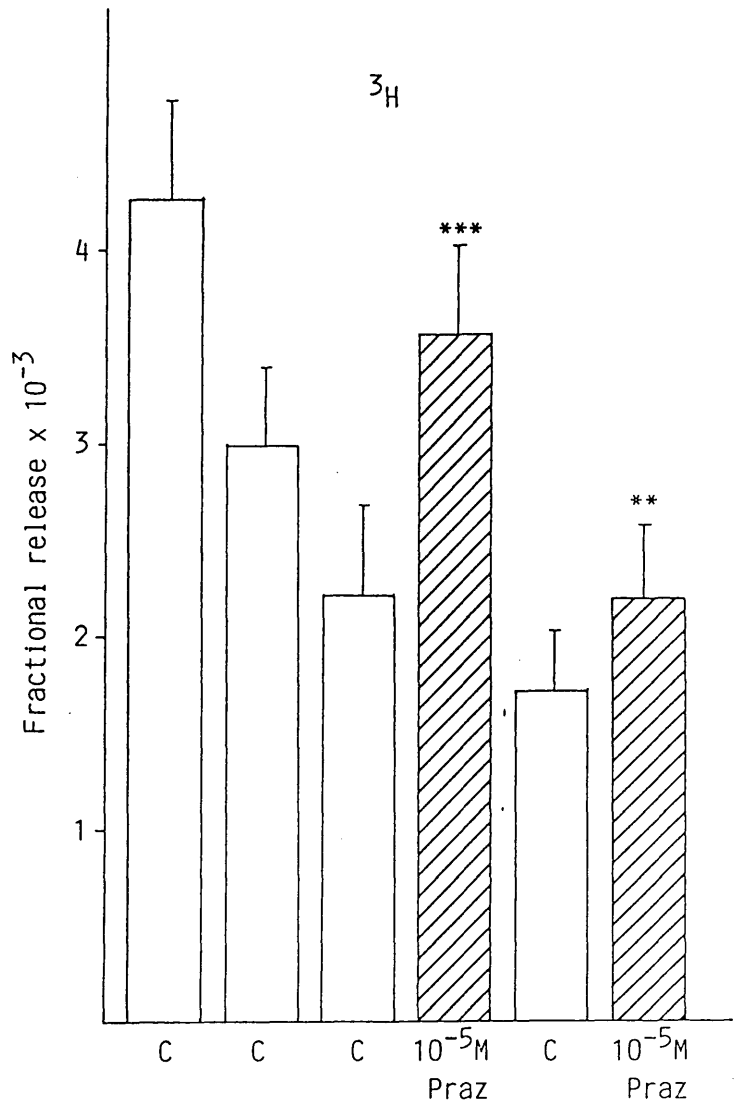
FIGURE 36. Effect of prazosin (10^{-5} M) on the field-
stimulation-induced overflow of ^{3}H in atria. The first 3
open histograms show control responses prior to drug
addition. The first diagonal striped histogram shows the
potentiating effect of prazosin on this response. The
next open histogram is a control response and the
potentiating effect of prazosin is abolished. The second
diagonal striped histogram again shows the potentiating
effect of prazosin on the overflow of ^{3}H. Each column
represents the mean ($\pm$ S.E. mean) of 4 observations.
** $0.01 > P > 0.001$,   *** $P < 0.001$ for comparison with control
prior to drug addition.
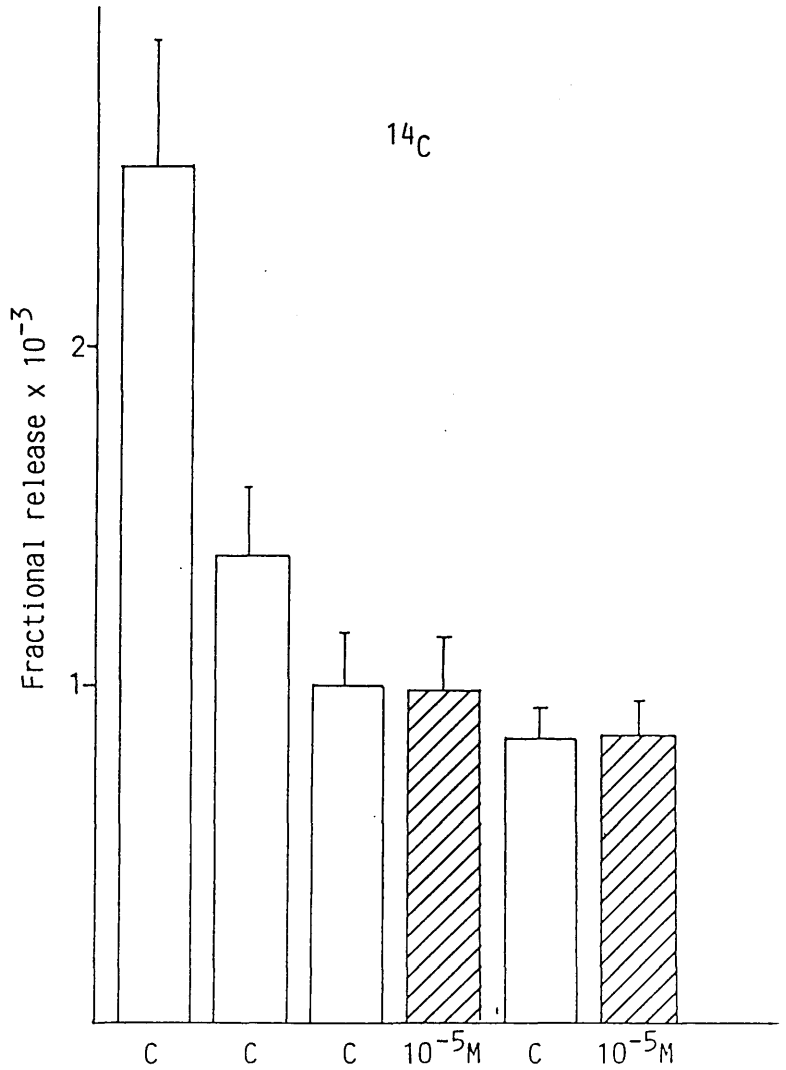
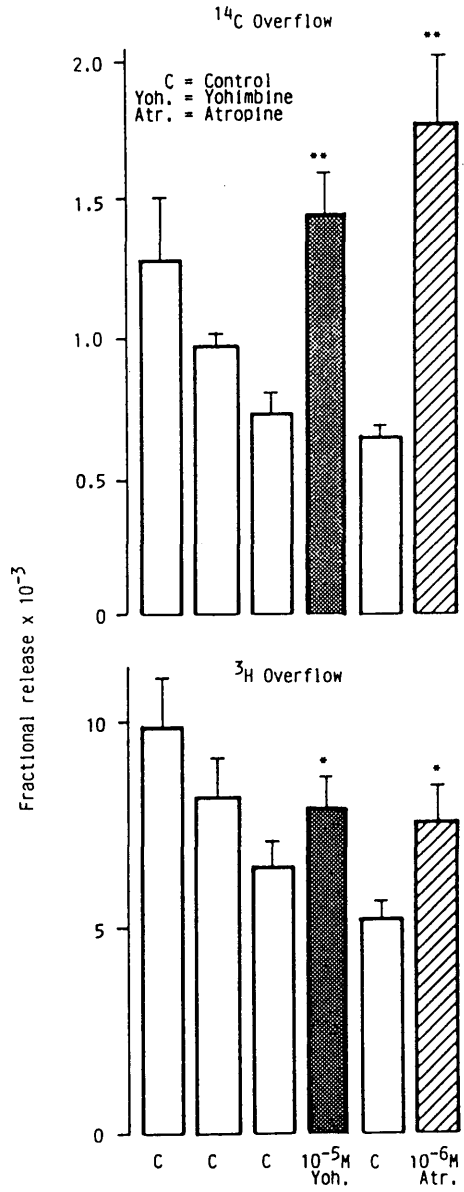FIGURE 37. Effect of prazosin $(10^{-5}$ M) on the field
stimulation-induced overflow of ^{14}C in the atria. The
first 3 open histograms show control responses prior to
drug addition. The first diagonal striped histogram shows
the lack of effect of prazosin on this response. The next
open histogram is a control response. The second diagonal
striped histogram again shows the lack of effect of
prazosin on the stimulation-evoked overflow of ^{14}C. Each
column represents the mean ($\pm$ S.E. mean) of 4
observations.

PART V

FURTHER INVESTIGATION OF THE EFFECTS OF DRUGS ON THE STIMULATION-EVOKED RELEASE OF ^{3}H FROM ATRIA PREVIOUSLY INCUBATED IN [^{3}H]-NA

Phentolamine (10^{-6} M) potentiated the stimulation-evoked overflow of ^{3}H. Acetylcholine (10^{-7} M – 10^{-5}M) inhibited the overflow of ^{3}H in the presence of phentolamine (Fig. 38). The presence of phentolamine did not affect the ability of acetylcholine to inhibit ^{3}H overflow at 10^{-7} M or 10^{-6} M. At 10^{-5} M acetylcholine was more effective at inhibiting ^{3}H overflow in the absence than in the presence of phentolamine (Fig. 39).

Clonidine (10^{-8} M – 10^{-5} M) was more effective at inhibiting ^{3}H overflow in the absence than in the presence of atropine (10^{-6} M) (Fig. 40).

The combined inhibitory effects of clonidine (10^{-7} M) and acetylcholine (10^{-7} M) on ^{3}H overflow were partially additive (Fig. 41).
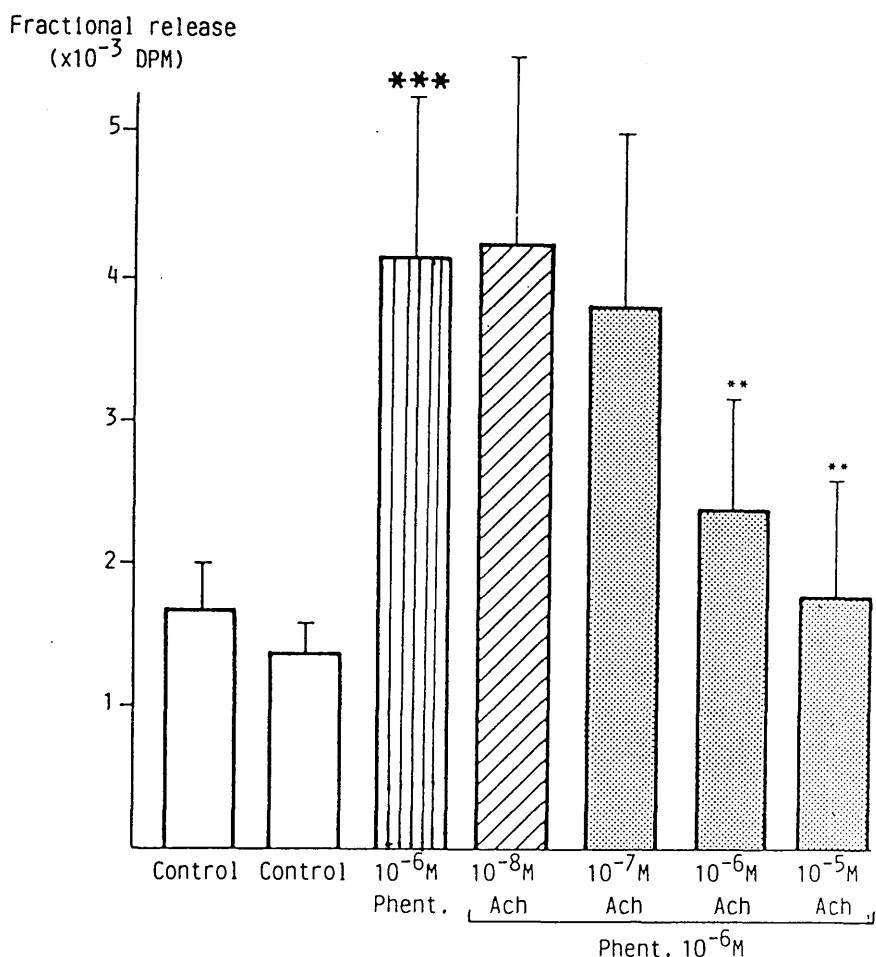
FIGURE 38. Effect of phentolamine and ACh in the presence of phentolamine on the stimulation-evoked release of ^{3}H in atria. The open histograms show control responses prior to drug addition. The vertical striped histogram shows the potentiating effect of phentolamine (10^{-6} M) on this response. The diagonal striped histogram shows the effect of ACh (10^{-8} M) in the presence of phentolamine. The succeeding 3 shaded histograms show the inhibitory effect of ACh added in increasing concentrations (10^{-7} M - 10^{-5} M) in the presence of phentolamine. Each column represents the mean ($\pm$ S.E. mean) of 6 observations. ** $0.01 > P > 0.001$ for comparison with phentolamine prior to ACh addition. *** $P < 0.001$ for comparison with control prior to phentolamine addition.
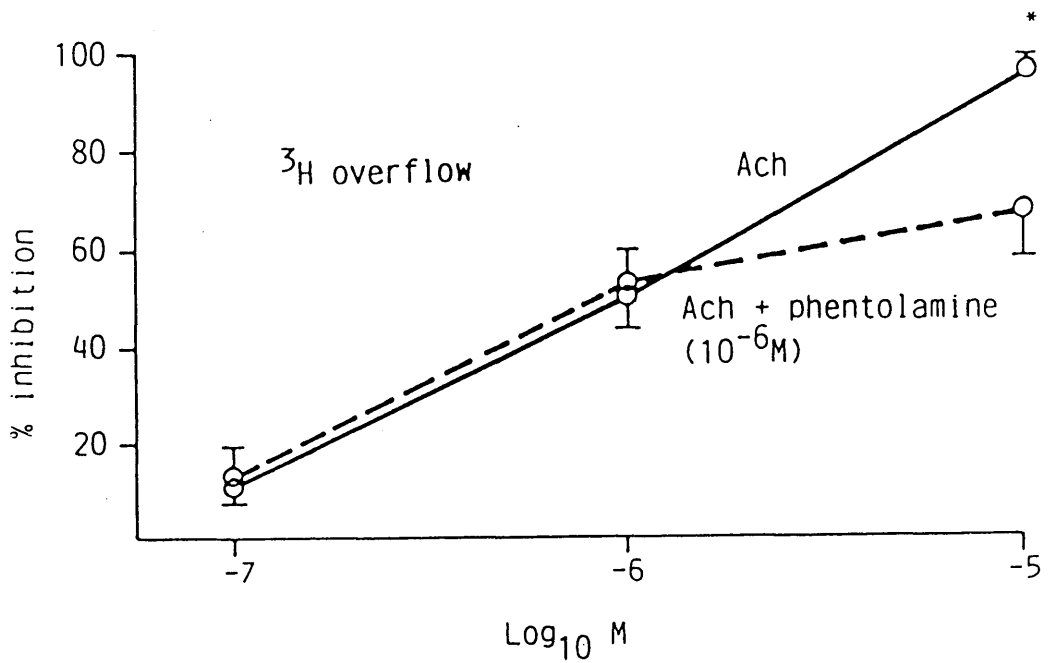
FIGURE 39. Dose %-response curves showing the inhibitory effects of ACh in the absence (O————O) and presence (O- - - -O) of phentolamine (10^{-6} M) on the stimulation-evoked overflow of ^3H in atria. Each point is the mean ($\pm$ S.E. mean) of ∤ 4 observations.

*   0.05>P>0.01.
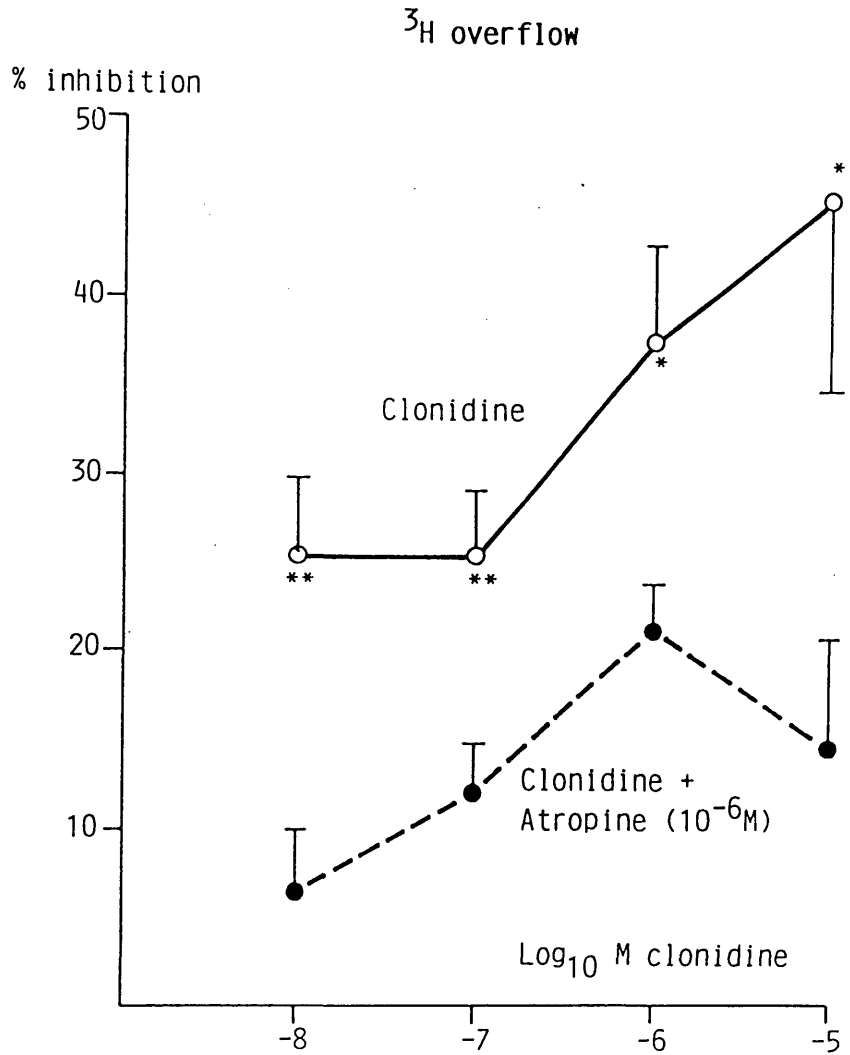
FIGURE 40. Dose %-response curves showing the inhibitory effects of clonidine (10^{-8} M - 10^{-5} M) in the absence (O——O) and in the presence of (●---●) of atropine (10^{-6} M) on the stimulation-evoked overflow of ^{3}H in the atria. Each point is the mean ($\pm$ S.E. mean) of ≮ 4 observations.   * 0.05>P>0.01,   ** 0.01>P>0.001.
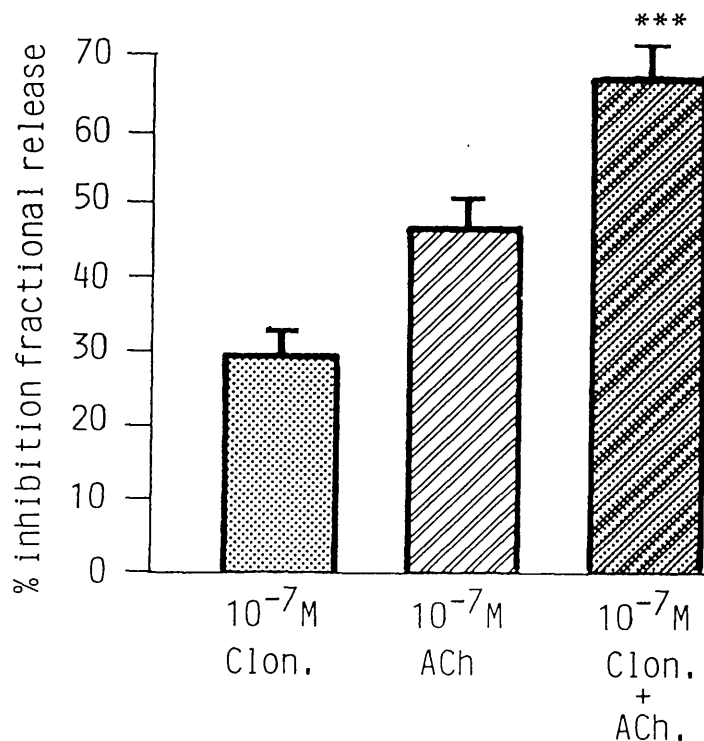
FIGURE 41. Inhibitory effects of clonidine and ACh alone
and combined on the stimulation-evoked overflow of ^{3}H
from atria. The stippled histogram shows the inhibitory
effect of clonidine (10^{-7} M) on ^{3}H overflow. The diagonal
striped histogram shows the inhibitory effect of ACh
(10^{-7} M) on ^{3}H overflow. The final diagonal striped
stippled histogram shows the additive effect of combining
clonidine (10^{-7} M) and ACh (10^{-7} M) on ^{3}H overflow. Each
column represents the mean ($\pm$ S.E. mean) of 4
observations. *** P<0.001 for comparison with ACh or
clonidine effect.

Isoprenaline (10^{-9} M - 10^{-6} M) potentiated the stimulation-evoked overflow of ^{3}H from atria previously incubated in [^{3}H]-NA (Fig. 42).

Isobutylmethylxanthine (IBMX) (10^{-5} M) potentiated the stimulation-evoked release of ^{3}H from atria previously incubated in [^{3}H]-NA (Fig. 43).

A single dose (10^{-4} M) of 8-Bromo-cAMP potentiated the stimulation-evoked release of ^{3}H from atria previously incubated in [^{3}H]-NA (Fig. 44).

A single dose (10^{-4} M) of 8-Bromo-cAMP, in the presence of atropine (10^{-5} M), retained its ability to potentiate the stimulation-evoked release of ^{3}H from atria previously incubated in [^{3}H]-NA (Fig 45).

Dimethylsulphoxide (DMSO) produced an increase in the stimulation-evoked overflow of ^{3}H. Forskolin (10^{-5} M), dissolved in DMSO, produced no further enhancement of ^{3}H overflow above that produced by DMSO alone (Fig. 46). Conversely, DMSO produced no enhancement of the positive inotropic component of the post-stimulus response in spontaneously beating paired atria, while forskolin (10^{-5} M), dissolved in DMSO, did potentiate this response (Table 3).

Animal pretreatment with pertussis toxin resulted in the loss of ability of clonidine (10^{-8} M) to inhibit the overflow of ^{3}H from atria. The ability of clonidine to inhibit ^{3}H overflow at higher concentrations (10^{-7} M - 10^{-5} M) was not affected by pertussis toxin pretreatment (Fig. 47). Pretreatment with pertussis toxin resulted in the abolition of the negative inotropic component of the post-stimulus response in spontaneously beating paired atria (Table 4).
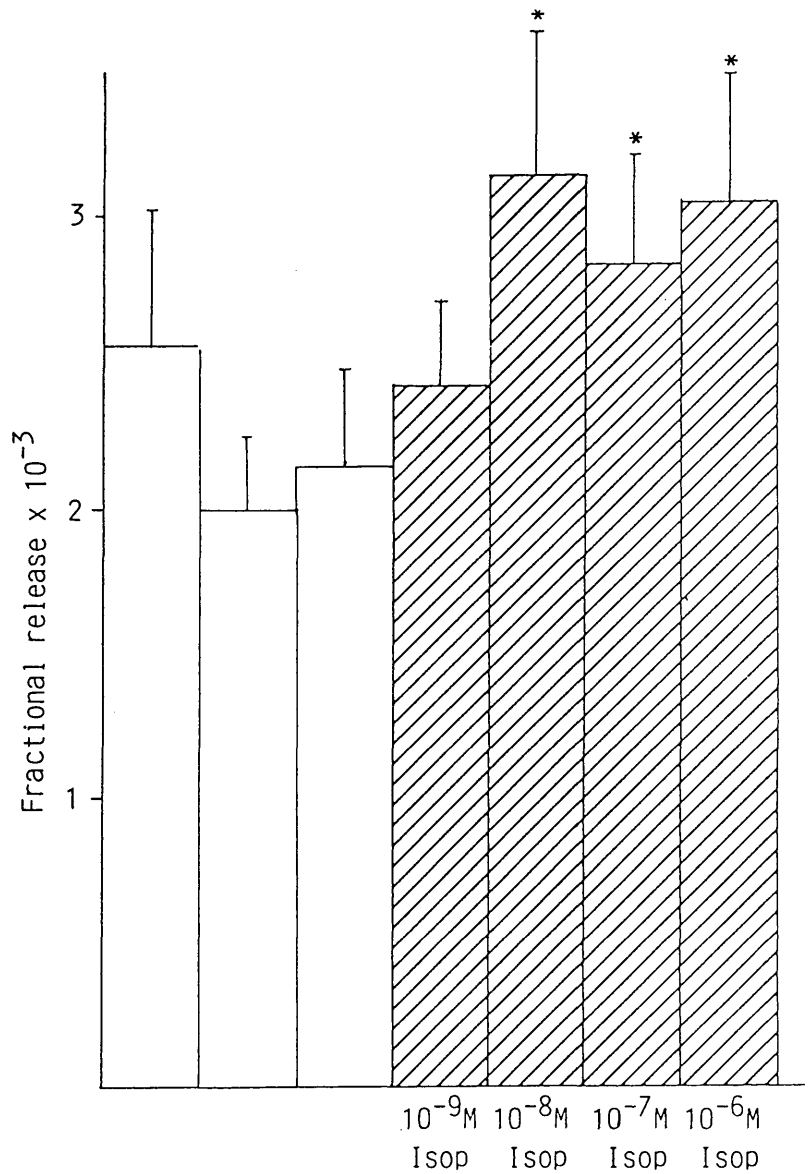
FIGURE 42. Effect of isoprenaline on field stimulation-
evoked overflow of ^{3}H in atria. The open histograms show
successive control responses prior to drug addition. The
succeeding 4 diagonal striped histograms show the
potentiating effect of isoprenaline added in increasing
concentrations (10^{-9} M - 10^{-5} M) in the same experiments.
Each column represents the mean ($\pm$ S.E. mean) of 4
observations.
* $0.05 > P > 0.01$ for comparison with control prior to drug
addition.
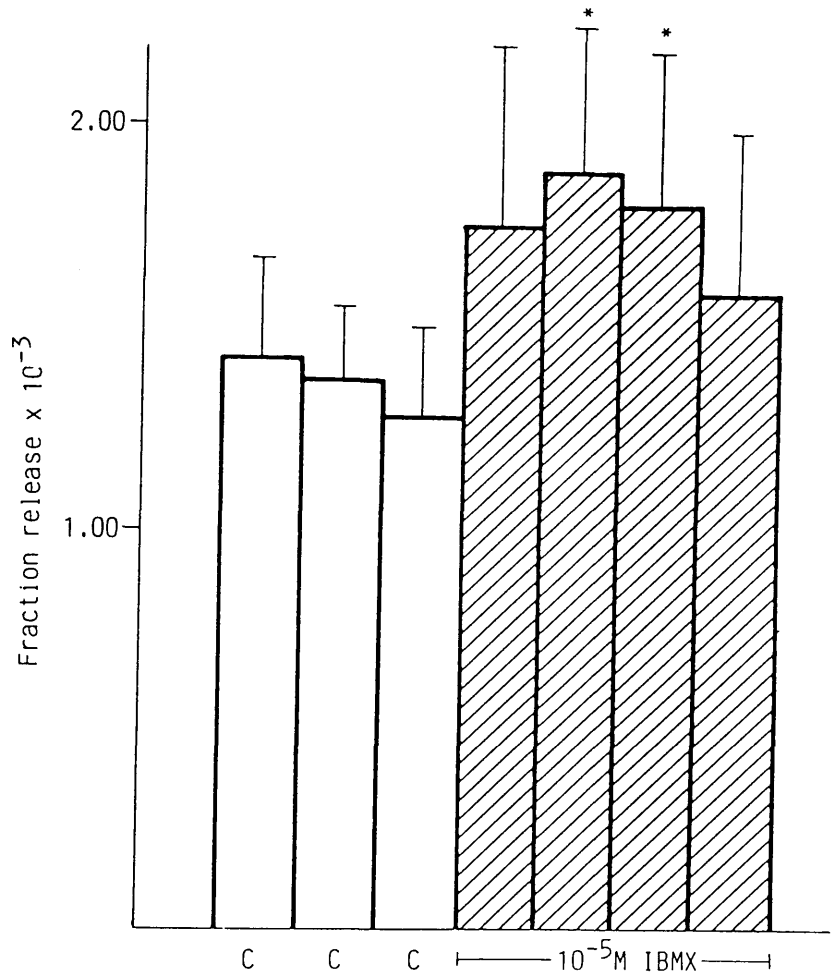
FIGURE 43. Effect of IBMX on field stimulation-induced overflow of ^{3}H in atria. The open histograms show successive control responses prior to drug addition. The succeeding 4 diagonal striped histograms show the potentiating effect of IBMX (10^{-5} M) present throughout this latter part of the experiment. Each column represents the mean ($^{\pm}$ S.E. mean) of 6 observations. * 0.05>P>0.01 for comparison with control prior to drug addition.

Fractional release × 10^{-3}

2.0 —

1.0 —

*

C   C 10^{-4}M   C
          8 Br cAMP
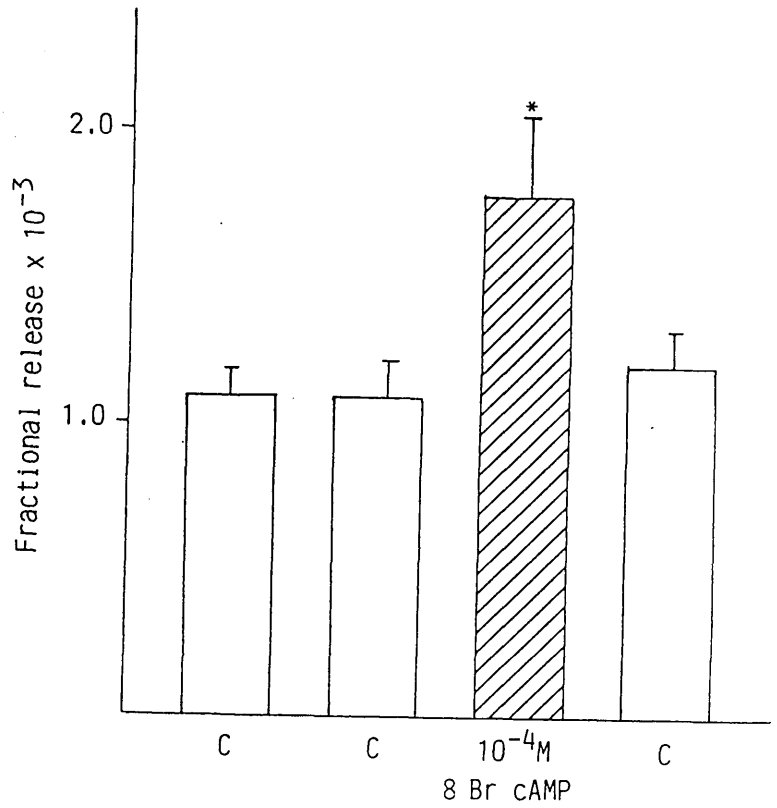
FIGURE 44. Effect of a single dose of 8—Bromo—cAMP on
field stimulation-evoked overflow of ^{3}H in atria. The
first 2 open histograms show control responses prior to
drug addition. The diagonal striped histogram shows the
potentiating effect of 8-Bromo-cAMP (10^{-4} M) on
stimulation-evoked ^{3}H overflow. The final open histogram
is a control response and the potentiating effect of
8 Bromo cAMP is abolished. Each column represents the
mean ($\pm$ S.E. mean) of 6 observations.
*   0.05>P>0.01 for comparison with control prior to drug
addition.
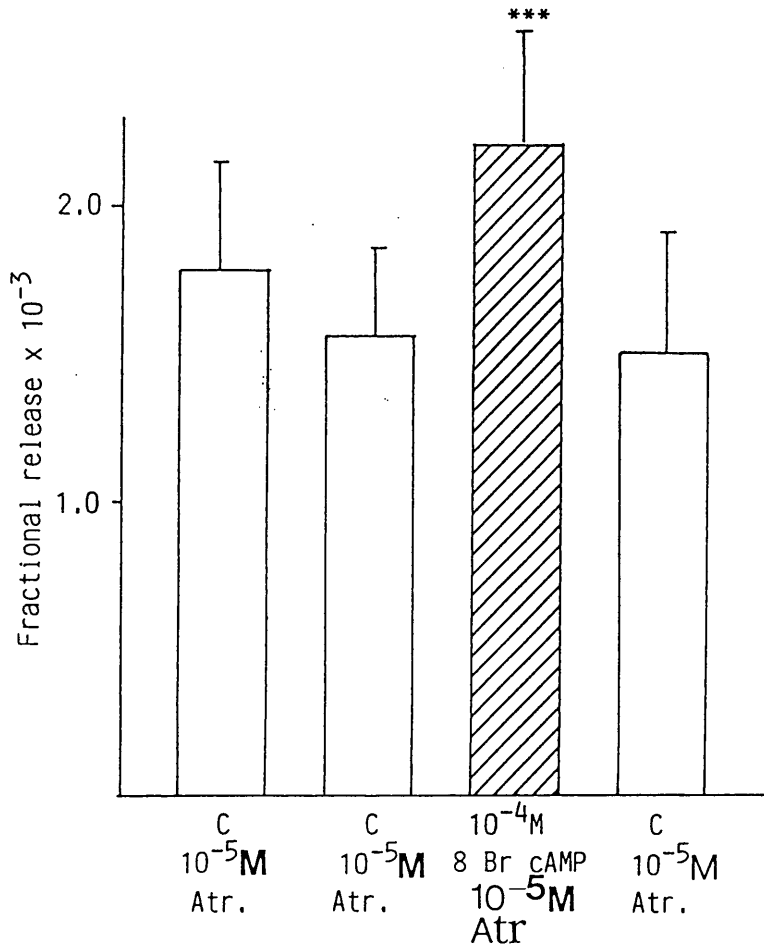
FIGURE 45. Effect of a single dose of 8-Bromo—cAMP, in the presence of atropine (10^{-5} M), on field stimulation-evoked overflow of ^{3}H in atria. The first 2 open histograms show control responses carried out in the presence of atropine. The diagonal striped histogram shows the potentiating effect of 8-Bromo—cAMP (10^{-4} M), in the presence of atropine, on stimulation-evoked ^{3}H overflow. The final open histogram is a control response carried out in the presence of atropine and the potentiating effect of 8—Bromo—cAMP is abolished. Each column represents the mean ($\pm$ S.E. mean) of $\not{<}$ 6 observations.

*** P<0.001 for comparison with atropine control prior to 8-Bromo-cAMP addition.
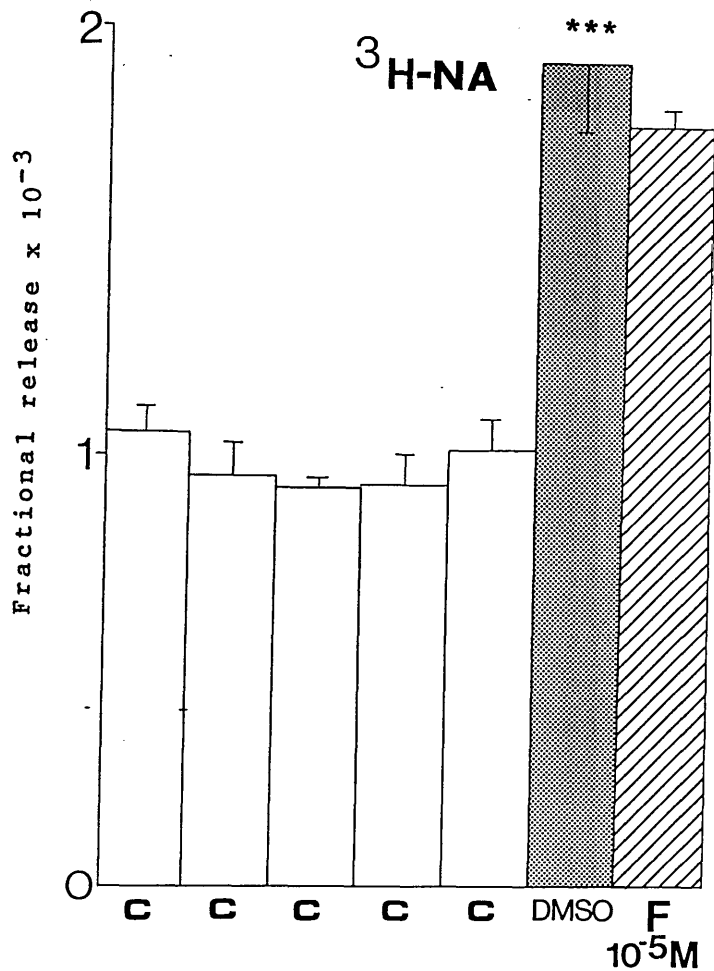
FIGURE 46. Effect of DMSO and forskolin dissolved in DMSO on field stimulation-evoked ^{3}H overflow in atria. The 5 open histograms show successive control stimulations prior to drug addition. The shaded histogram shows the potentiating effect of the vehicle DMSO (1% v:v) on this response. The diagonal striped histogram shows the lack of additive effect of forskolin (10^{-5} M) in comparison with DMSO alone. Each column represents the mean ($\pm$ S.E. mean) of ≮ 4 observations.

*** P<0.001 for comparison with control prior to drug addition.
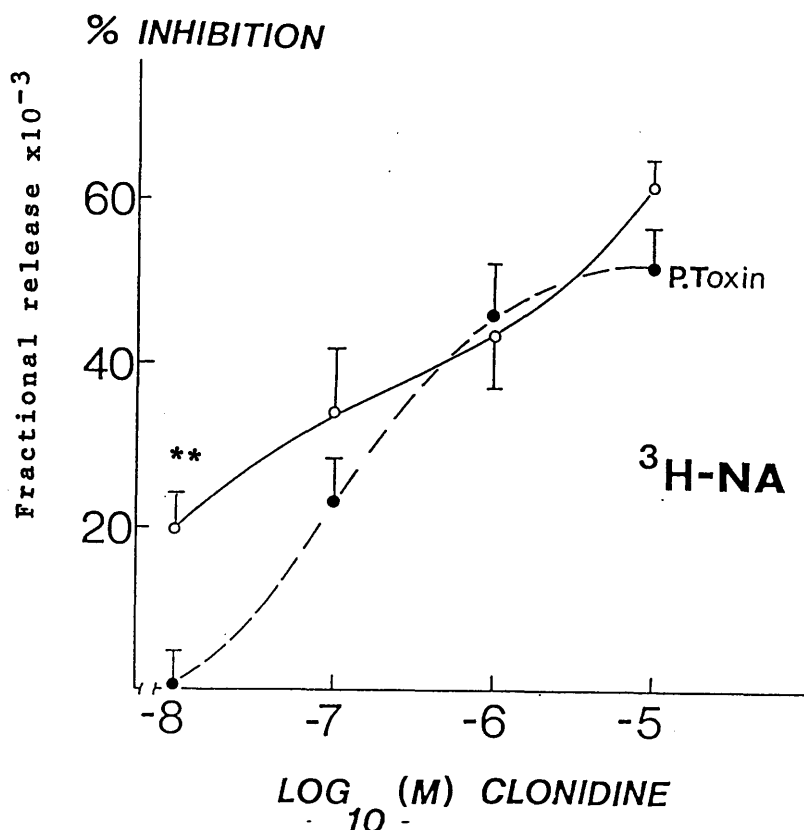
FIGURE 47. Dose %-response curves comparing the inhibitory effect of clonidine on ^3H overflow in atria from control (O———O) and pertussis toxin pretreated (●- - -●) rats. Pertussis toxin pretreatment has abolished the ability of clonidine to inhibit ^3H overflow at a concentration of clonidine of 10^{-8} M. At higher concentrations of clonidine (10^{-7} M - 10^{-5} M) there is little or no difference in the ability of clonidine to inhibit ^3H overflow in atria from control or pertussis toxin treated rats. Each column represents the mean ($\pm$ S.E. mean) of ≮ 5 observations.
** $0.01 > P > 0.001$.

Table 3. Effect of DMSO and forskolin dissolved in DMSO on the
          positive inotropic component of the post-stimulus
          response in spontaneously beating paired atria.

Treatment	Mean Percentage ($\pm$ S.E. Mean) Increase of Positive IPSR.
Control stimulation (2Hz x 30s)	41.3 $\pm$ 5.2 (n=6)
DMSO (1% v:v)	39.8 $\pm$ 3.6 (n=6)
Forskolin (10^{-5} M)/DMSO (1% V:V)	69.7 $\pm$ 5.9 (n=6) ***

*** P<0.001 for comparison between stimulation in the presence of

forskolin dissolved in DMSO and DMSO alone.

Table 4. Effect of pertussis toxin pretreatment on the negative
          inotropic component of the post-stimulus response of the
          spontaneously beating paired atria.

Pre-treatment	Mean Percentage ($\pm$ S.E. Mean) Decrease of Negative IPSR.
Control.Stimulation (2Hz x 30s)	35.7 $\pm$ 4.1 (n=6)
Pertussis toxin.	0.0 $\pm$ 0.0 (n=5) ***

*** P<0.001 for comparison between response from control and
pretreated atria.

Sodium nitroprusside (10^{-8} M – 10^{-5} M), (Fig. 48) and (10^{-7} M – 10^{-5} M), (Fig. 49) produced a dose-dependent increase in the stimulation-evoked overflow of radioactivity from atria incubated in [^{3}H]-NA.

Administration of 8-Bromo-cGMP (10^{-6} M – 10^{-4} M) also resulted in a potentiation of the stimulation-evoked release of ^{3}H (Fig. 50).

Sodium nitroprusside (10^{-5} M) was able to reverse the inhibitory effect of clonidine (10^{-5}M) on the overflow of ^{3}H. In contrast, sodium nitroprusside (10^{-5} M) was unable to reverse the inhibitory effect of acetylcholine (10^{-5} M) on ^{3}H overflow (Fig. 51).

In the presence of atropine (10^{-7} M), which potentiated ^{3}H overflow, sodium nitroprusside (10^{-7} M) was able further to enhance the overflow of ^{3}H (Fig. 52). In contrast, in the presence of a higher concentration of atropine (10^{-5} M), which also potentiated ^{3}H overflow, a higher dose of sodium nitroprusside (10^{-5} M) was unable to further enhance the overflow of ^{3}H (Fig. 53).
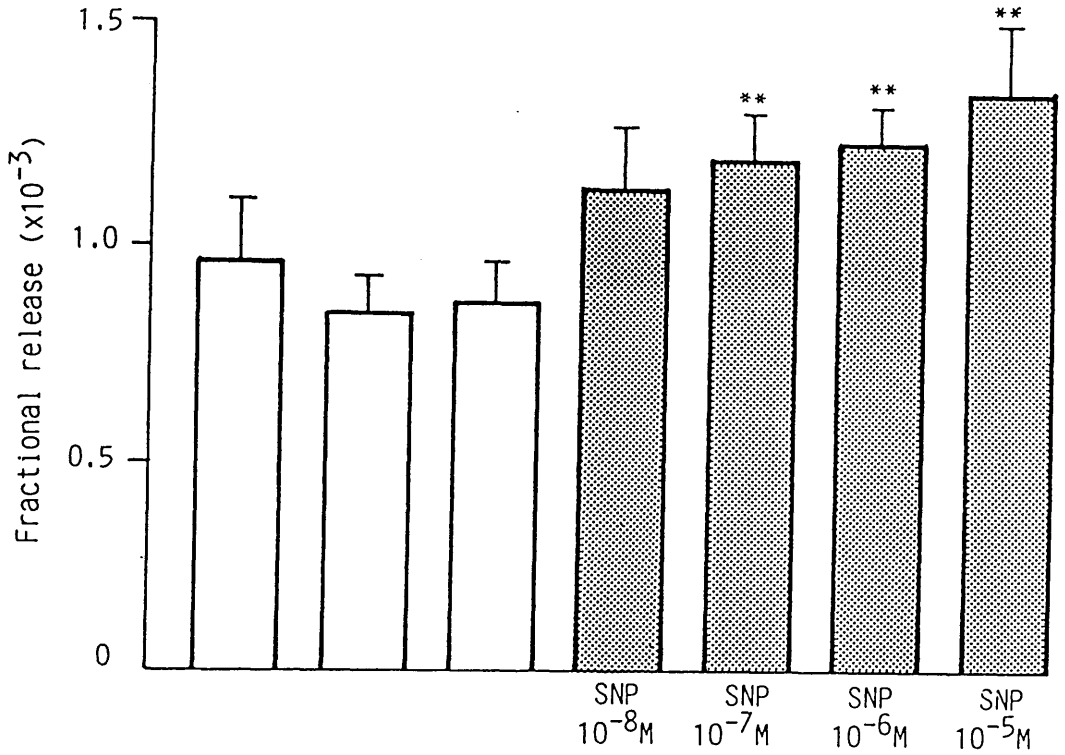
FIGURE 48. Effect of sodium nitroprusside (SNP) on field stimulation-evoked overflow of ^{3}H in atria at a frequency of 2Hz for a period of 30 sec. The open histograms show successive control responses prior to drug addition. The succeeding 4 shaded histograms show the potentiating effect of SNP added in increasing concentrations (10^{-8} M - 10^{-5} M) in the same experiment. Each column represents the mean ($\pm$ S.E. mean) of ξ 4 observations.

** $0.05 > P > 0.01$ for comparison with control prior to drug addition.
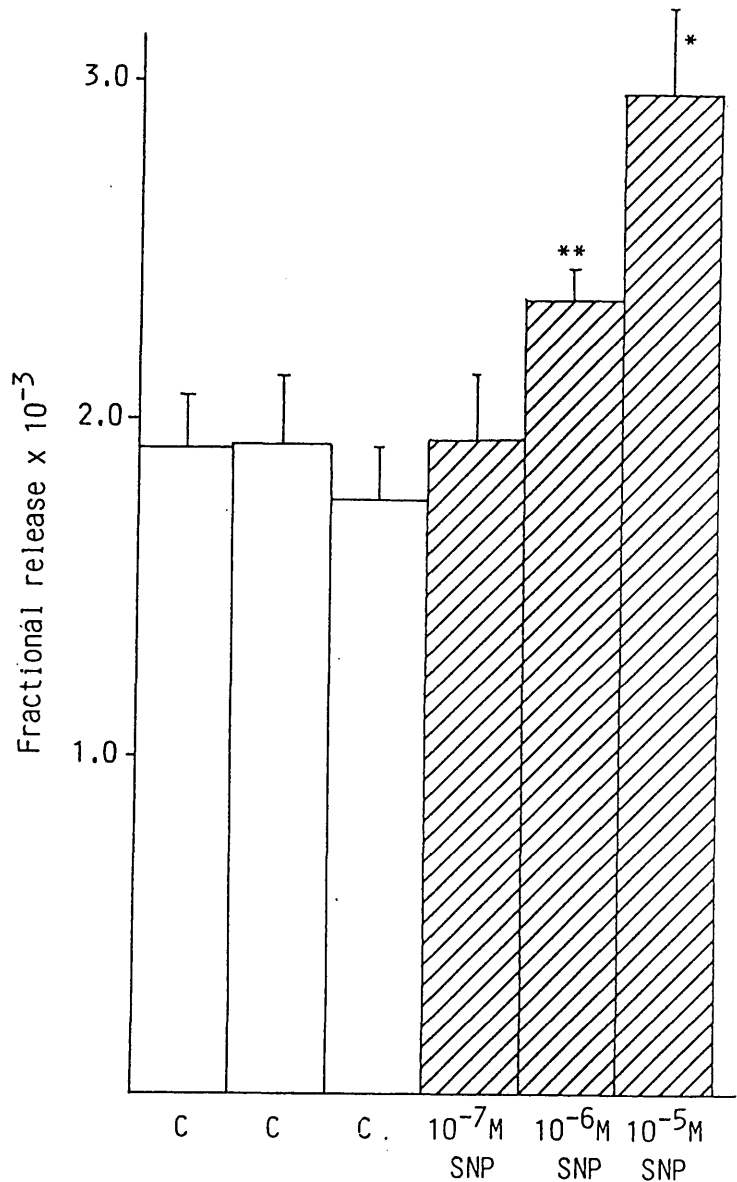
FIGURE 49. Effect of SNP on field stimulation-induced overflow of ^{3}H in atria at a frequency of 5Hz for a period of 12 sec. The open histograms show successive control responses prior to drug addition. The succeeding 3 diagonal striped histograms show the potentiating effect of SNP added in increasing concentrations (10^{-7} M – 10^{-5} M) in the same experiment. Each column represents the mean ($\pm$ S.E. mean) of { 4 observations.
* $0.05 > P > 0.01$, ** $0.01 > P > 0.001$ for comparisons with control prior to drug addition.
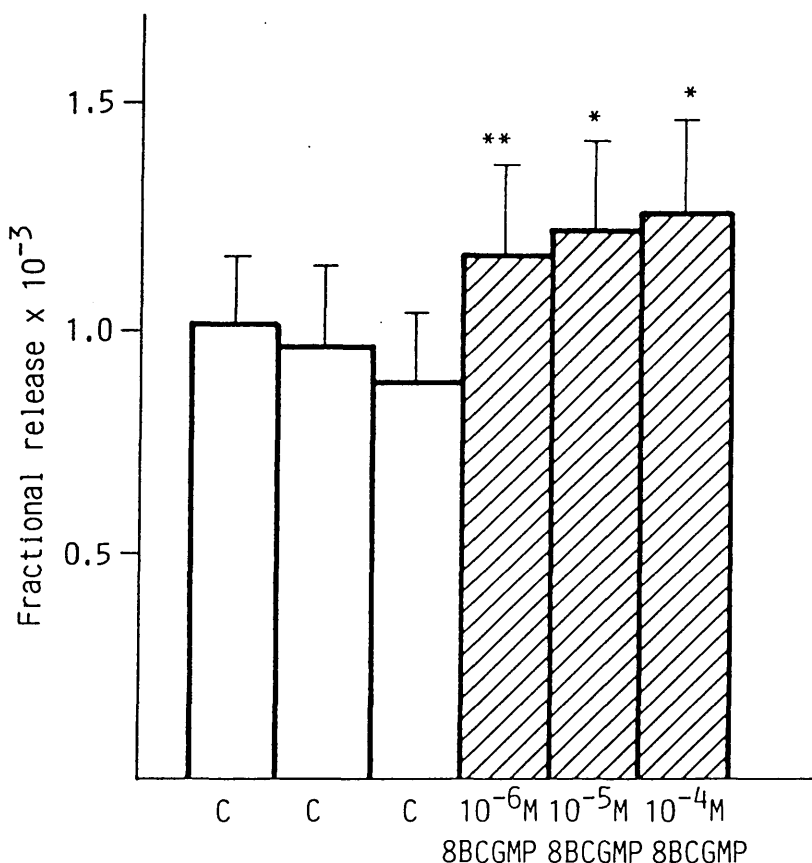
FIGURE 50. Effect of 8-Bromo-cGMP on field stimulation-evoked overflow of ^{3}H in atria. The open histograms show successive control responses prior to drug addition. The succeeding 3 diagonal striped histograms show the potentiating effect of 8-Bromo-cGMP added in increasing concentrations (10^{-6} M - 10^{-4} M) in the same experiment. Each column represents the mean ($\pm$ S.E. mean) of 4 observations.

* 0.05>P>0.01, ** 0.01>P>0.001 for comparison with control prior to drug addition.
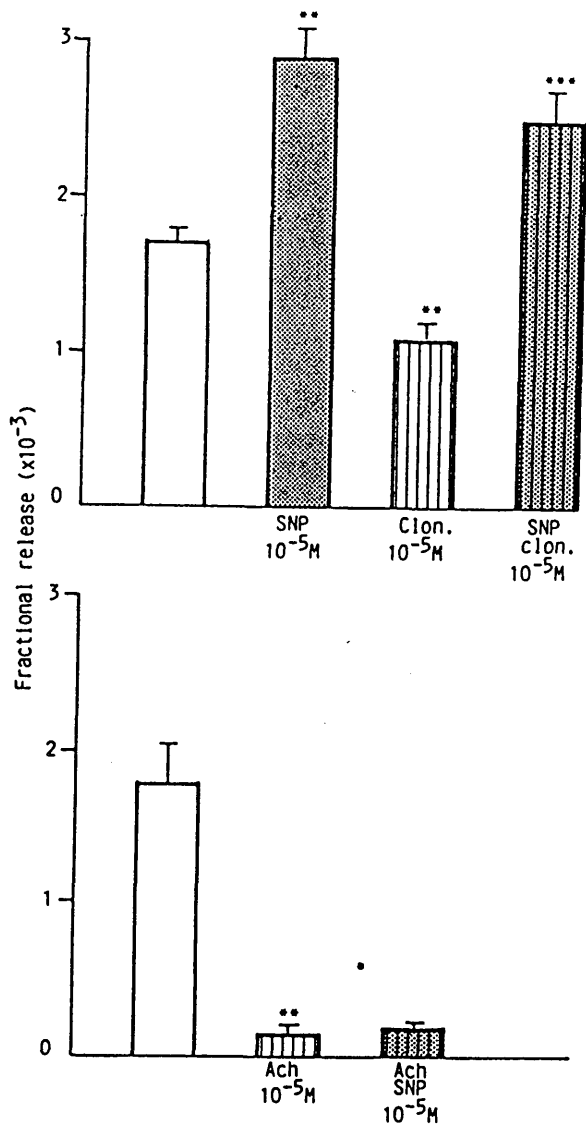
FIGURE 51. Effect of SNP on the inhibitory effect of
clonidine and ACh on field stimulation-evoked overflow of ^{3}H
in atria. In the top panel the open histogram is a control
response. The shaded histogram shows the potentiating effect
of SNP (10^{-5} M) on this response. The vertical striped
histogram shows the inhibitory effect of clonidine (10^{-5} M)
on this response. The shaded vertical striped histogram
shows the ability of SNP to reverse the inhibitory effect of
clonidine. In the lower panel the open histogram is a
control response. The vertical striped histogram shows the
inhibitory effect of ACh (10^{-5} M) on this response. The
shaded vertical striped histogram shows the inability of SNP
(10^{-5} M) to reverse this effect of ACh. Each column
represents the mean ($\pm$ S.E. mean) of 4 observations.
** 0.01>P>0.001, *** P<0.001.
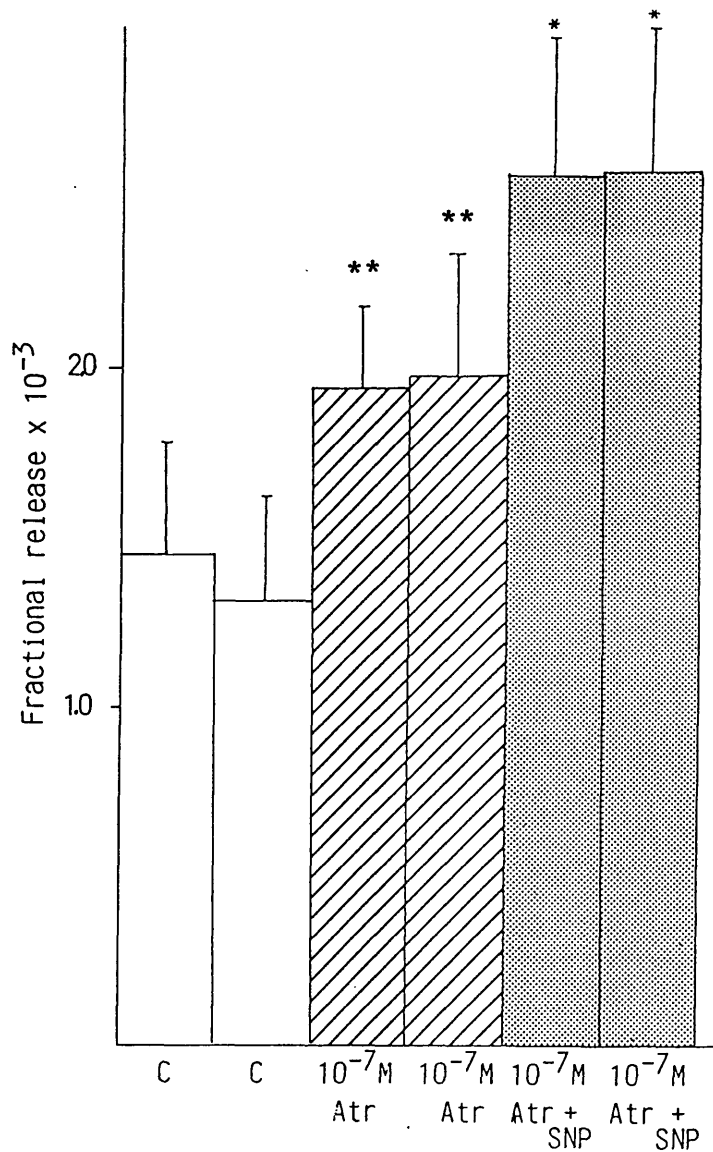
FIGURE 52. Effect of SNP in the presence of atropine on
the field stimulation-evoked release of ^{3}H in atria.
The open histograms show successive control responses
prior to drug addition. The succeeding 2 diagonal
striped histograms show the potentiating effect of
atropine (10^{-7} M) on this response. The final 2 shaded
histograms show the significant additional potentiating
effect of SNP (10^{-7} M) on this response. Each column
represents the mean ($\pm$ S.E. mean) of 4 observations.
* $0.05 > P > 0.01$ for comparison with atropine and
atropine/SNP response. ** $0.01 > P > 0.001$ for comparison
with control prior to atropine addition.
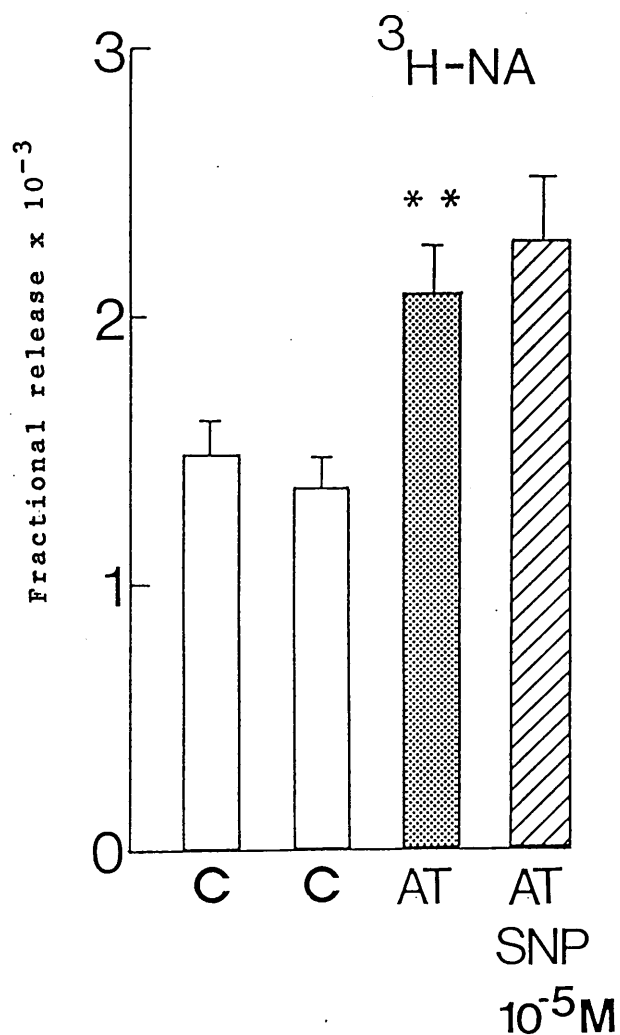
FIGURE 53. Effect of SNP in the presence of atropine on the field stimulation-evoked release of ^{3}H in atria. The open histograms show successive control responses prior to drug addition. The shaded histogram shows the potentiating effect of atropine (10^{-5} M) on this response. The diagonal striped histogram shows the inability of SNP (10^{-5} M) to further potentiate the response in the presence of atropine. Each column represents the mean ($\pm$ S.E. mean) of 4 observations. ** 0.01>P>0.001 for comparison with control prior to atropine addition.

PART VI

**FURTHER INVESTIGATION OF THE EFFECTS OF DRUGS ON THE STIMULATION-EVOKED RELEASE OF ^{14}C FROM ATRIA PREVIOUSLY INCUBATED IN [^{14}C]-CHOLINE**

Sodium nitroprusside (10^{-6} M, 10^{-5} M) inhibited the stimulation-evoked release of ^{14}C from atria previously incubated in [^{14}C]-choline (Fig. 54).

A combination of prazosin (10^{-5} M) and yohimbine (10^{-5} M) potentiated the stimulation-evoked overflow of ^{14}C. In the presence of these alpha-adrenoceptor antagonists sodium nitroprusside (10^{-6} M) retained its ability to inhibit the stimulation-evoked overflow of ^{14}C (Fig. 55).
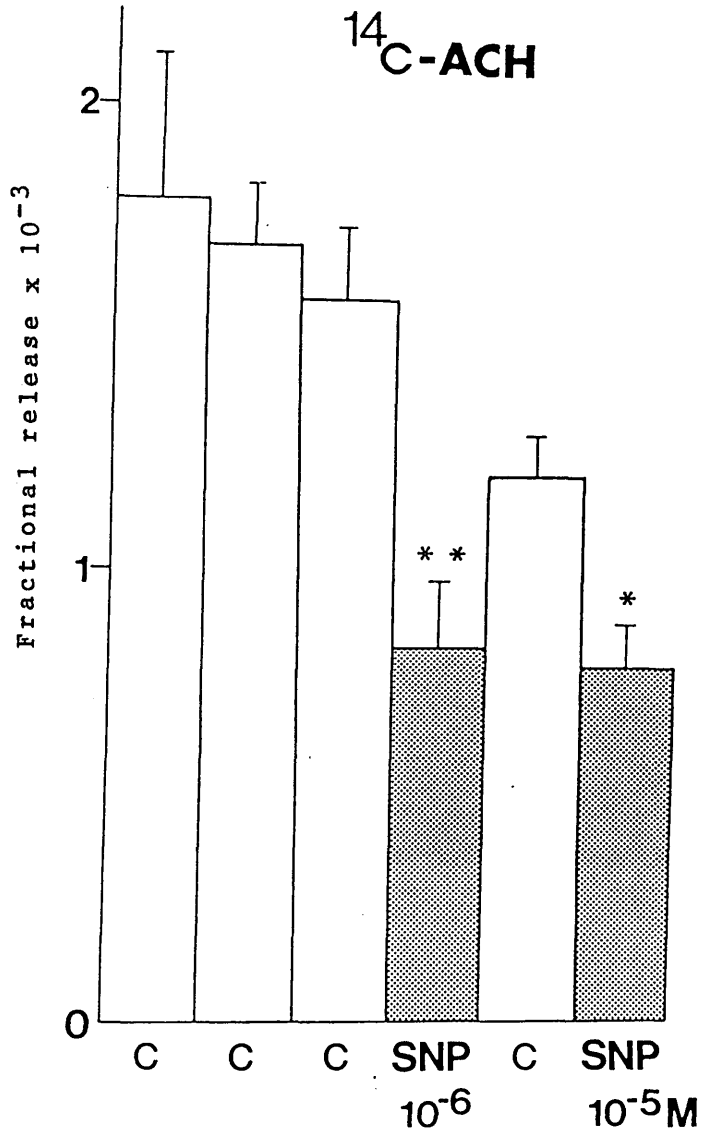
FIGURE 54. Effect of SNP on the field stimulation-
evoked release of ^{14}C in atria. The 3 open histograms
show successive control responses prior to drug
addition. The first shaded histogram shows the
inhibitory effect of SNP (10^{-6} M) on this response. The
following open histogram is a control response and the
inhibitory effect of SNP is abolished. The final shaded
histogram again shows the inhibitory effect of SNP
(10^{-5} M) on this response. Each column represents the
mean ($\pm$ S.E. mean) of 6 observations.
* 0.05>P>0.01, ** 0.01>P>0.001 for comparison with
control prior to drug addition.
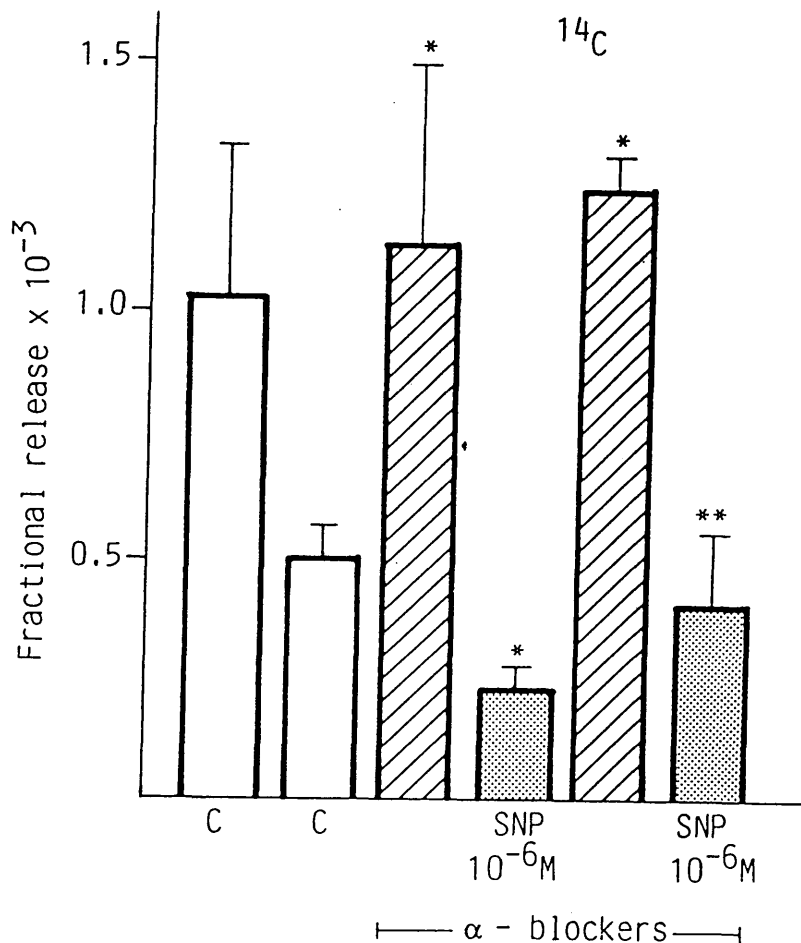
FIGURE 55. Effect of SNP in the presence of prazosin
(10^{-5} M) and yohimbine (10^{-5} M) on the field
stimulation-evoked release of ^{14}C in atria. The 2 open
histograms show successive control responses prior to
drug addition. The first diagonal striped histogram
shows the significant potentiating effect of prazosin
and yohimbine on this response. The first stippled
histogram shows the significant inhibitory effect of
SNP (10^{-6} M) in the presence of prazosin and
yohimbine.The second diagonal striped histogram again
shows the potentiating effect of prazosin and
yohimbine. The second stippled histogram again shows
the significant inhibitory effect of SNP (10^{-6} M) in
the presence of prazosin and yohimbine. Each column
represents the mean ($\pm$ S.E. mean) of 4 observations.
* $0.05 > P > 0.01$, **$0.01 > P > 0.001$.

PART VII

EFFECTS OF SODIUM NITROPRUSSIDE ON THE CHRONOTROPIC RESPONSES OF THE HEART TO NERVE STIMULATION IN ANAESTHETISED AND PITHED RATS AND ON THE POSITIVE INOTROPIC RESPONSE OF FIELD STIMULATED SPONTANEOUSLY BEATING PAIRED ATRIA

Stimulation (10Hz x 5s) of the vagus nerve in anaesthetised rats resulted in a decrease in the heart rate. Sodium nitroprusside (60 µg Kg^{-1}) did not affect this response (Fig. 56). The response to vagal stimulation, with these parameters, was potentiated by administration of prazosin (100 µg Kg^{-1}) and yohimbine (100 µg Kg^{-1}). Under these circumstances sodium nitroprussude inhibited the vagally-induced bradycardia (Figs. 57, 58).

In the anaesthetised rat intravenous administration of acetylcholine (0.6 µg Kg^{-1}) produced a negative chronotropic effect on heart rate. Sodium nitroprusside (60 µg Kg^{-1}) did not affect this response (Fig. 59). Prazosin (100 µg Kg^{-1}) and yohimbine (100 µg Kg^{-1}) did not affect the response to exogenous acetylcholine, nor did a combination of prazosin, yohimbine and sodium nitroprusside affect the response to exogenous acetylcholine (Fig. 60).

Stimulation (5Hz X 10s) of the vagus nerve in pithed rats resulted in a decrease in heart rate. Sodium nitroprusside (60 µg Kg^{-1}) produced an attenuation of this vagally-induced bradycardia (Figs. 61, 62).

FIGURE 56. Effect of SNP on the response of the heart rate to vagus nerve stimulation (10Hz x 5 sec) in the anaesthetised rat. The open histogram shows that vagal stimulation results in a decrease in heart rate. The diagonal striped histogram shows that SNP does not affect this response. Each column represents the mean ($\pm$ S.E. mean) of 7 observations.

100µg
Praz. + Yoh.

60µg
SNP

100
B/min

10s

10Hz   10Hz   10Hz
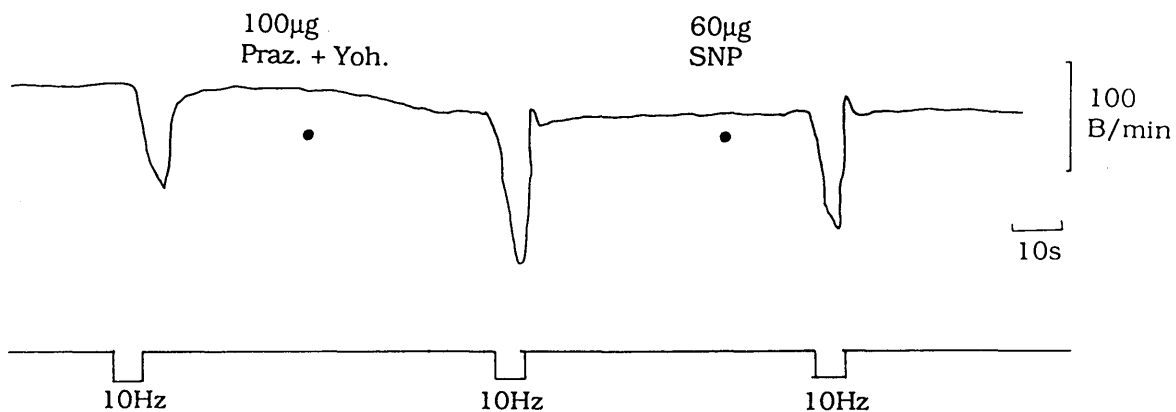
FIGURE 57. Recording of the effects of vagal
stimulation (10Hz x 5 sec) on heart rate in the
anaesthetised rat and the effects of prazosin and
yohimbine and SNP on this response. Stimulation of the
vagus resulted in a decrease in heart rate.
Administration of prazosin and yohimbine potentiated
this response. SNP inhibited the response in the
presence of prazosin and yohimbine.
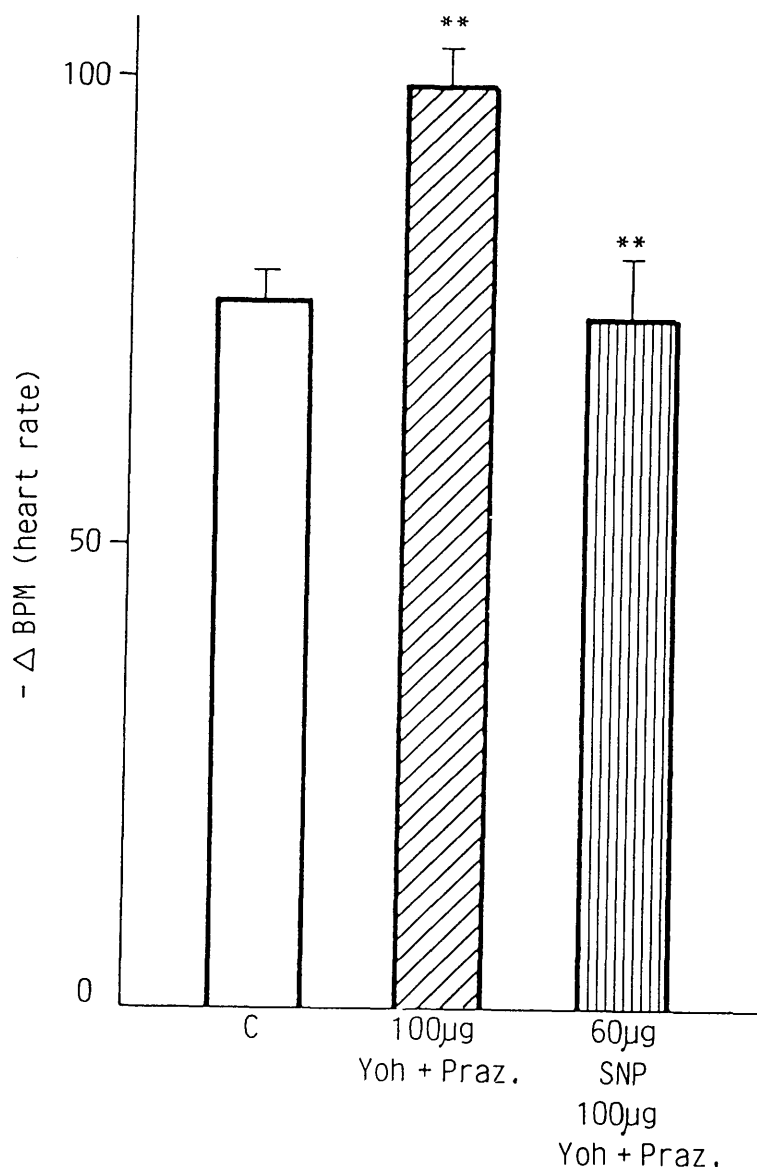
FIGURE 58. Potentiating effect of prazosin and
yohimbine and reversal by SNP on the response of the
heart rate to vagal stimulation (10Hz x 5s) in the
anaesthetised rat. The open histogram shows that
stimulation of the vagus nerve results in a decrease in
heart rate. The diagonal striped histogram shows the
significant potentiating effect of prazosin and
yohimbine on this response. The vertical striped
histogram demonstrates the ability of SNP to
significantly inhibit the response to vagal stimulation
in the presence of prazosin and yohimbine. Each column
represents the mean ($\pm$ S.E. mean) of 6 observations.
** 0.01>P>0.001.
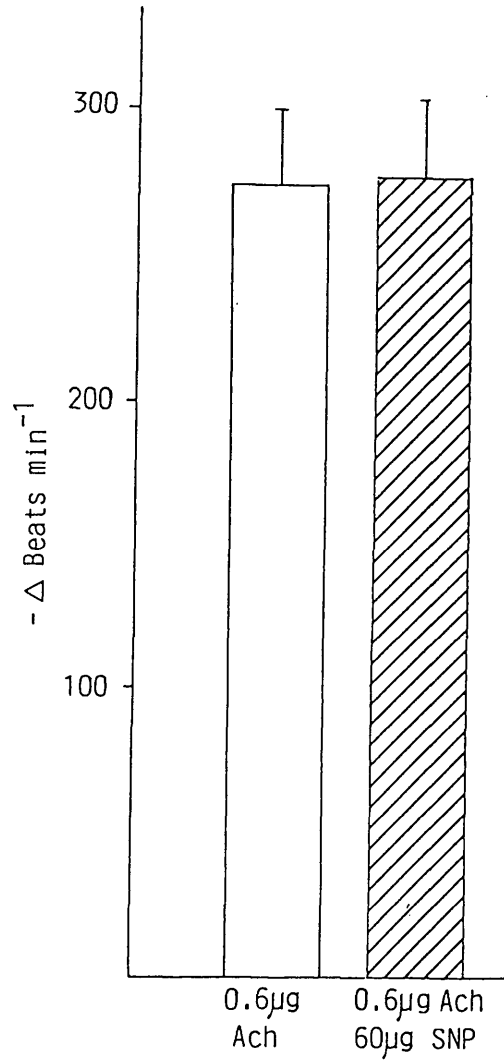
FIGURE 59. Effect of SNP on the response of the heart
rate to exogenous ACh administration in the
anaesthetised rat. The open histogram shows the
inhibitory effect of ACh on the heart rate. The
diagonal striped histogram shows that SNP does not
affect the response to ACh administration. Each column
represents the mean ($\pm$ S.E. mean) of 6 observations.
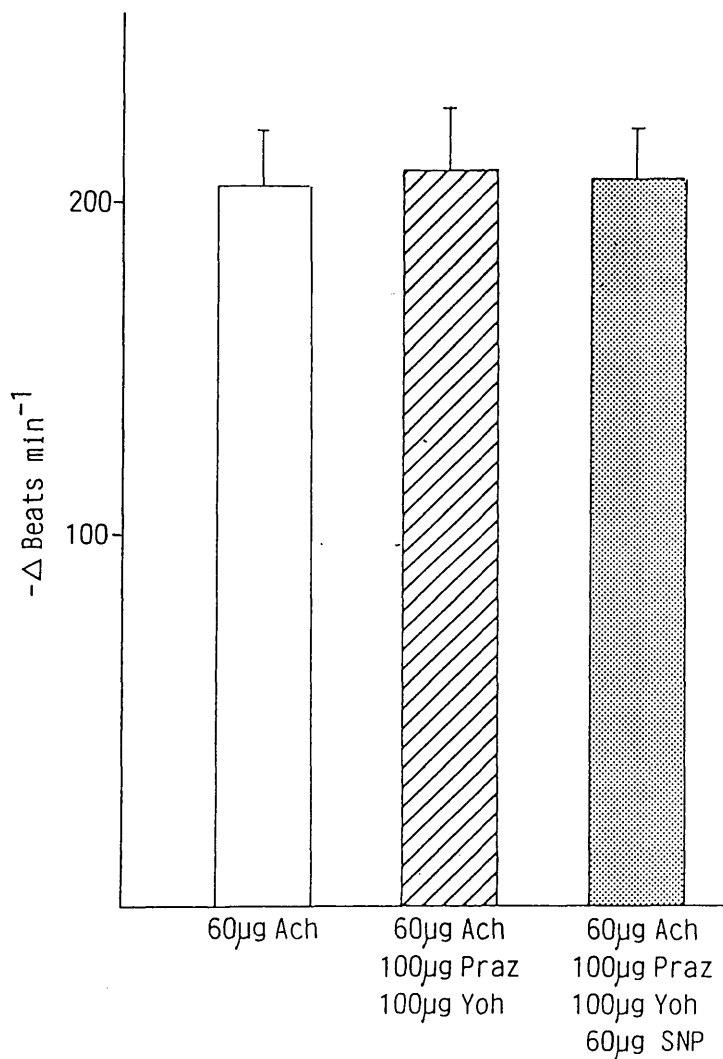
FIGURE 60. Effect of prazosin and yohimbine, and
prazosin, yohimbine and SNP on the response of the
heart rate to exogenous ACh administration in the
anaesthetised rat. The open histogram shows that ACh
administration results in a decrease in heart rate. The
diagonal striped histogram shows that prazosin and
yohimbine do not affect this response. The stippled
histogram shows that prazosin, yohimbine and SNP do not
affect the response to exogenous ACh administration.
Each column represents the mean ($\pm$ S.E. mean) of 6
observations.

FIGURE 61. Recording of the effects of vagal stimulation (5Hz x 10s) on heart rate in the pithed rat and the effect of SNP on this response. Two control stimulations result in a decrease in heart rate. Administration of SNP results in an attenuation of the response of the heart rate to vagal stimulation.
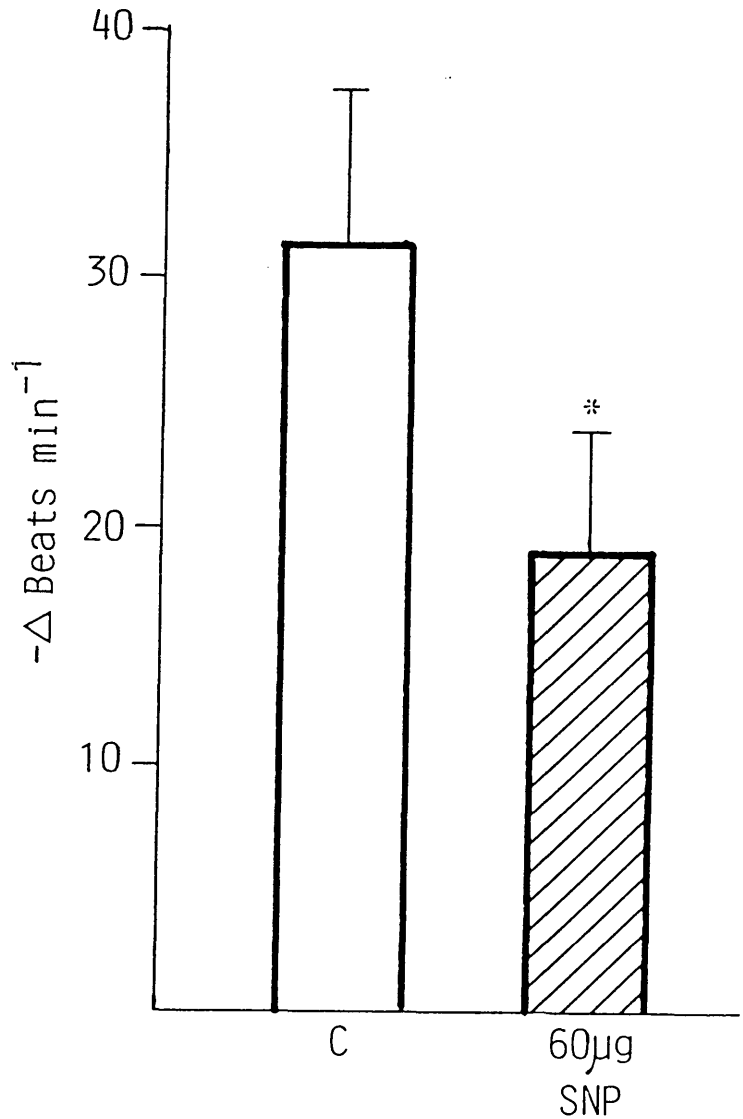
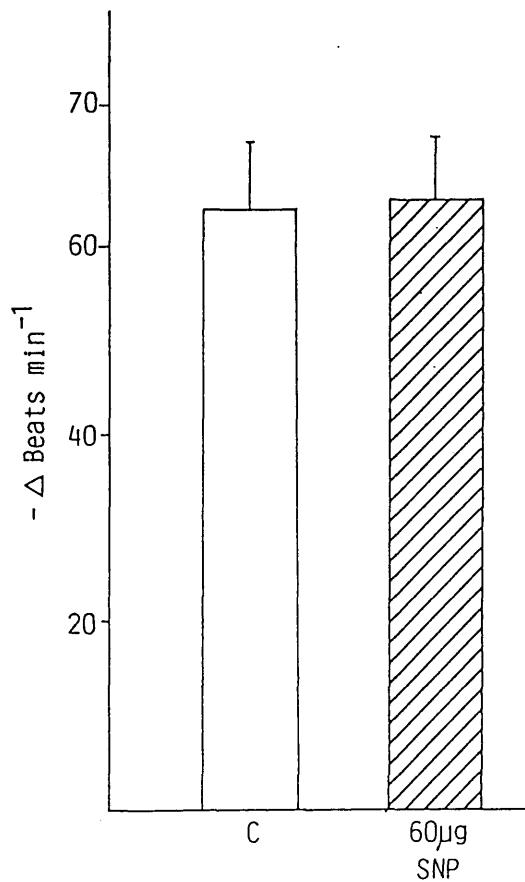FIGURE 62. Effect of SNP on the response of the heart
rate to vagal stimulation (5Hz x 10s) in the pithed
rat. The open histogram shows that vagal stimulation
results in a decrease in heart rate. The diagonal
striped histogram shows that SNP inhibited this
response. Each column represents the mean ($\pm$ S.E. mean)
of 4 observations.
* 0.05>P>0.01 for comparison with control prior to drug
addition.

Stimulation (10Hz x 10s) of the vagus nerve in pithed rats frequently resulted in a biphasic chronotropic response (Fig. 63). This response consisted of a transient negative chronotropic component and a positive chronotropic component. The former, negative component could be abolished by atropine (50 μg Kg^{-1}) administration (Table 5a), while the latter, positive component could be abolished by propranolol (50 μg Kg^{-1}) administration (Table 5b).

Sodium nitroprusside (60 μg Kg^{-1}) administration resulted in a small attenuation of the negative component of the biphasic response (Fig. 63). Sodium nitroprusside potentiated and prolonged the positive chronotropic component of the response (Figs. 63, 64, 65).

Intravenous administration of NA (0.1 μg Kg^{-1}) in pithed rats resulted in an increase in the rate of beating of the heart. Sodium nitroprusside (60 μg Kg^{-1}) did not affect this response (Fig. 66).

Stimulation (2Hz x 30s) of the cardio-accelerator nerves in the spinal column resulted in an increase in the rate of beating of the heart. This response was not affected by administration of sodium nitroprusside (60 μg Kg^{-1}) (Fig 67).

Field stimulation of isolated spontaneously beating paired atria resulted in an increase in the force of contraction of the beating atria. This response was potentiated by sodium nitroprusside (10^{-6} M) and by atropine (10^{-5} M). A combination of sodium nitroprusside and atropine did not result in a further enhancement in the response of the atria above that produced by either drug alone(Fig 68).
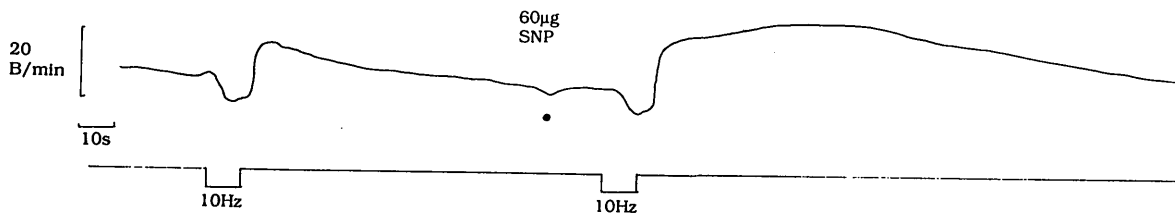
FIGURE 63. Recording of the effects of vagal

stimulation (10Hz x 10s) on heart rate in the pithed

rat and the effects of SNP on this response.

Stimulation of the vagus results in a biphasic effect

on the heart rate. This consists of a decrease in heart

rate which is transient as the response rapidly

develops into an increase in heart rate. Administration

of SNP results in a small attenuation of the negative

chronotropic response and a marked potentiation and

prolonging of the positive chronotropic response.

Table 5a. Effect of atropine on the negative component of the
           chronotropic response following vagal stimulation
           in the pithed rat.

Treatment	Mean Decrease ($\pm$ S.E. Mean) In Heart Rate (BPM).
Control. Stimulation (10Hz x 10s)	15.4 $\pm$ 2.1 (n=8)
Atropine (50μg Kg^{-1})	0.0 $\pm$ 0.0 (n=8) ***

*** P<0.001 for comparison between response in the absence and
presence of atropine.

Table 5b. Effect of propranolol on the positive component of the
           chronotropic response following vagal stimulation
           in the pithed rat.

Treatment	Mean Increase ($\pm$ S.E. Mean) In Heart Rate (BPM).
Control. Stimulation (10Hz x 10s)	40.3 $\pm$ 7.4 (n=8)
Propranolol (50μg Kg^{-1})	3.1 $\pm$ 1.5 (n=8) ***

*** P<0.001 for comparison between response in the absence and
presence of propranolol.
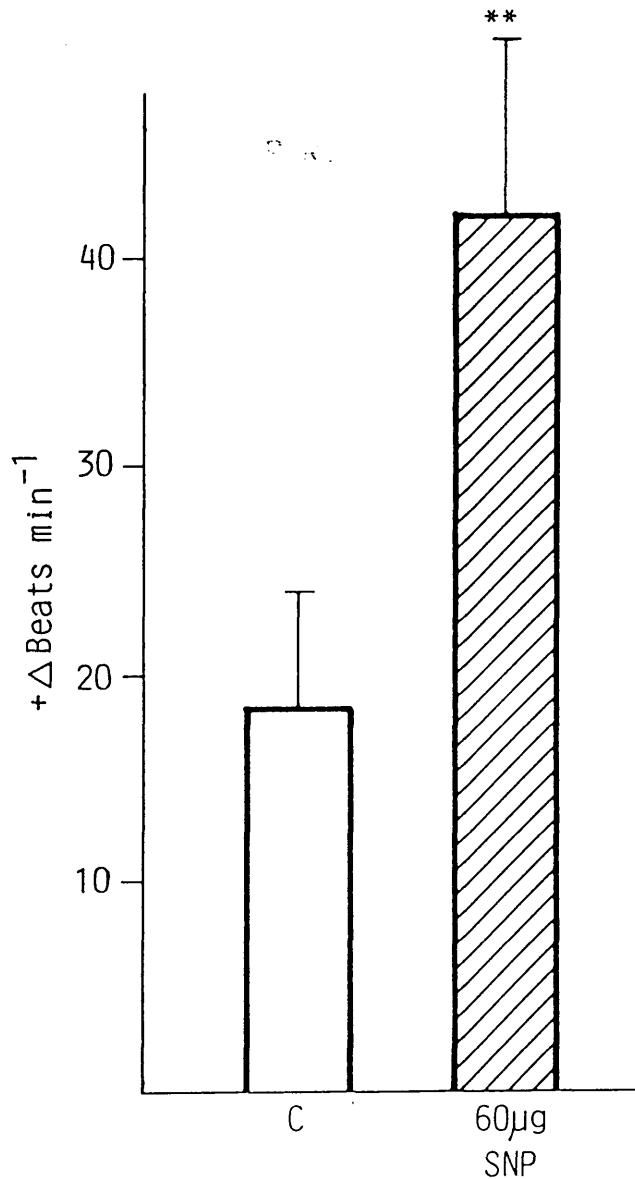
FIGURE 64. Effect of SNP administration on the positive
chronotropic response of the heart rate produced by
vagal stimulation (10Hz x 10s) in the pithed rat. The
open histogram shows that stimulation of the vagus at
these parameters can result in an increase in heart
rate. The diagonal striped histogram shows that
administration of SNP can potentiate this response to
vagal stimulation. Each column represents the mean
($\pm$ S.E. mean) of 8 observations.
** $0.01>P>0.001$.
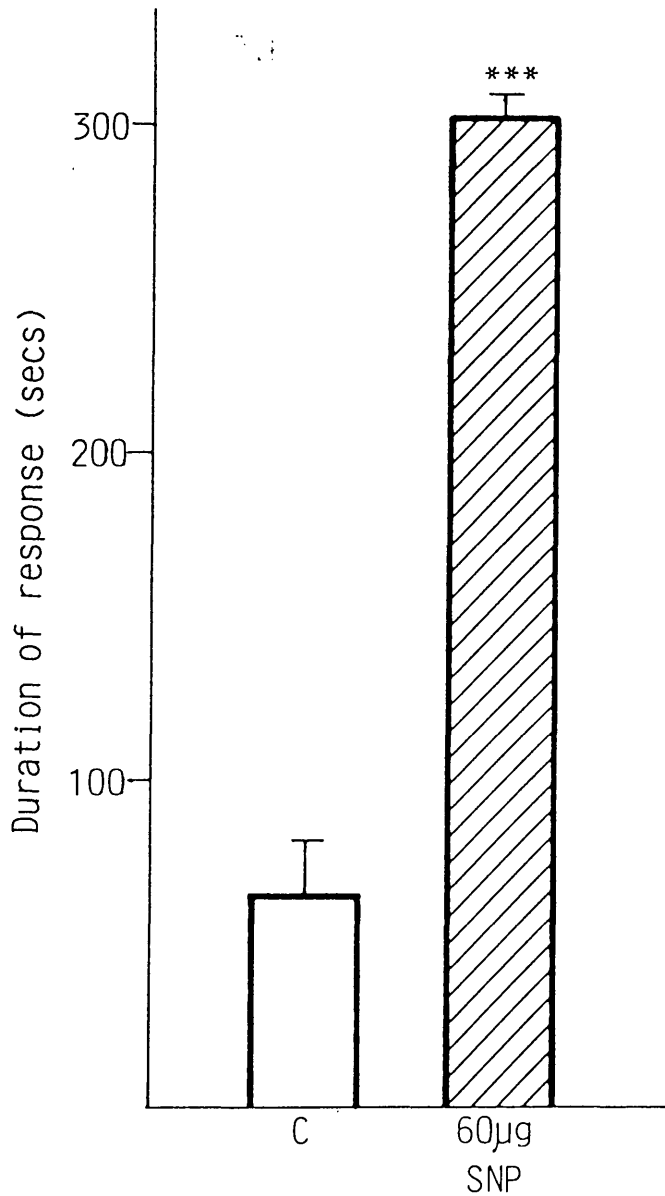
FIGURE 65. Effect of SNP administration on the duration
of the positive chronotropic response of heart rate
produced by vagal stimulation (10Hz x 10s) in the
pithed rat. The open histogram shows the duration of
the positive chronotropic component of the response.
The diagonal striped histogram demonstrates the ability
of SNP to prolong this response. Each column represents
the mean ($\pm$ S.E. mean) of 8 observations.
*** P<0.001.
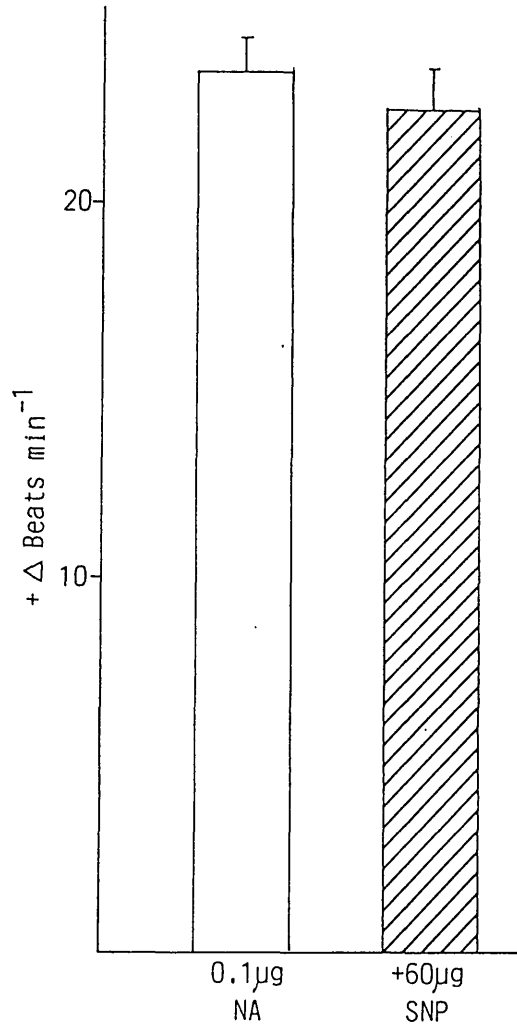
FIGURE 66. Effect of SNP on the response of the heart
rate to exogenous NA administration in the pithed rat.
The open histogram shows the potentiating effect of NA
on the heart rate. The diagonal striped histogram shows
that SNP does not affect the response to exogenous NA
administration. Each column represents the mean
($\pm$ S.E. mean) of 4 observations.

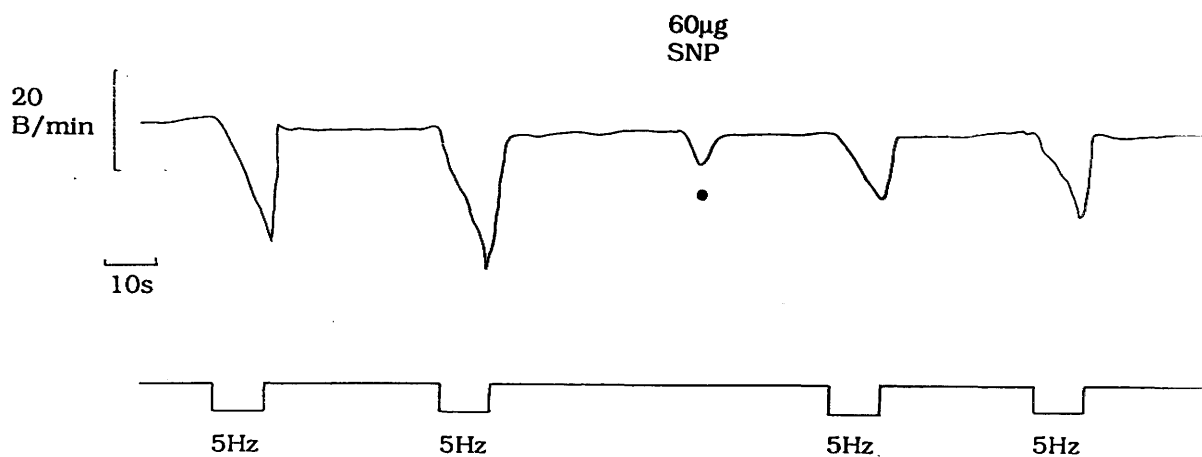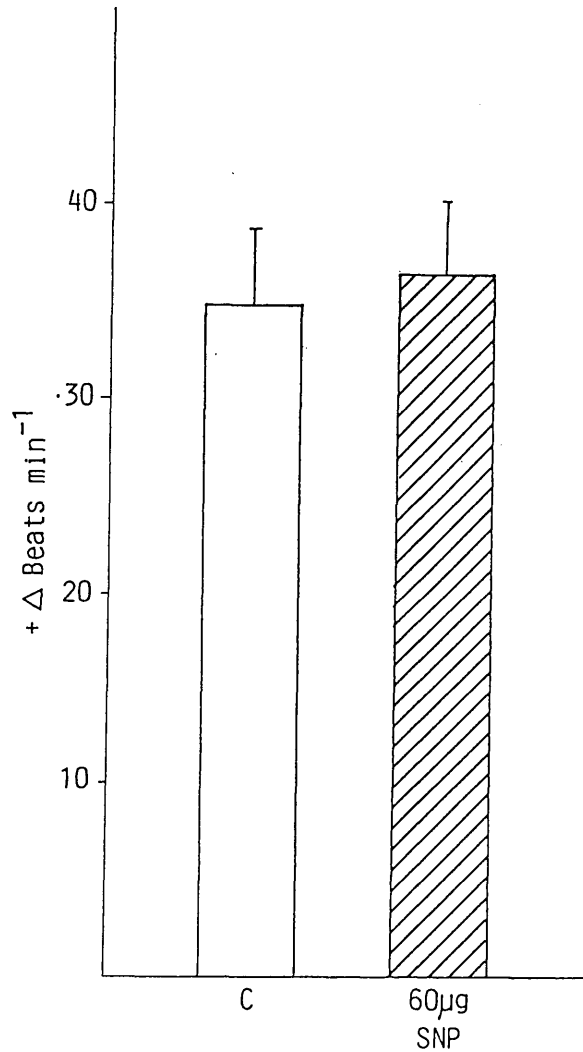FIGURE 67. Effect of SNP on the response of the heart rate to cardio-accelarator nerve stimulation (2Hz x 30s) in the pithed rat. The open histogram shows that cardio-accelarator nerve stimulation results in an increase in heart rate. The diagonal striped histogram shows that SNP did not affect this response. Each column represents the mean ($\pm$ S.E. mean) of 4 observations.
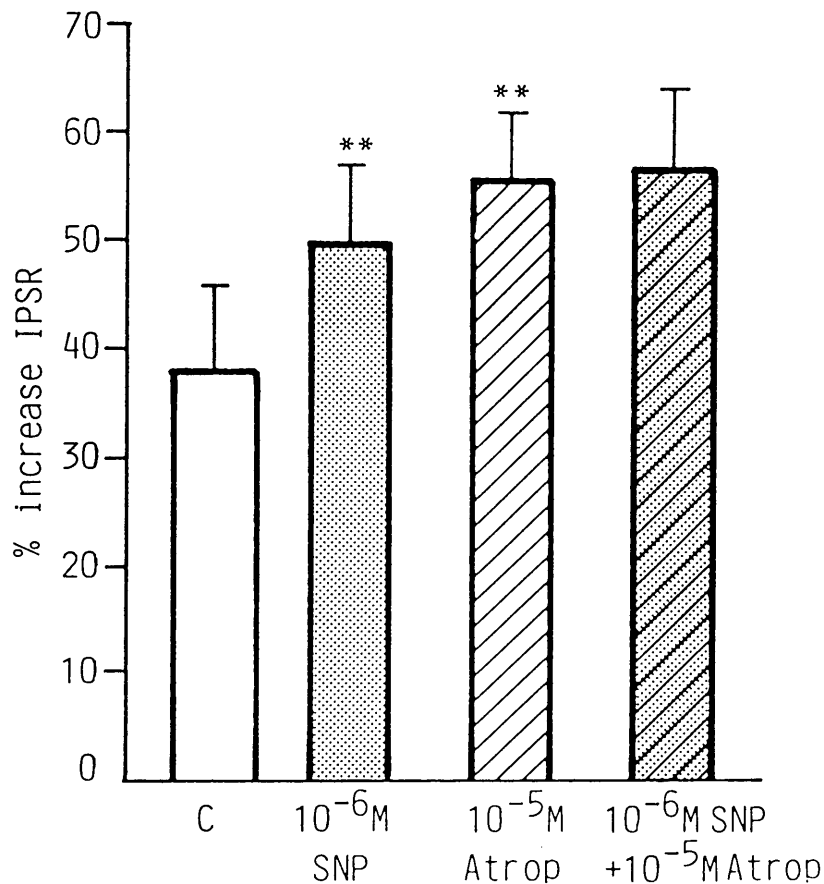
FIGURE 68. Effect of SNP in the absence and presence of atropine on the positive inotropic component of the post-stimulus response of the spontaneously beating atria of the rat. The open histogram shows that field stimulation (2Hz x 30s) results in an increase in the force of contraction of the beating atria. The stippled histogram shows that SNP (10^{-6} M) can potentiate this response. The diagonal striped histogram shows that atropine (10^{-5} M) can also potentiate this response. The stippled diagonal striped histogram shows that a combination of SNP and atropine does not produce a further enhancement of the response. Each column represents the mean ($\pm$ S.E. mean) of 6 observations. ** 0.01>P>0.001 for comparison with control prior to drug addition.

PART VIII

EFFECTS OF THYROXINE PRETREATMENT ON PRE- AND POST-SYNAPTIC RECEPTOR

SENSITIVITY IN ISOLATED ATRIA

Thyroxine (T_4) pretreatment increased serum free T_4 levels (Fig. 69). This change was accompanied by alterations in the sensitivity of the atria to agonists acting at pre- and post-synaptic sites.

T_4 pretreatment potentiated the inherent rate of beating of the isolated atria. The pretreatment enhanced the ability of isoprenaline (10^{-10} M - 10^{-5} M) to increase the rate of beating of isolated spontaneously beating paired atria but had little effect on the maximum percentage increase in rate produced by the drug (Figs. 70, 71, 72). Control atrial rate: 251 beats min^{-1}. T_4 pretreated atrial rate: 375 beats min^{-1}.

T_4 pretreatment did not affect the sensitivity of the atria to carbachol. Carbachol (10^{-9} M - 10^{-5} M) inhibited the rate of beating of the atria in a dose-dependent manner and the pretreatment had little effect on the maximum percentage inhibition produced by this drug (Figs. 73, 74, 75)

In paced left atria, phenylephrine (10^{-9} M - 10^{-4} M) produced a dose-dependent increase in the force of contraction of the atria. This response was blocked by prazosin (10^{-6} M) (Fig. 76). T_4 pretreatment reduced the percentage increase inotropic response produced by phenylephrine (Fig. 77).
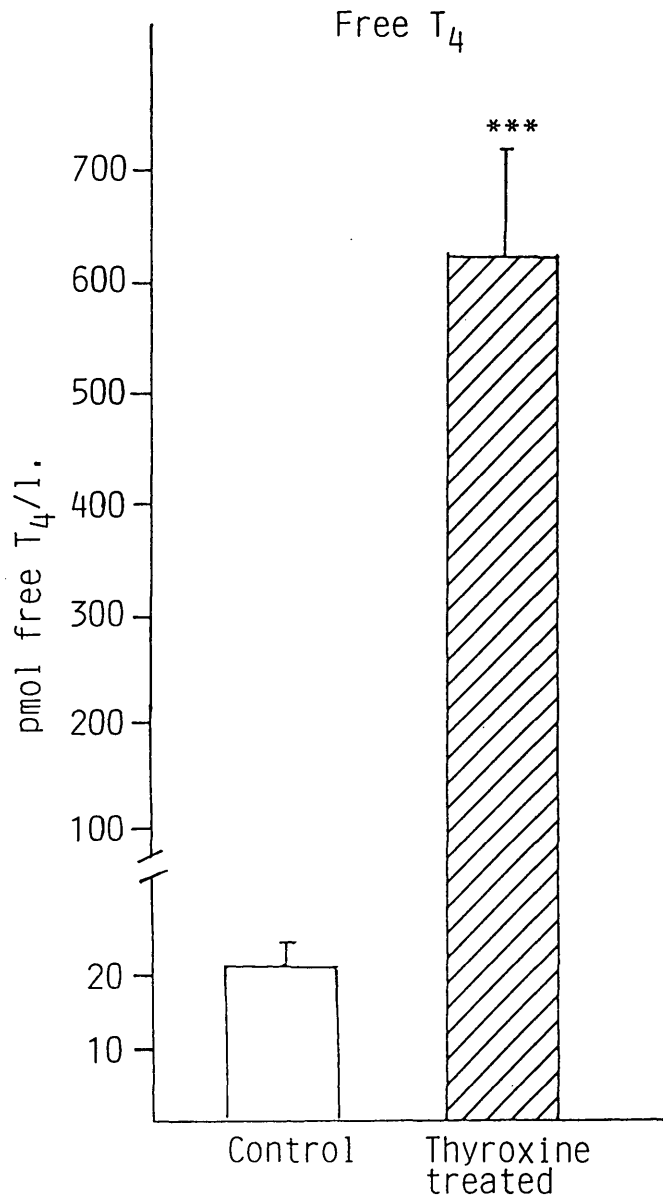
FIGURE 69. Effect of T_4 pretreatment in rats on the unbound T_4 in plasma, measured in pmol free T_4/l. Each column represents the mean ($\pm$ S.E. mean) of 9 observations. *** P<0.001.
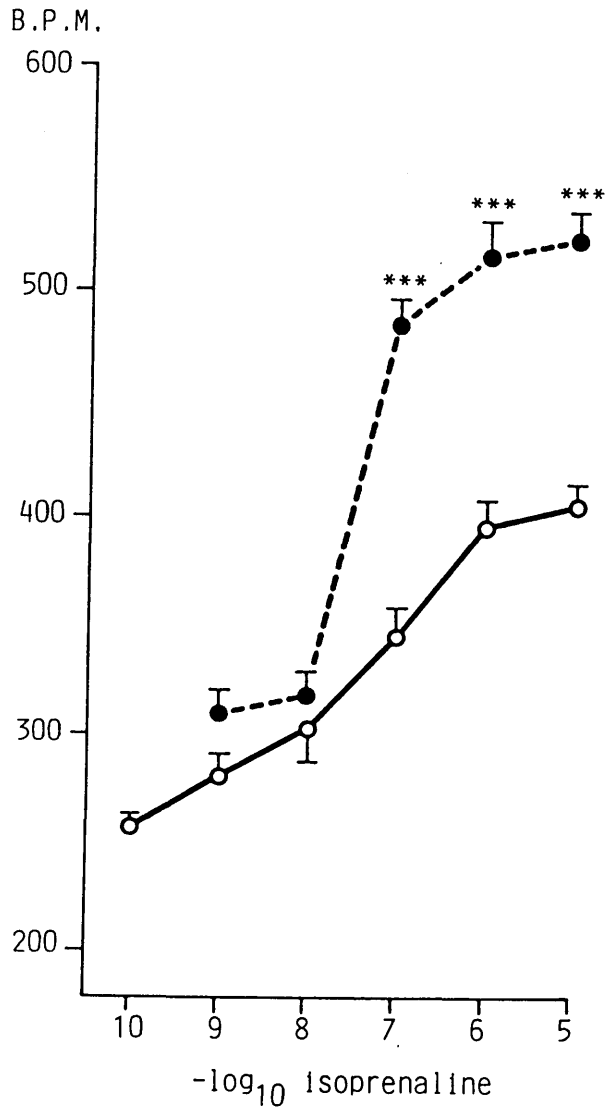
FIGURE 70. Dose-response curves showing the positive
chronotropic effect of isoprenaline in spontaneously
beating paired atria from control (0————0) and T_4 pre-
treated (•———•) rats. The vertical axis expresses the
effects of isoprenaline in absolute B.P.M. Each point is
the mean ($\pm$ S.E. mean) of ≮ 8 observations.
*** P<0.001.
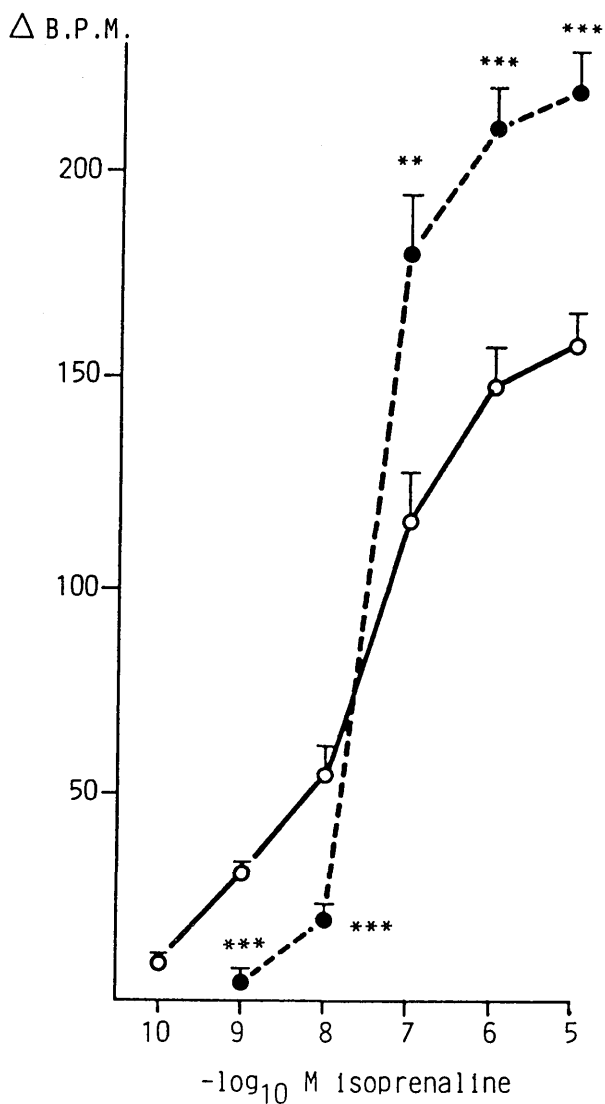
FIGURE 71. Dose-response curves showing the positive
chronotropic effect of isoprenaline in spontaneously
beating paired atria from control (O———O) and T_4 pre-
treated (●----●) rats. The vertical axis expresses the
effects of isoprenaline as the change in rate ($\triangle$ B.P.M)
above the resting level of beating. Each point is the
mean ($\pm$ S.E. mean) of k 8 observations.
** 0.01>P>0.001, *** P<0.001.

% maximum increase
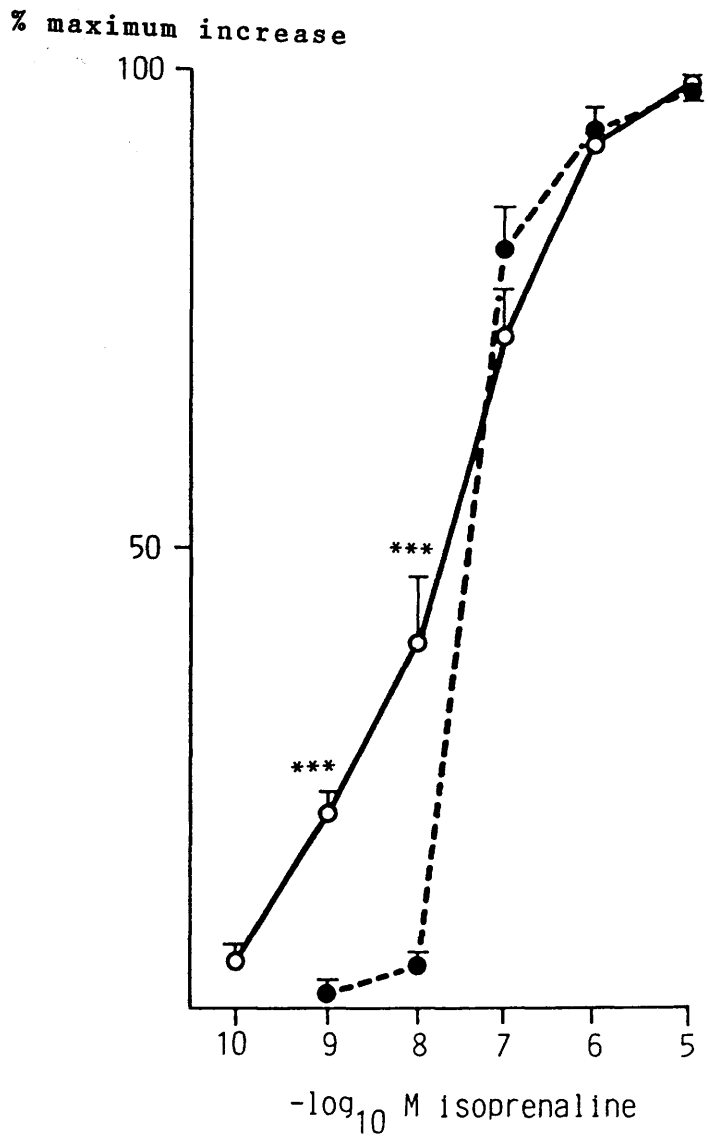
FIGURE 72. Dose %-response curves showing the positive
chronotropic effect of isoprenaline in spontaneously
beating paired atria from control (O———O) and T_4 pre-
treated (●— — —●) rats. Each point is the mean ($\pm$ S.E.
mean) of ⟨8 observations.

*** P<0.001.
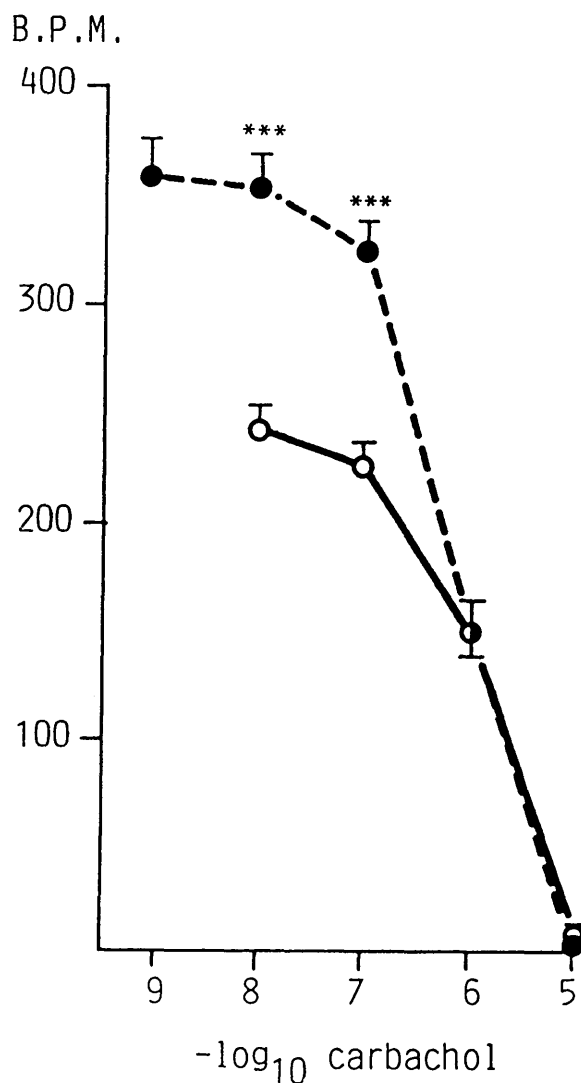
FIGURE 73. Dose-response curves showing the negative
chronotropic effect of carbachol in spontaneously
beating atria from control (O——O) and T_4 pre-treated
(●- - -●) rats. The vertical axis expresses the effects
of carbachol in absolute B.P.M. Each point is the mean
($\pm$ S.E. mean) of 10 observations.
*** P<0.001.
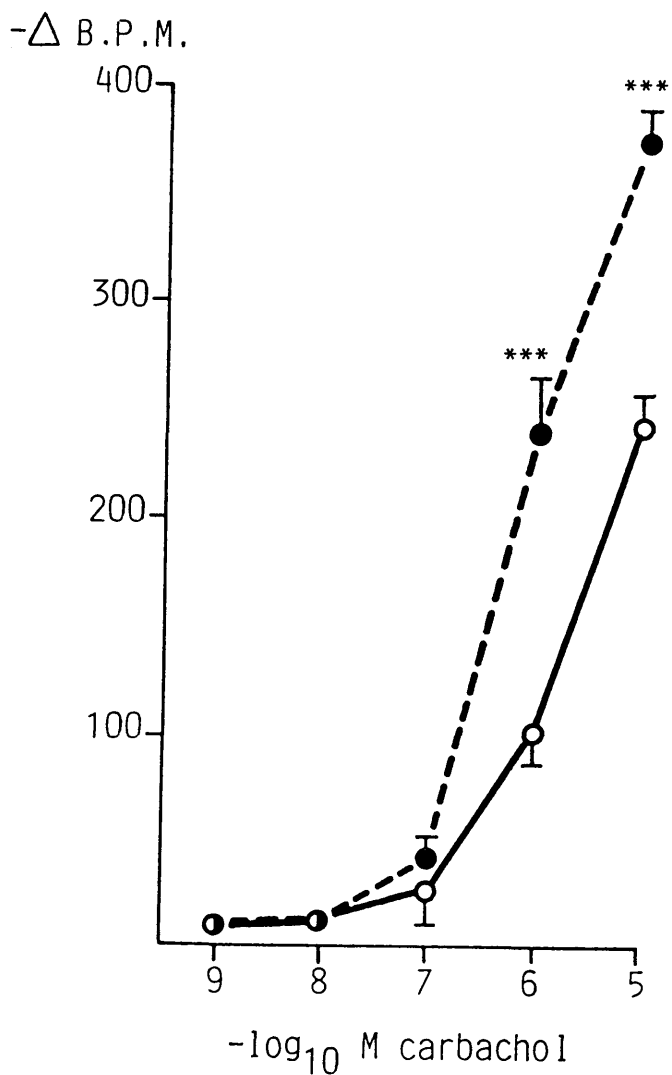
FIGURE 74. Dose-response curves showing the negative chronotropic effect of carbachol in spontaneously beating paired atria from control (O———O) and T_4 pretreated (●———●) rats. The vertical axis expresses the effects of carbachol as the change in rate ($\triangle$B.P.M.) below the resting level of beating. Each point is the mean ($\pm$ S.E. mean) of 10 observations.
*** P<0.001.

% maximum inhibition
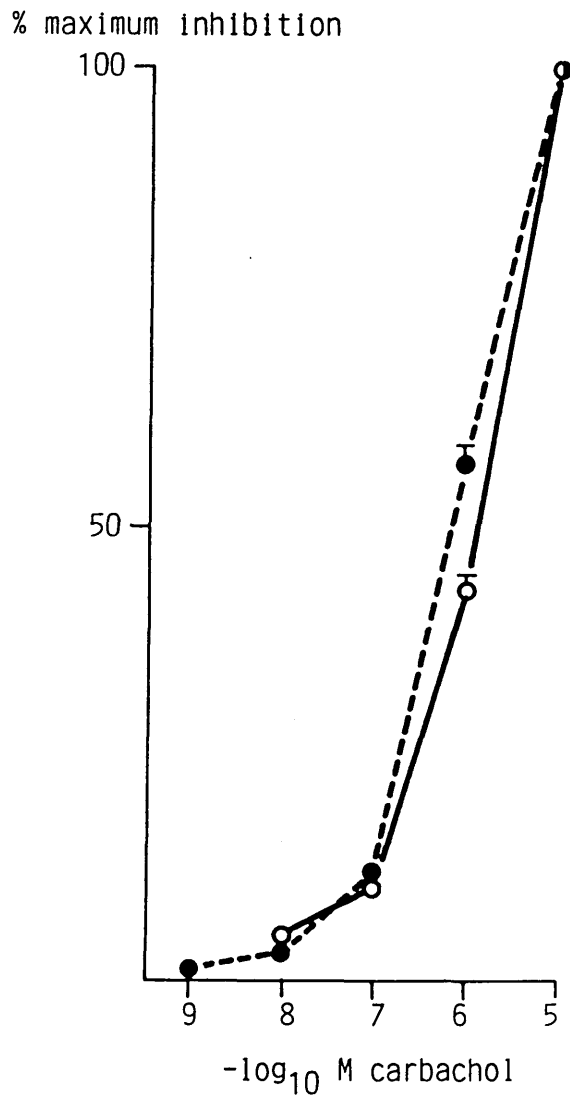
FIGURE 75. Dose %-response curves showing the negative
chronotropic effect of carbachol in spontaneously
beating paired atria from control (O———O) and T_4 pre-
treated (●---●) rats. Each point is the mean ($\pm$ S.E.
mean) of 10 observations.
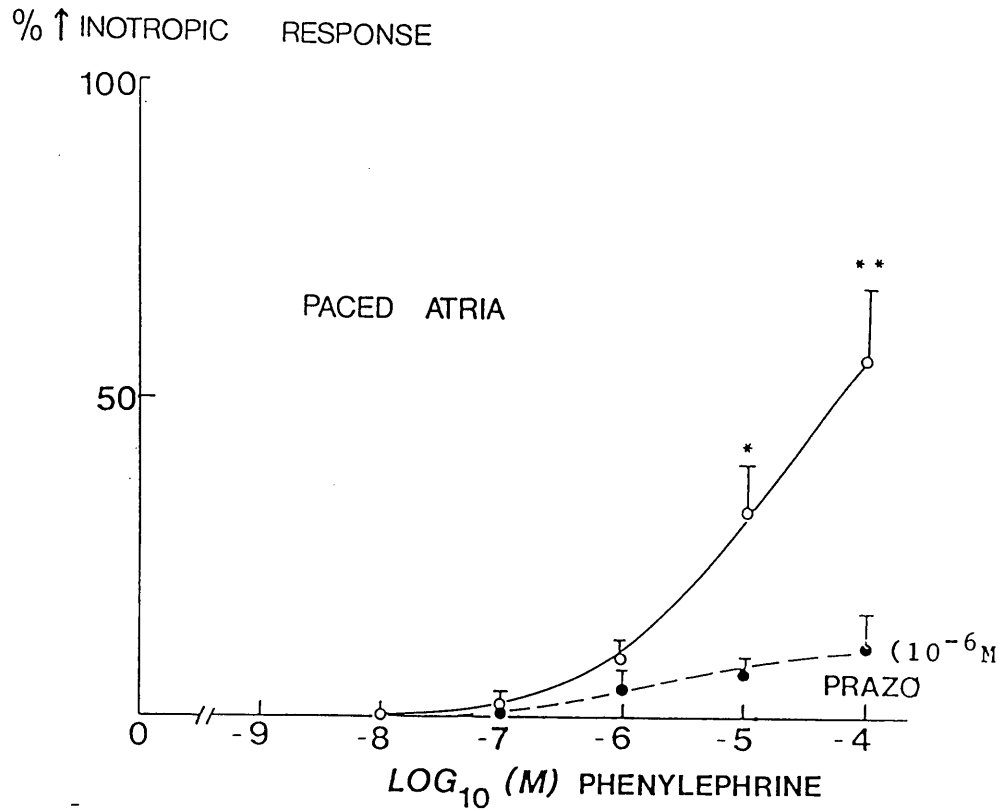
FIGURE 76. Dose %-response curve showing the inotropic
effect of phenylephrine in the absence (O———O) and in
the presence (●-- -●) of prazosin in the electrically
paced left atria of the rat. Each point is the mean
($\pm$ S.E. mean) of 6 observations.
* 0.05>P>0.01, ** 0.01>P>0.001.
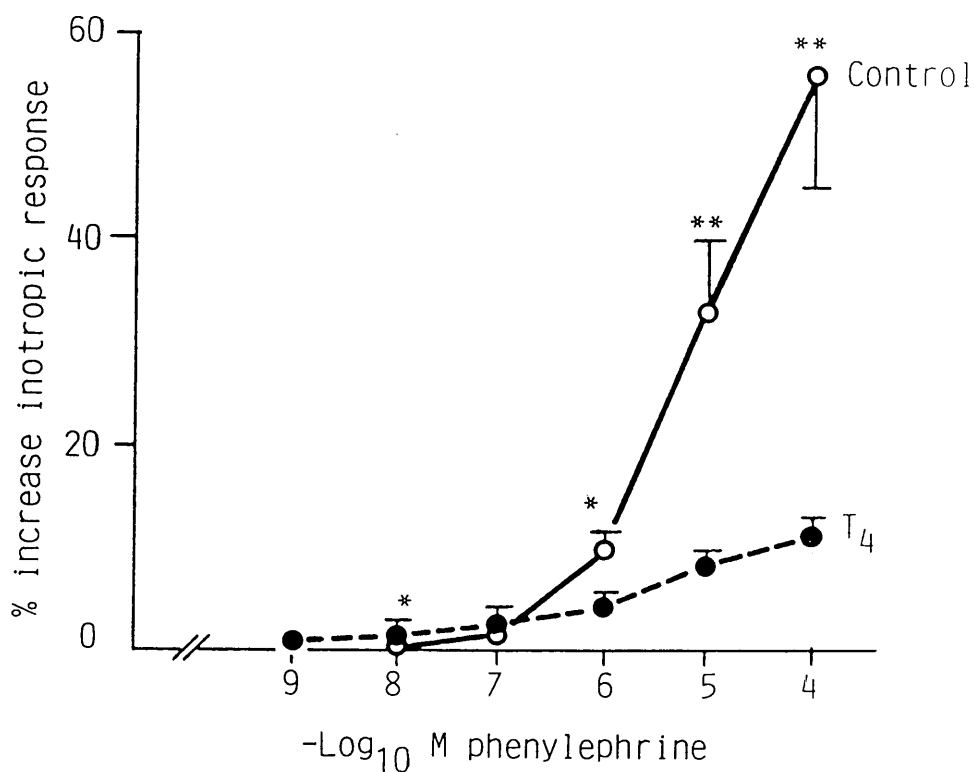
FIGURE 77. Dose %-response curves showing the inotropic
effects of phenylephrine on the electrically paced left
atria from control (O————O) and T_4 pre-treated (●–––●)
rats. Each point is the mean ($\pm$ S.E. mean) of 6
observations.

* 0.05>P>0.01, ** 0.01>P>0.001, *** P<0.001.

T_4 pretreatment increased the positive inotropic component of the post-stimulus response in isolated spontaneously beating paired atria (Fig. 78). In addition, T_4 pretreatment reduced the negative inotropic component of the post-stimulus response in isolated spontaneously beating paired atria (Fig. 79).

T_4 pretreatment reduced the ability of clonidine (10^{-9} M - 10^{-5} M) to inhibit the positive inotropic component of the post-stimulus response in the presence of atropine (10^{-6} M) (Fig. 80).

T_4 pretreatment reduced the ability of clonidine (10^{-8} M - 10^{-5} M) to inhibit the stimulation-evoked overflow of ^{3}H from atria previously incubated in [^{3}H]-NA (Fig. 81).

T_4 pretreatment did not affect the ability of acetylcholine (10^{-8} M - 10^{-5} M) to inhibit the stimulation-evoked overflow of ^{3}H from atria previously incubated in [^{3}H]-NA (Fig. 82).

In atria previously incubated in [^{14}C]-choline and [^{3}H]-NA, T_4 pretreatment reduced the ability of clonidine (10^{-5} M) to inhibit the stimulation-evoked overflow of ^{3}H. T_4 pretreatment did not affect the ability of acetylcholine (10^{-5} M) to inhibit ^{3}H overflow, or the ability of clonidine (10^{-5} M) or acetylcholine (10^{-5} M) to inhibit the simultaneous evoked overflow of ^{14}C (Fig. 83).
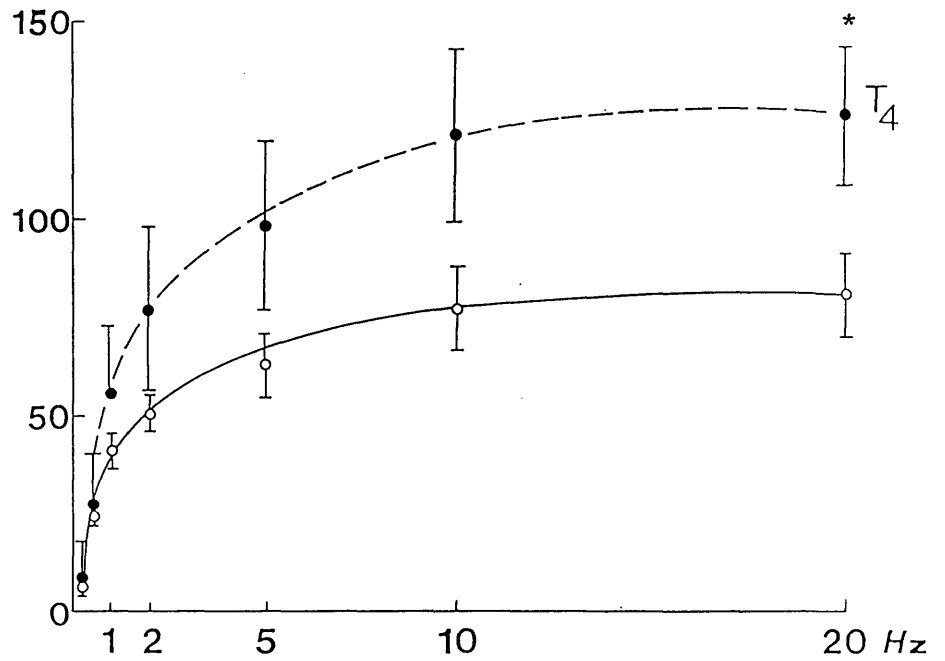
FIGURE 78. Effect of T_4 pre-treatment on the positive inotropic component of the post-stimulus response in spontaneously beating paired atria. Frequency response curves were constructed on atria from control (O————O) and from T_4 pre-treated (●— — —●) rats. Each point represents the mean (± S.E. mean) of ≮ 4 observations. * 0.05>P>0.001.
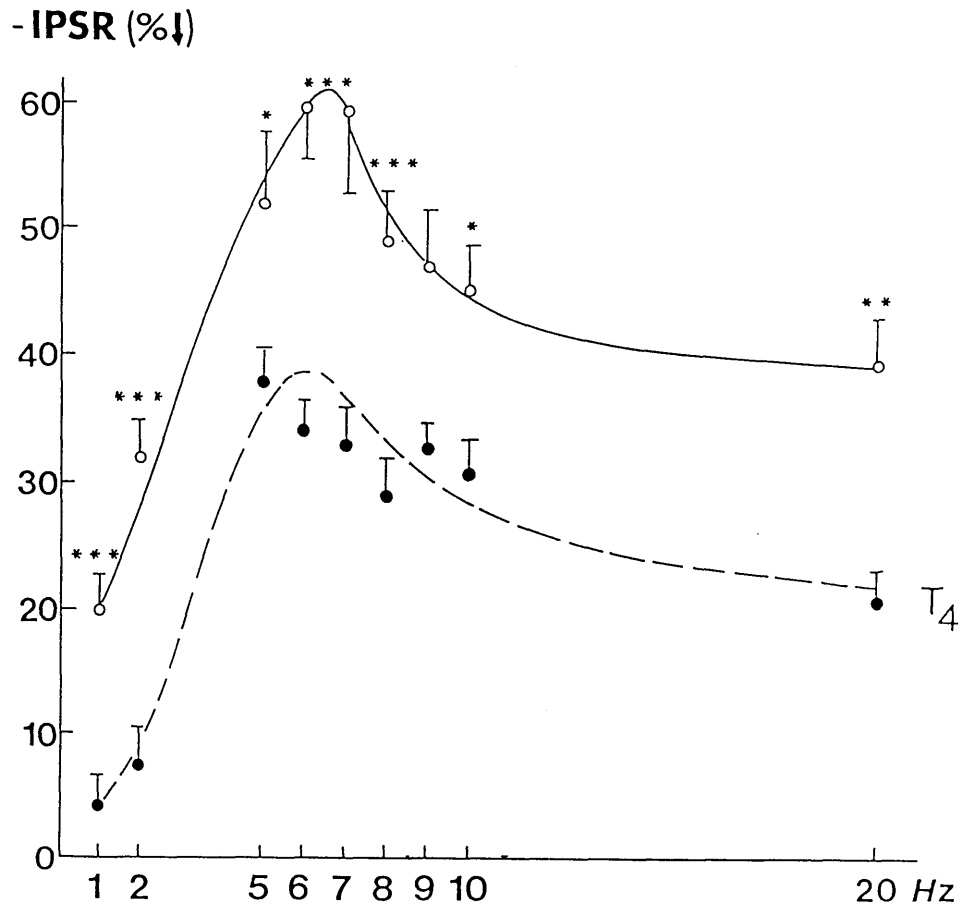
FIGURE 79. Effect of T_4 pre-treatment on the negative
inotropic component of the post-stimulus response in
spontaneously beating paired atria. Frequency response
curves were constructed in atria from control (O——O)
and from T_4 pre-treated (●———●) rats. Each column is
the mean (± S.E. mean) of ≮6 observations.

* 0.05>P>0.01, **0.01>P>0.001, *** P<0.001.

% INHIBITION OF IPSR

LOG_{10} *(M)* CLONIDINE
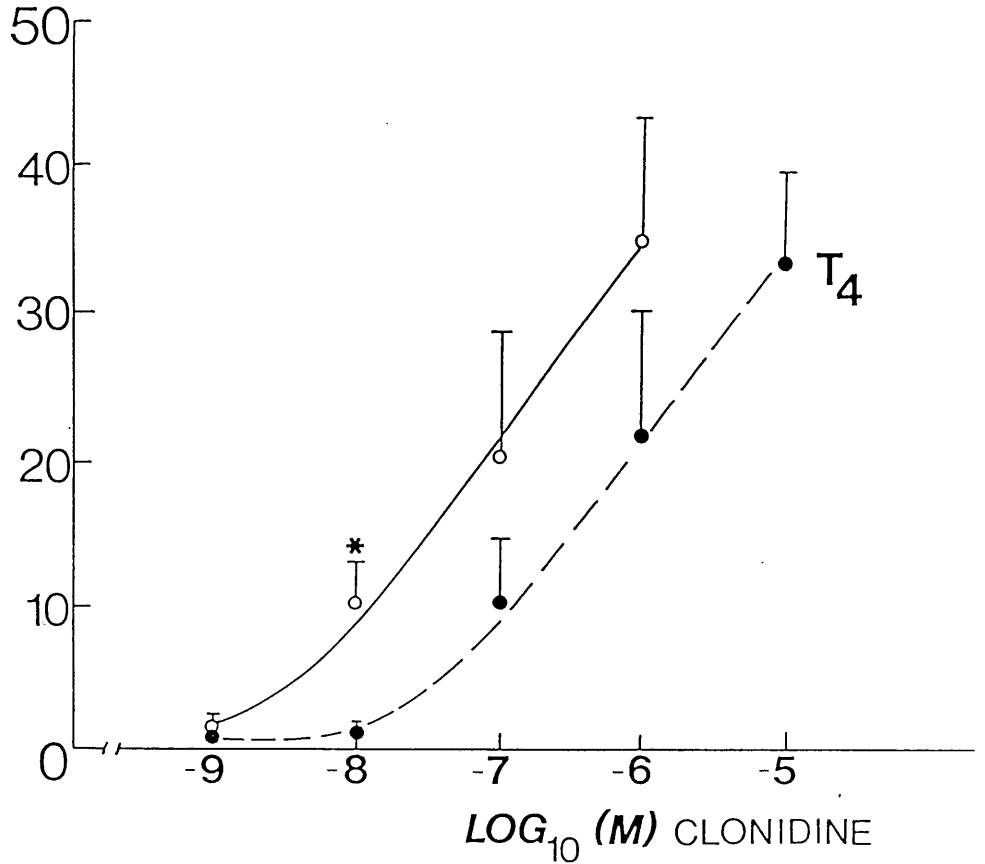
FIGURE 80. Dose %-response curves showing the inhibitory effects of clonidine (in the presence of atropine (10^{-6} M)) on the positive inotropic component of the post-stimulus response in atria from control (0———0) and from T_4 pretreated (●− − −●) rats. Atria were stimulated at 5Hz for a period of 4 seconds. Each point is the mean ($\pm$ S.E. mean) of ‹ 5 observations. * 0.05>P>0.01.
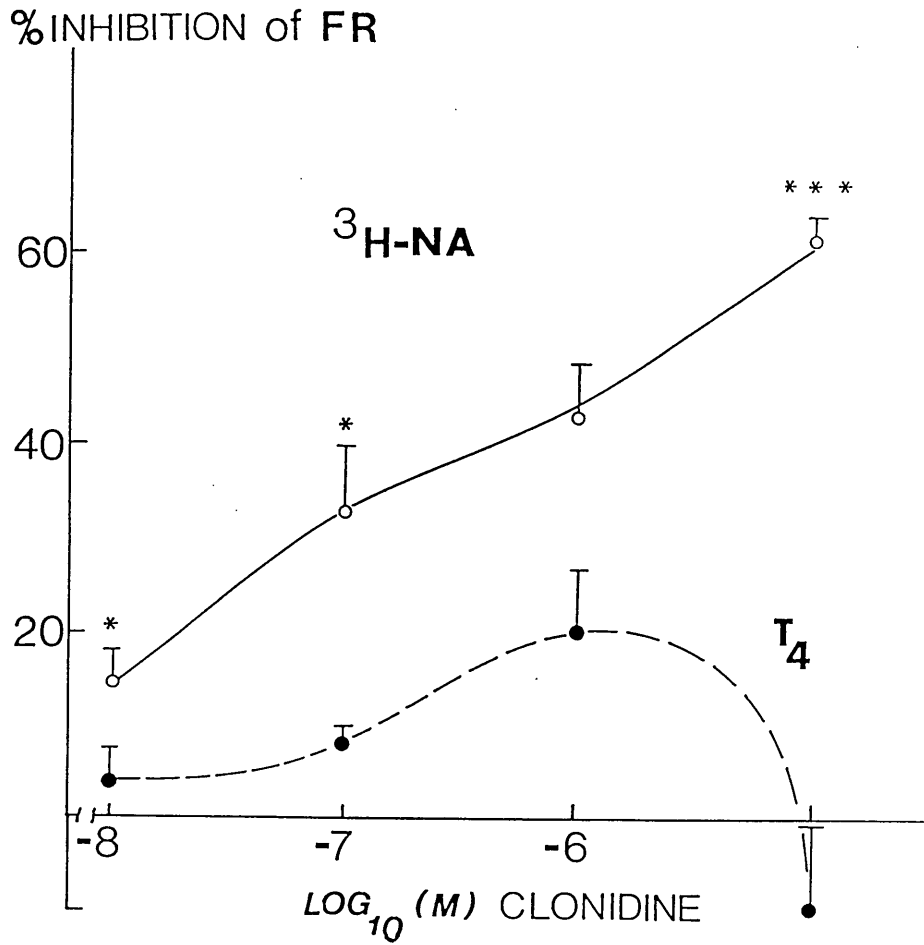
FIGURE 81. Dose %-response curves showing the effects of
clonidine on the stimulation-evoked overflow of ^{3}H in
atria from control (o———o) and from T_4 pre-treated
(•– – –•) rats. Each point is the mean ($\pm$ S.E. mean) of
≮ 6 observations.

* 0.05>P>0.01, ***P<0.001.
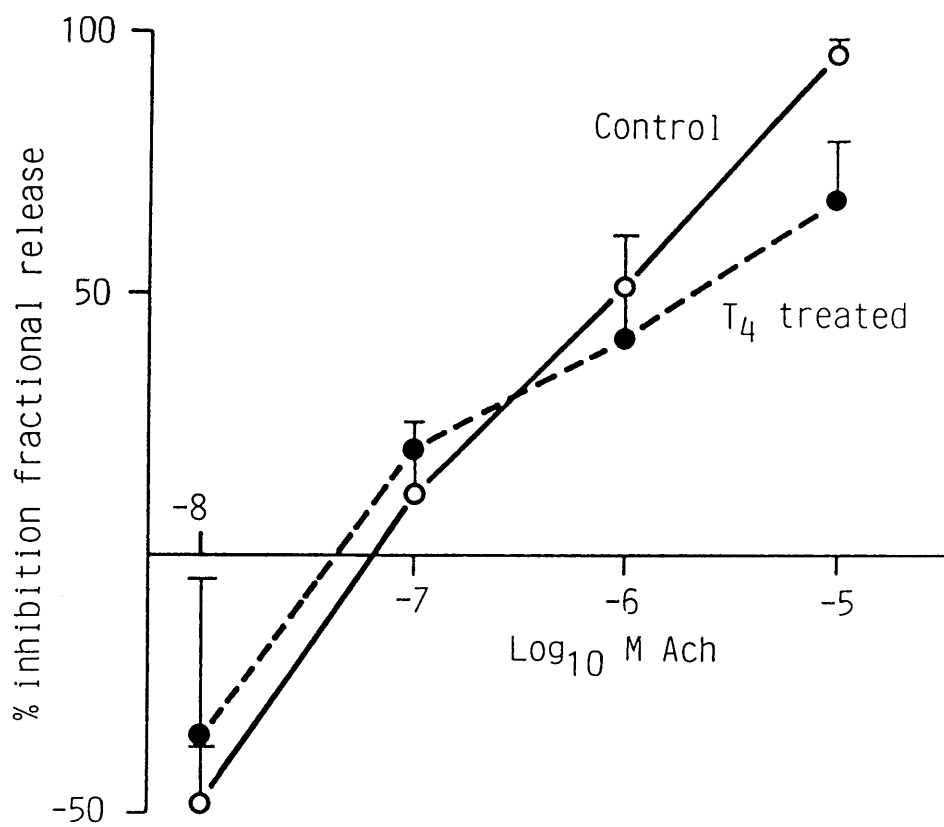
FIGURE 82. Dose %-response curves showing the effects of ACh on the stimulation-evoked overflow of ^{3}H in atria from control (O———O) and from T_4 pre-treated (●– – –●) rats.

Each point is the mean ($\pm$ S.E. mean) of 4 observations.
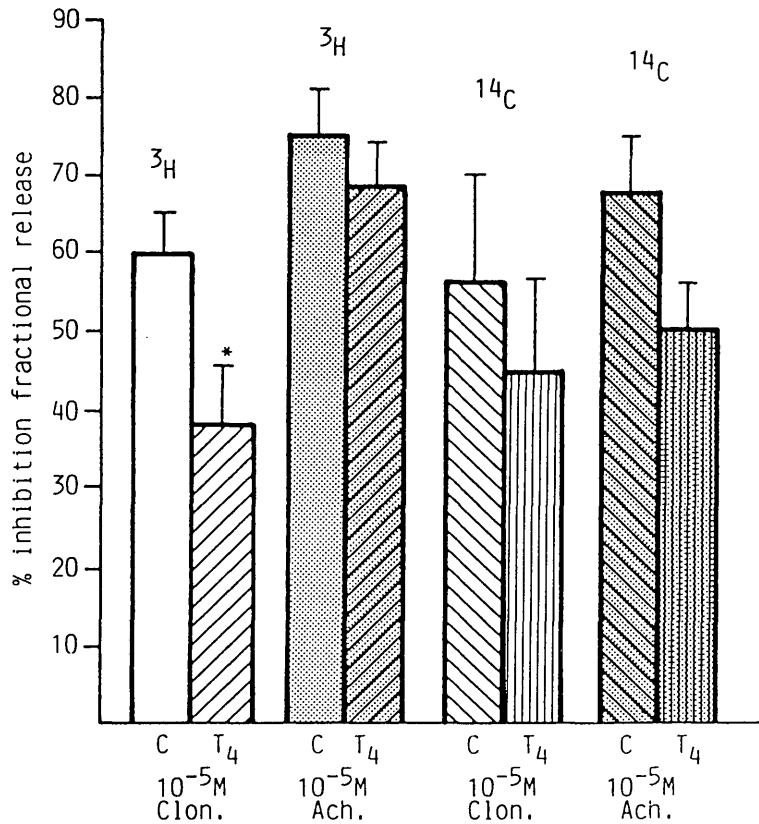
FIGURE 83. Effect of clonidine and ACh on the stimulation-evoked (5Hz x 105s) release of ^{3}H and ^{14}C in atria from control and T_4 pre-treated rats. The first 2 histograms show the significant attenuation in the ability of clonidine (10^{-5} M) to inhibit ^{3}H overflow in T_4 pre-treated atria. The second pair of histograms show the lack of effect of T_4 pre-treatment on the ability of ACh (10^{-5} M) to inhibit ^{3}H overflow. The third pair of histograms show the lack of effect of T_4 pre-treatment on the ability of clonidine (10^{-5} M) to inhibit ^{14}C overflow. Finally, the fourth pair of histograms show the lack of effect of T_4 pre-treatment on the ability of ACh (10^{-5} M) to inhibit ^{14}C overflow. Each column represents the mean ($\pm$ S.E. mean) of ≮ 3 observations. * 0.05>P>0.01.

DISCUSSION

PRESYNAPTIC INTERACTIONS OF THE ADRENERGIC AND CHOLINERGIC NERVES

The heart receives an excitatory adrenergic innervation and an inhibitory cholinergic innervation (Randall, 1977). Many of the terminal fibres of the sympathetic and parasympathetic systems lie in close proximity to each other in the heart (Jacobowitz et al, 1967; Ehinger et al, 1970). This anatomical arrangement provides an opportunity to study the complex interactions between the peripheral neurones of these two subdivisions of the autonomic nervous system. This study sought to confirm the existence of such interactions and to examine the nature of the presynaptic control exerted by these nerves.

The study, by examining the effect of nerve stimulation to the heart in situ and also by field stimulating spontaneously-beating isolated rat atria in vitro provided strong evidence for neural interactions. The pithed rat preparation was employed as this allowed independent and simultaneous stimulation of the cardio-accelerator nerves in the spinal column and of the vagus nerve in the neck of the pithed animal.

Stimulation of the cardio-accelerator nerves via an electrode in the spinal column produced an increase in heart rate; an effect that was abolished by propranolol and TTX. Stimulation of the vagus nerve in the neck produced a decrease in heart rate; an effect that was abolished by atropine and TTX. If the two sets of nerves were stimulated simultaneously or if the vagus nerve was stimulated in the presence of a dose of exogenous NA, both of which caused an increase in heart rate, then the response to vagal stimulation was enhanced.

This enhancement of a response to vagal stimulation, with respect to heart rate, against a background of raised sympathetic activity has been termed "accentuated antagonism" (Levy, 1971; 1984).

Accentuated antagonism is a common phenomenon which is widely recognised and can be produced in a variety of species (Samman, 1935; Warner & Cox, 1962; Warner & Russell, 1969; Levy & Zieske, 1969). The study also found that the response to vagal stimulation was greater in anaesthetised animals, in which, there exists a basal sympathetic tone and consequently an increased basal heart rate, than in pithed animals which have no sympathetic tone and consequently a decreased basal heart rate.

It is generally accepted that the inhibitory influence of vagal stimulation or exogenous ACh predominates over adrenergically-induced cardio-stimulation. It has been demonstrated in this study that the effects of vagal stimulation are exaggerated against a raised sympathetic tone.

Further evidence of interaction between the sympathetic and parasympathetic nerves was again derived from stimulation of the vagus nerve in the pithed rat and the consequent effect on heart rate. At a frequency of 10Hz, stimulation of the vagus nerve produced a biphasic effect on heart rate. This response consisted of a decrease in heart rate followed by an increase in heart rate. The fall in heart rate could be abolished by administration of atropine, while the increase in heart rate could beinhibited by propranolol. This observation suggests that, at this frequency, stimulation of the vagus nerve results not only in excitation of inhibitory cholinergic fibres but also of stimulatory adrenergic fibres contained in the vagosympathetic

trunk. Such observations have previously been encountered (Loewi, 1921; Iano et al, 1973); however, different explanations have been offered to explain the secondary cardioaccelerator component of vagal stimulation. In the case of the observations of Loewi (1921), as with this study, the vagal excitation appeared to be due to unavoidable excitation of sympathetic nerves in the vagosympathetic trunk. However, unlike the current study, that of Iano et al (1973), produced a vagal excitation which was not propranolol-sensitive but was atropine-sensitive. This accelerator component of parasympathetic control of the heart was postulated to be due to an action of ACh on sino-atrial nodal cells.

The evidence of sympathetic-parasympathetic interactions in the heart in situ was supplemented with experiments carried out on the isolated, spontaneously-beating paired atria from the rat. Field stimulation of such atria produced a complex post-stimulus response. During the period of stimulation the atria were driven electrically if the frequency of stimulation was faster than inherent rate of beating. If the frequency of stimulation was lower than the inherent rate of beating then the inherent rate was interrupted by the field stimulation. Immediately following stimulation a transient negative inotropic response arose that lasted about 2-3 seconds, before developing into a positive chronotropic and inotropic response. This preparation has been employed previously (Idowu & Zar, 1977), as has the equivalent preparation in the guinea-pig (Vizi et al, 1973). In the study by Idowu & Zar, the positive chronotropic response of rat atria to field stimulation was studied. This response was found to be blocked by propranolol and inhibited by clonidine suggesting that the increase in atrial rate after electrical stimulation was due to the release of NA.

The present study examined the effects of field stimulation of the isolated atria in detail. The effects of field stimulation of the atria were complex since they appeared to be the resultant not only of the postsynaptic excitatory effects of NA and the postsynaptic inhibitory effects of ACh but also because they reflected, in part, the presynaptic interactions of NA and ACh, which together modulated transmitter release. It is clear that the post-stimulation chronotropic and inotropic responses were neurally-mediated since TTX blocked both responses but allowed pacing of the atria to continue during stimulation. The positive chronotropic effects reached a maximum response, in terms of percentage increase, that was 50% less than the maximum response produced for the concomitant positive inotropic response. Thus, field stimulation of atria produced a minimal effect on heart rate but a large effect on the force of contraction.

The positive chronotropic response could be blocked by propranolol and guanethidine. However, the positive chronotropic response was insensitive to addition of atropine. The insensitivity of the chronotropic response to atropine is surprising, particularly since atropine inhibited the negative inotropic response and powerfully potentiated the positive inotropic component. Since the chronotropic response was measured in the transitional period from negative to positive inotropy and since this was lost in the presence of atropine, it is possible that the effect of atropine on the chronotropic response was masked due to the loss of this transitional phase.

The negative inotropic response reached a maximum percentage inhibition of force of contraction of 7-8Hz and was abolished by atropine. This response was not significantly affected by addition of

propranolol. These results suggest that the negative inotropic component of the post-stimulus response is due to the release of ACh. The post-stimulus positive inotropic response was sensitive to a variety of drugs. This response was abolished by reserpine pretreatment and inhibited by guanethidine and propranolol suggesting that field stimulation released NA from sympathetic nerves. The positive inotropic response was potentiated by atropine suggesting that neurally-released ACh normally acted to attenuate the response. Atropine potentiated this response by two possible mechanisms. First, atropine probably blocked postsynaptic muscarinic receptors, so that the ACh-mediated physiological antagonism of the effects of released NA was abolished. Secondly, atropine may have blocked presynaptic muscarinic receptors on sympathetic nerves, where ACh may have had a negative influence on NA release. The existence of presynaptic muscarinic receptors inhibiting NA release is well documented (for review see Muscholl, 1980).

Since the positive inotropic response was also potentiated by the alpha$_2$-adrenoceptor antagonists yohimbine, and the less specific phenoxybenzamine, which also blocks neuronal and extraneuronal uptake of NA, apparently there was considerable endogenous negative feedback of NA release in the atria. In spite of the apparent ability to block presynaptic alpha$_2$-adrenoceptors with antagonists as reflected in a increased mechanical response of the tissue following field stimulation, clonidine the alpha$_2$-adrenoceptor agonist did not inhibit the positive inotropic post-stimulus response. This was a surprising result since clonidine might have been expected to inhibit the release of NA by acting on alpha$_2$-adrenoceptors on the sympathetic nerves. The lack of effect of clonidine together with the potentiating effect of yohimbine perhaps suggests that in the atria, with no clonidine

present, there is already substantial alpha$_2$-adrenoceptor-mediated feedback restricting NA release so that the addition of clonidine has little or no superimposed effect.

This hypothesis cannot, however, be sustained since, in the presence of atropine which increased the response of the atria, clonidine inhibited the positive inotropic post-stimulus component. The fact that a ten-fold higher dose of clonidine was employed in the presence of atropine is of no relevance since the hypothesis argues that the presynaptic alpha$_2$-adrenoceptor is being maximally stimulated. It is possible, however, that the initial dose of clonidine is too small to initiate activation of presynaptic alpha$_2$-adrenoceptors and this required further investigation (see below). However, the presence of atropine should further increase the release of NA, by blocking presynaptic muscarinic receptors on the sympathetic nerve terminal thus leading to more NA acting on the presynaptic alpha$_2$-adrenoceptor and if anything diminishing a potential effect of clonidine as opposed to unmasking one.

A possible explanation of the anomalous effects of clonidine in the absence and presence of atropine is that clonidine does indeed act on presynaptic alpha$_2$-adrenoceptors however, these are situated not only on the adrenergic nerve terminal but also on the cholinergic nerve terminal. Such an arrangement is plausible because otherwise there would be an asymmetrical distribution of presynaptic control of transmitter release. There is considerable pharmacological evidence for the existence of presynaptic alpha-adrenoceptors on the parasympathetic nerves in the gut, that act to modulate the release of ACh (Knoll & Vizi, 1970; Wikberg, 1978 a,b; Manber & Gershon, 1979; Davey, 1980) and also in the trachea (Baker & Don, 1987).

If alpha-adrenoceptors do exist on the parasympathetic nerves in the atria and if they regulate ACh as they regulate NA release in the sympathetic nerves then clonidine would not affect the positive inotropic post-stimulus response because clonidine would inhibit the release of ACh and NA simultaneously, so that the postsynaptic consequences would be negated. More importantly, if clonidine acted on alpha-adrenoceptors on parasympathetic nerves, inhibition of ACh release would also remove the inhibitory effects of ACh on presynaptic cholinoceptors on sympathetic nerves so that more NA would be released. This would be balanced by the inhibitory effects of clonidine at the $alpha_2$-adrenoceptor on the sympathetic nerves so that it is unlikely that the positive inotropic response would be affected and almost certainly would not be reduced. Thus, the inhibitory effect of clonidine on NA release is only seen when the inhibitory effect of clonidine on ACh release is masked by the presence of atropine. It is, therefore, suggested that there are presynaptic inhibitory alpha-adrenoceptors not only on the sympathetic nerve terminals but also on the cholinergic nerve terminals in the atria.

The above evidence for adrenergic-cholinergic interactions and the existence of a presynaptic inhibitory alpha-adrenoceptor situated on the parasympathetic nerve terminal is circumstantial in so far as it attempts to interpret the effects of drugs thought to act presynaptically (Drew, 1976; Starke, 1977) to inhibit the release of transmitter by monitoring postsynaptic events. Although the atria has previously been used to study transmitter release by assaying the postsynaptic effects of drugs which act prejunctionally to affect transmitter release (Idowu & Zar, 1977; Lew & Angus, 1983; Loiacono & Story, 1986) a more direct method of examining presynaptic receptors

would be to measure the output of transmitter. This eliminates the involvement of post-synaptic events in the analysis of results.

Since NA is known to be a transmitter in atria, part of this study investigated the effects of a variety of drugs on the release of NA from atria pre-incubated with [^{3}H]-NA. Field stimulation of such atria increased the amounts of radioactivity released into the bathing solution. TTX abolished, in a dose-dependent manner, [^{3}H]-NA overflow indicating it was neurally-mediated. Separation of [^{3}H]-NA from its metabolites confirmed that the overflow collected was principally NA.

The inactivation of neurally-released NA is carried out by neuronal uptake, which reduces the concentration of released transmitter in the synaptic cleft (Langer, 1977). This study has shown that a combination of tranylcypromine, desmethylimipramine and 17, Beta-oestradiol, which block, respectively, monoamine oxidase (MAO) and the neuronal and extraneuronal uptake of NA, produced a significant increase in the overflow of [^{3}H]-NA. It has been proposed (Langer, 1977) that blockade of neuronal uptake enhances the amount of transmitter available to activate presynaptic alpha-adrenoceptors leading to enhanced feedback inhibition. It is perhaps surprising, therefore, that uptake blockers, such as those employed, enhanced transmitter overflow. However, in the atria, cocaine, a neuronal uptake agent has been seen to increase transmitter overflow (McCulloch et al, 1972, Story & McCulloch, 1974) suggesting that despite the increased concentration of NA in the synaptic cleft the alpha$_2$-adrenoceptor feedback mechanism is not being fully activated. Two results from this study support this. First, clonidine still inhibits [^{3}H]-NA release in the presence of tranylcypromine, desmethylimipramine and 17, Beta-oestradiol and secondly, yohimbine

still enhances [3H]-NA release in the presence of these drugs. Thus, it appears as if the uptake blockers do enhance the stimulation-induced overflow of transmitter, but this has little effect on the presynaptic alpha-adrenoceptor feedback mechanism governing NA release.

The situation is remarkably complex if there is indeed a presynaptic alpha-adrenoceptor situated on the cholinergic nerve terminals. Since the presynaptic mechanisms are so sophisticated the increased concentration of NA, caused by the uptake blockers, would presumably further inhibit ACh release. This would result in a removal of ACh restraint on NA release via the presynaptic muscarinic receptor on the adrenergic nerve resulting in an even greater increase in NA overflow.

Clonidine inhibited [3H]-NA overflow and yohimbine potentiated [3H]-NA overflow thus confirming the presence of presynaptic alpha$_2$-adrenoceptors on the adrenergic nerve and also that endogenous inhibition of NA release was mediated via these receptors. ACh inhibited [3H]-NA release but at a lower concentration potentiated [3H]-NA release. The potentiating action of ACh is surprising. The concentrations of ACh used were less than one-hundredth of those required to have any appreciable effect on the uptake of NA (Allen, 1973) or to have any stimulatory effect on nicotinic receptors (Muscholl, 1980). Furthermore, nicotinic cholinoceptors do not appear to modulate the release of [3H]-NA from rat isolated atria evoked by sympathetic nerve stimulation (Fuder et al, 1982). An enhancing effect of very low concentrations of ACh on sympathetic transmission has been demonstrated in the mesenteric artery. The response was inconsistent and small. A similar enhancement of sympathetic stimulation after low concentrations of ACh release was reported for

the rabbit ear artery (Rand & Varma, 1970). This effect was confirmed by Allen et al (1975) whose studies involved measuring [³H]-NA overflow. The phenomenon was found to be highly sensitive to changes in extracellular calcium ion concentration (Hope et al, 1978). Some reservation would be justified in attaching general significance to the phenomenon since the effect has been sought but not found in rabbit pulmonary artery (Rand et al, 1975; Endo et al, 1977) in rabbit heart (Muscholl, 1973) in guinea-pig atria (Story et al, 1975) and in several blood vessels from the dog (Vanhoutte, 1977).

Atropine enhanced the release of [³H]-NA confirming the existence of an endogenous ACh-mediated restraint on NA release. The ability of atropine and yohimbine to potentiate field stimulation-induced [³H]-NA overflow confirmed what was suggested by the effects of these drugs on the mechanical response of the field-stimulated atria. The inhibitory effect of clonidine of [³H]-NA overflow also confirmed the observation that clonidine could inhibit the inotropic post-stimulus response although only in the presence of atropine.

In this atrial preparation there are presynaptic $alpha_2$-adrenoceptors and muscarinic cholinoceptors on the sympathetic nerve terminals and through these receptors, endogenous, neurally-released NA and ACh act simultaneously to inhibit further NA release. It seems clear that the ability of yohimbine to potentiate field-stimulation induced [³H]-NA overflow by blocking NA-mediated auto-inhibition via presynaptic $alpha_2$-adrenoceptors on the sympathetic nerves would be restrained by the simultaneous blocking of the hypothetical presynaptic alpha-adrenoceptors on the parasympathetic nerve mediating regulation of ACh release. The increased amount of ACh thus released by yohimbine would, through an action on presynaptic muscarinic cholinoceptors on

the sympathetic nerves, inhibit NA release. The study has shown that yohimbine and atropine had additive and at some concentrations synergistic effects on [^{3}H]-NA overflow. This synergistic potentiation of field-stimulation-induced transmitter release is compatible therefore, with the existence of alpha-adrenoceptors on the cholinergic nerves as well as on the noradrenergic nerves.

These results confirm the initial observations obtained from the mechanical responses produced by field stimulated atria. They provide further evidence suggesting the existence of an inhibitory presynaptic alpha-adrenoceptor on the cholinergic nerve terminal but they do not permit direct measurement of the release of ACh and examination of the effects of presynaptic agonists and antagonists on this parameter.

In the course of this study atria were incubated in [^{14}C]-choline. It has been rigorously shown that the immediate precursors of ACh are acetyl-CoA and choline, which is taken up into the parasympathetic nerves by means of a high-affinity Na$^+$-dependent uptake mechanism (Wetzel & Brown, 1983; Loffelholz et al, 1984). It has been shown that incubation in radiolabelled choline results in incorporation of such choline as to form radiolabelled ACh. This method ensures that radiolabelled ACh synthesis occurs in the cholinergic neurones since non-neuronal cardiac cells do not contain choline acetyltransferase, the enzyme responsible for the synthesis of ACh in cholinergic neurones (Roskoski et al, 1977). With the addition, after incubation, of hemicholinium-3, which prevents the reuptake of choline (Jope, 1979) and eserine, which inhibits the breakdown of ACh, field stimulation results in an overflow of [^{14}C] which is principally ACh.

In the present study the procedure used to radiolabel the cholinergic transmitter pools was essentially that of Fosbraey & Johnson (1980). No attempt was made to account for radioactivity in terms of acetylcholine and its metabolite choline. Presumably, were it not for the presence of the cholinesterase inhibitor, eserine, a large proportion of transmitter ACh released from the atrial cholinergic nerves would have been subject to enzymatic destruction. The stimulation-evoked ACh overflow from hearts of chicks, cats, guinea-pigs and rabbits is markedly increased in the presence of eserine, a cholinesterase inhibitor (Dieterich et al, 1976; 1978).

In rat isolated atria previously incubated in [3H]-choline most of the efflux of radioactivity released by stimulation is present as [3H]-ACh, whereas [3H]-choline accounts for most of the efflux during resting periods (Wetzel & Brown, 1985). Similar results have been obtained with tissues other than the heart which have been incubated in [3H]-choline to study cholinergic transmitter processes (Wikberg, 1977). The field stimulation-induced overflow in this study was assumed, therefore, to represent ACh release, presumably from both pre-and post-ganglionic parasympathetic terminals within the atria. The [14C]- radiolabel was chosen to allow simultaneous incubation with [3H]-NA thus permitting both neurotransmitters to be collected and measured separately. This allowed the effects of drugs on the field stimulation-induced release of both radiolabels to be examined in the same experiments.

Preliminary experiments were carried out in which the Krebs solution bathing the atria did not include eserine and hemicholinium. Under these circumstances no increase in [14C] overflow was observed. Only in the presence of these drugs was an appreciable increase in [14C]

overflow observed following stimulation. This effect has been observed on $[^3H]$-choline overflow studies in the guinea-pig ileum (Fosbraey & Johnson, 1980). Field stimulation of atria incubated in $[^3H]$-NA and $[^{14}C]$-choline results in an increase in overflow of both these radiolabels.

With the parameters used in this study a descending level of overflow resulted with each consecutive stimulation at 10 minute intervals. TTX inhibited the stimulation-induced overflow of $[^{14}C]$ suggesting that this was a neurally-mediated response. This study has shown that the alpha-adrenoceptor agonist, clonidine, can inhibit the field-stimulation-induced release of $[^{14}C]$ and $[^3H]$ simultaneously. In addition, the muscarinic receptor agonist, ACh, can also inhibit the field-stimulated release of $[^{14}C]$ and $[^3H]$. These results suggests that there exists on the cholinergic nerve terminal a presynaptic alpha-adrenoceptor and a muscarinic receptor that when activated by exogenous agonists inhibit the field-stimulated release of ACh. The muscarinic receptor antagonist, atropine, potentiated the release of $[^{14}C]$. These observations suggest that field stimulation of atria releases ACh, which acts on a presynaptic muscarinic receptor on the cholinergic nerve terminal to inhibit its own release; a system analogous to that of NA-mediated feedback inhibition via presynaptic alpha-adrenoceptors on adrenergic nerve terminals.

As previously suggested, there does appear to exist on the cholinergic nerve terminal an alpha-adrenoceptor which when activated by clonidine inhibits the release of ACh. Clonidine simultaneously inhibits NA-release. The study examined the effect of yohimbine and prazosin on $[^3H]$-NA and $[^{14}C]$ overflow. It was found that yohimbine potentiated the stimulation-induced overflow of $[^3H]$-NA and $[^{14}C]$. Prazosin, an

alpha$_1$-adrenoceptor antagonist failed to potentiate [^{14}C] overflow but did potentiate [^{3}H]-NA overflow. The results suggest that field stimulation of atria results in the release of NA.

It appears this NA acts on a presynaptic alpha-adrenoceptor on the cholinergic nerve terminal to inhibit release of ACh. The NA-mediated restraint of ACh release can be prevented by yohimbine but not by prazosin. This result partially confirms the observations of Wetzel et al (1985) and McDonough et al (1986). In these studies [^{3}H]-ACh release was promoted by potassium depolarisation. Under these circumstances exogenous ACh could inhibit the potassium-induced release of [^{3}H]-ACh overflow, while atropine could potentiate the potassium-induced release of [^{3}H]-ACh. In addition, these studies, have characterised an inhibitory adrenoceptor on the cholinergic nerve as being of the alpha$_1$-subtype. Contrary to the results in this study, it was found that clonidine was unable to inhibit [^{3}H]-ACh overflow. Also, in contrast, these studies showed that selective alpha$_1$-adrenoceptor antagonists more powerfully potentiated [^{3}H]-ACh overflow than selective alpha$_2$-adrenoceptor antagonists. Although, there remains doubt as to the nature of alpha-adrenoceptor situated on the cholinergic nerve terminal, there is little doubt as to its existence and its ability to inhibit the release of ACh.

The ability of the alpha$_1$-adrenoceptor antagonist, prazosin, to potentiate the overflow of [^{3}H]-NA is consistent with evidence obtained for alpha$_1$-adrenoceptor-mediated inhibition of NA released obtained in the rat (Kobinger & Pichler, 1982; Docherty, 1984) and the dog (Uchida et al, 1984).

This study and recent studies clearly demonstrate that there exists on the cholinergic nerve muscarinic cholinoceptors and alpha-adrenoceptors which when activated by respective agonists inhibit the release of ACh. Previous studies concerned with the regulation of cardiac parasympathetic neurotransmission have assessed ACh release indirectly by examining physiological responses to vagal stimulation (Vincenzi & West, 1965; Hadhzy et al, 1973; Glitsch & Pott, 1978 a,b). Two such studies measuring chronotropic responses to vagal stimulation suggested that the release of ACh in the mammalian heart is regulated presynaptically by cholinergic (Yonehara et al, 1979) and adrenergic agents (Chassaing et al, 1983). These conclusions are consistent with the reported effects of cholinergic and adrenergic agents on ACh release in non-cardiac tissues (Wikberg, 1978(b); Koketsu & Yamada, 1982).

Thus, while this study has provided evidence for adrenergic modulation of ACh release, a functional and physiological response demonstrating such modulation would be desirable. Therefore, rats were anaesthetised or pithed and the effects of vagal stimulation on heart rate in situ was monitored. In anaesthetised rats, in which there exists a basal sympathetic tone (McGrattan et al, 1987), the alpha-adrenoceptor antagonists prazosin and yohimbine significantly potentiated vagally-induced bradycardia. The bradycardia produced by exogenous ACh was unaffected by these drugs. These results suggest that vagal stimulation in anaesthetised rats caused release of ACh and that the release of ACh is tonically inhibited by noradrenaline presumably by activation of presynaptic adrenoceptors on the cholinergic nerve terminals. In addition, the study showed that phenylephrine, the alpha$_1$-selective adrenoceptor agonist, could attenuate vagally-induced bradycardia and that this effect could be reversed by prazosin.

Similarly, clonidine could also attenuate vagally-induced bradycardia and this attenuation could be partially reversed by yohimbine. In the pithed rat, clonidine was again able to attenuate vagally-induced bradycardia and as before yohimbine was only partially able to reverse this effect.

These results provide further evidence for the existence of an alpha-adrenoceptor inhibiting the neural release of ACh. The ability of phenylephrine to inhibit vagally-induced bradycardia and the reversal of this effect by prazosin has been reported elsewhere (McGrattan et al, 1987) and is believed to be further evidence in support of the adrenergic receptor on the cholinergic nerve terminal to be of the $alpha_1$-subtype. However, doubt must still remain as to the nature of this receptor, since in this in vivo study, clonidine was able to attenuate the vagally-induced bradycardia, an effect which could be reversed by yohimbine. The results of the present study provide evidence in support of the speculative hypothesis proposed by Rand et al (1975, 1980) which suggested that the transmitters ACh and NA may act not only by having opposite effects on the post-junctional effector cells but also on transmitter release from the nerves with the opposing action. Thus, this study would suggest there exists presynaptic autoinhibition of transmitter release and also presynaptic mutual cross inhibition of transmitter release in the heart (Fig 84).

	Presynaptic	
Postsynaptic receptor activation	Auto-inhibition	Mutual cross inhibition
Cardiac muscle	Autonomic nerves	
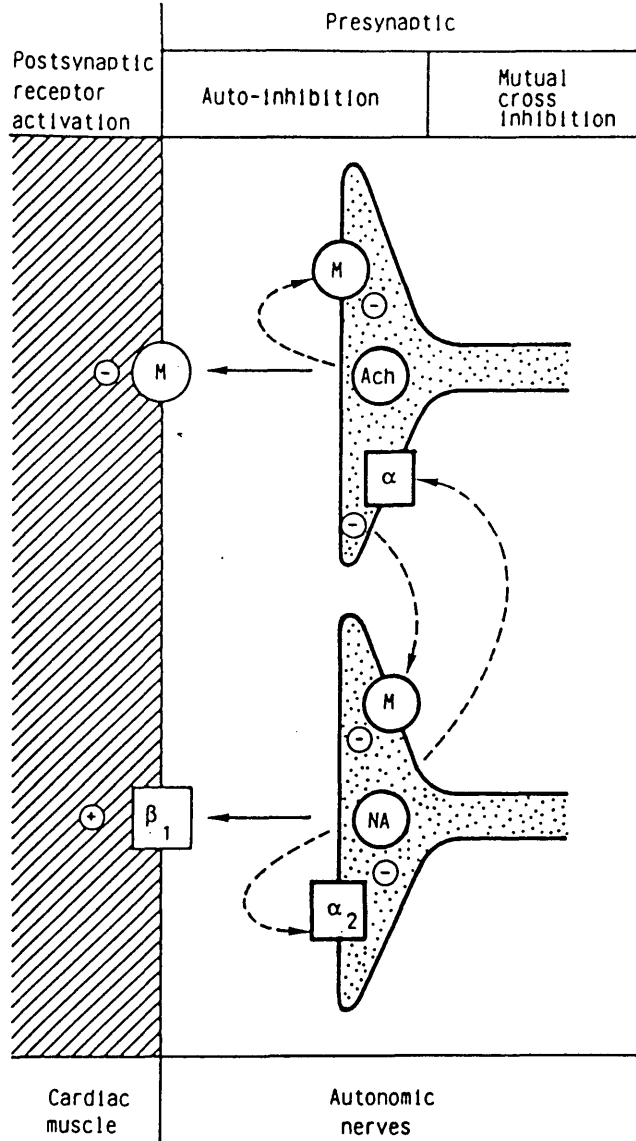
FIGURE 84. Schematic representation of the presynaptic
interactions between the adrenergic and cholinergic nerves
in the atria. Field stimulation of the atria results in the
simultaneous release of both ACh and NA, which act on a
post-synaptic muscarinic receptor and beta-adrenoceptor
respectively. ACh also acts to auto-inhibit its own release
by acting on an inhibitory pre-synaptic muscarinic receptor
on the cholinergic nerve terminal. ACh also inhibits the
release of NA by acting on an inhibitory muscarinic receptor
on the adrenergic nerve terminal. NA auto-inhibits its own
release by acting on an inhibitory presynaptic $alpha_2$-
adrenoceptor on the adrenergic nerve terminal. In addition,
NA inhibits the release of ACh by acting on an inhibitory
alpha-adrenoceptor situated on the cholinergic nerve
terminal. Such a system would result in a sophisticated
control of the release of the 2 transmitters.

INVOLVEMENT OF CYCLIC NUCLEOTIDES IN MODULATING THE RELEASE OF ACh AND NA

The release of NA from sympathetic nerves can be modulated by endogenous and exogenous substances acting on receptors situated on the nerve terminals (Langer, 1977; Starke, 1977; Muscholl, 1980). These receptors include beta-adrenoceptors, alpha-adrenoceptors, muscarinic cholinoceptors, dopamine receptors, opiate receptors and purinergic receptors among others (Langer, 1981). The presynaptic receptors which have been investigated most frequently are the inhibitory $alpha_2$-adrenoceptor, the inhibitory muscarinic cholinoceptor and the facilitatory beta-adrenoceptor. Activation of the beta-adrenoceptor results in an enhancement of NA release. The ultimate effects of activation of these receptors are well documented however, the intraneuronal mechanisms through which these effects are mediated are still unknown.

The present study examined the hypothesis postulated by Loiacono et al (1985), who suggested that presynaptic muscarinic cholinoceptors and $alpha_2$-adrenoceptors on the sympathetic nerve terminal may interact so that activation of one type of receptor interferes with the effectiveness of an agonist acting at the other. The evidence for such a reciprocal interaction was the observation that phentolamine blocked $alpha_2$-adrenoceptors and consequently increased NA release but paradoxically also enhanced the ability of exogenous ACh to inhibit NA release. It was suggested that the reciprocal interaction was due to the fact that both these receptors are competing for a final common intracellular pathway which ultimately regulates the availability of calcium ions for stimulus-response coupling (Gothert, 1977).

Such an interaction between presynaptic receptors competing for a common intracellular pathway have previously been proposed. Thus, it has been shown that activation of prejunctional beta-adrenoceptors in enhancing NA release is inversely related to the degree of activation of the prejunctional alpha-adrenoceptors (Majewski & Rand, 1981). In addition, it has been suggested that $alpha_2$-adrenoceptors and opiate receptors located on nerve terminals in the vas deferens may be linked to a common mechanism that inhibits transmitter release (McCulloch & Pollock, 1985).

The present study re-examined the possibility of such a reciprocal interaction between the presynaptic alpha-adrenoceptor and the cholinergic muscarinic receptor on the sympathetic nerve. Repetition of the original experiments, whereby, the effectiveness of ACh at inhibiting NA release was examined in the absence and presence of phentolamine did not suggest that ACh was more effective at inhibiting NA release when the $alpha_2$-adrenoceptor was blocked. Rather, at low concentrations ACh was equally effective at inhibiting NA release in the absence or in the presence of phentolamine. At a higher dose of ACh, phentolamine actually inhibited the ability of ACh to attenuate NA release. The discrepancy between the studies is interesting. The parameters of stimulation and concentration of drugs used were identical although the interval between stimulation differed as was the duration of exposure of the atria to drugs. The author and Loiacono can find no other explanation other than those cited for the discrepancy (Loiacono, personal communication).

However, the present study provided further evidence which suggested that the muscarinic and $alpha_2$-adrenoceptor did not feed into the same intracellular pathway. If the original hypothesis were true then the

ability of an alpha-adrenoceptor agonist to inhibit NA release should be enhanced in the absence of endogenous ACh-mediated restraint. Thus, since field stimulation of isolated atria releases both ACh and NA then atropine should prevent endogenous ACh inhibiting NA release in a manner analogous to that of phentolamine preventing NA from inhibiting its own release. The present study compared the ability of clonidine to inhibit NA release in the absence and in the presence of atropine. Therefore, the converse experiment to that performed by Loiacono et al (1985) was carried out. Thus, when the muscarinic receptor was blocked, by atropine, to remove ACh-mediated restraint of NA release the $alpha_2$-adrenoceptor agonist, clonidine, was less effective at inhibiting ^3H overflow. This result is contrary to that predicted by the hypothesis of Loiacono et al, (1985). A possible explanation of the result is that clonidine is less effective in the ,presence of atropine because the ACh-mediated restraint on NA release is removed so that there is enhanced activation of $alpha_2$-adrenoceptors and thus the scope for clonidine to act on the $alpha_2$-adrenoceptors is reduced.

The previous study (Loiacono et al, 1985) found that in arterial preparations both exogenous ACh and clonidine inhibited NA overflow. Combining ACh with clonidine did not alter the inhibitory effect of clonidine. The present study also found that, in the atria, exogenous ACh and clonidine inhibited NA overflow, however, it also showed that the inhibitory effect of clonidine was enhanced in the presence of ACh. Indeed, the ability of clonidine and ACh to inhibit NA release were additive.

Finally, the ability of atropine and yohimbine to produce at some doses a synergistic potentiation of field stimulation-induced NA overflow may also indicate that the two inhibitory pathways, activated respectively by ACh and NA, affect different stages in the process of excitation-secretion coupling.

The present study disagrees with the observation of Loiacono et al (1985) that the presynaptic muscarinic cholinoceptor and the presynaptic alpha$_2$-adrenoceptor interact in such a way that they feed into a common pathway inhibiting the release of NA.

The nature of the intracellular pathways governing transmitter release are unknown. It has been proposed that activation of presynaptic adrenoceptors affects the release of NA from sympathetic nerves by altering adenylate cyclase activity and cyclic AMP formation (Rasmussen, 1970; Wooten et al, 1973; Cubeddu et al, 1975; Stjarne, 1979). It is possible that alterations in endogenous cAMP production may explain the modulatory effects of alpha- and beta-adrenoceptor agonists on NA release. Findings in non-neuronal tissue support this theory where beta-adrenoceptor agonists enhance adenylate cyclase activation leading to increased cAMP production (Perkins, 1973) and alpha$_2$-adrenoceptor agonists inhibit adenylate cyclase and decrease cAMP production (Jakobs, 1985). Thus, the ability of beta-adrenoceptor agonists to promote NA release and alpha$_2$-adrenoceptor agonists to inhibit NA release may be a reflection of the effects these drugs have on neuronal cAMP production. In most tissues the proportion of nerve to effector cell is so small as to make it experimentally impracticable to attribute to the neuronal tissue a change in the cyclic nucleotide level which is also present to a large extent in non-neuronal tissue (Gillespie, 1980). The inability to

measure cyclic nucleotide levels in neuronal tissue directly means that the role of cyclic nucleotides in modulating transmitter release has to be investigated indirectly by examining the effects of drugs, which alter cyclic nucleotide levels, on the release of transmitter.

The present study sought to determine whether cAMP was involved in modulating NA release from the sympathetic nerve terminals in the heart. The study also investigated the role of cGMP in modulating transmitter release in the atria. For these purposes, the effects of drugs that activate adenylate or guanylate cyclase, mimic cAMP or cGMP or inhibit the breakdown of these cyclic nucleotides were examined.

The ability of clonidine to inhibit NA release has previously been described. The study demonstrated the ability of isoprenaline the beta-adrenoceptor agonist to potentiate NA release. This facilitatory adrenoceptor, believed to be of the $beta_2$-subtype is thought not to be activated by endogenous NA but rather by adrenaline in the circulation (Majewski, 1983). It has been proposed that the facilitation of NA release evoked by activation of presynaptic beta-adrenoceptors may be mediated through increased levels of neuronal cAMP (Langer, 1979).

The ability of IBMX to potentiate field stimulation-induced $[^3H]$-NA overflow confirmed what previous studies have shown, namely that phosphodiesterase inhibitors enhance NA release during nerve stimulation (Cubeddu et al, 1975; Celuch et al, 1978; Johnston et al, 1987). The cell permeant analogue of cAMP, 8-bromo-cAMP enhanced the field-stimulation induced release of $[^3H]$-NA from the atria. However, since field stimulation results in release of both NA and ACh and since ACh exerts an inhibitory influence on NA release it was necessary to determine whether 8-bromo-cAMP was acting directly on the

sympathetic nerve terminal to facilitate the release of NA, or whether it was acting on the cholinergic nerve terminal to inhibit ACh release so that less ACh was available to inhibit the release of NA. In such circumstances 8-bromo-cAMP would increase NA overflow. If, however, 8-bromo-cAMP could still produce an increase in $[^3H]$-NA overflow when the cholinergic restraint on NA release was blocked by atropine then this would provide evidence for a facilitatory action of 8-bromo-cAMP on the adrenergic nerve terminal. The present study has shown that 8-bromo-cAMP does indeed potentiate field stimulation-induced $[^3H]$-NA overflow in the presence of atropine in the atria. Thus, the study also examined the effect of forskolin on the field-stimulated release of $[^3H]$-NA. Forskolin activates adenylate cyclase leading to increased cAMP production (Seaman & Daly, 1981). Thus, if cAMP is involved in promoting the release of NA then forskolin should potentiate the release of $[^3H]$-NA. Since it was necessary to dissolve forskolin in DMSO the effect of forskolin on $[^3H]$-NA release was compared with that of DMSO alone. It was found that DMSO produced an enhancement in the field-stimulated release of $[^3H]$-NA and that forskolin produced no further increase in release. The study is unable to produce evidence implicating forskolin in promotion of transmitter release. The vehicle, DMSO, possibly masked any potential effect of forskolin on $[^3H]$-NA overflow and perhaps a different vehicle, for example ethanol, might not have produced such an undesirable effect.

Forskolin did have a powerful postsynaptic excitatory effect on the force of contraction of the atria, presumably because of its effect on adenylate cyclase in the effector tissue but the vehicle, DMSO, had no such effect. Other workers have reported that forskolin does enhance the stimulation-induced release of $[^3H]$-NA (Hovevei-Sion et al, 1983).

There seems little doubt, that increasing the concentration of intraneuronal cAMP by pharmacological means, results in an increase in noradrenaline release (Wooten et al, 1973; Cubeddu et al, 1975; Hentrich et al, 1985). In addition, activation of the presynaptic beta-adrenoceptor has also been linked to increased cAMP production which promotes the release of noradrenaline (Langer, 1981; Johnston et al, 1987). However, the possibility that the presynaptic alpha$_2$-adrenoceptor on the adrenergic nerve terminal, in the atria, is linked to adenylate cyclase is more controversial.

It has been shown previously that in non-neuronal tissues occupation of an alpha$_2$-adrenoceptor causes dissociation of a trimeric guanine-nucleotide binding protein (Ni) and these subunits inhibit adenylate cyclase leading to a decrease in cAMP production (Dolphin, 1987). Since it appears that alpha$_2$-adrenoceptors inhibit adenylate cyclase when they are situated post-synaptically, this study investigated if the same action on adenylate cyclase occurred following activation of presynaptic alpha$_2$-adrenoceptors. Previous studies have examined this hypothesis indirectly by using either cell permeable cAMP analogues to saturate the cell with cAMP (Johnston et al, 1987) or phosphodiesterase inhibitors to prevent the breakdown of cAMP (Cubeddu et al, 1975; Johnston et al, 1987). These studies found little evidence to suggest that cAMP is involved in alpha$_2$-adrenoceptor-mediated presynaptic inhibition of transmitter release. A previous study by Johnston and Majewski (1986) had however, found evidence which suggested that presynaptic alpha-adrenoceptors were linked to adenylate cyclase.

A more direct approach is to use pertussis toxin purified from the bacteria Bordetella pertussis which abolishes alpha$_2$-adrenoceptor

agonist inhibition of adenylate cyclase in both membranes and intact cells (Katada et al, 1986). Pertussis toxin pretreatment leads to a loss of receptor-mediated inhibition of adenylate cyclase (Dolphin, 1987). Thus, if the presynaptic alpha$_2$-adrenoceptor is linked via a pertussis toxin sensitive guanine nucleotide binding protein to adenylate cyclase, then pretreatment with pertussis toxin should result in the abolition of the ability of clonidine to inhibit [^{3}H]-NA overflow. The present study compared the ability of clonidine to inhibit field stimulation-induced [^{3}H]-NA overflow from control atria and atria removed from rats pretreated with pertussis toxin.

The results showed that, in atria from pertussis toxin treated rats, the ability of a low dose of clonidine to inhibit [^{3}H]-NA overflow was abolished. At higher doses there was no difference in the ability of clonidine to inhibit overflow in control or pretreated atria. This anomalous result has three possible explanations. First, it is possible that the pertussis toxin pretreatment was ineffective and the apparent ability of pertussis toxin to abolish the effect of the low concentration of clonidine is an artefact. This possibility was examined by assessing the effectiveness of the pertussis toxin pretreatment on the cholinergically-mediated negative inotropic response to field stimulation of the atria. Pertussis toxin abolished this response to field stimulation and this is consistent with the ability of pertussis toxin to abolish the response to carbachol in rat atria (Endoh et al, 1985) and mouse atria (Musgrave et al, 1987). Secondly, since it appears that pertussis toxin pretreatment was effective, as indicated by the abolition of the cholinergic response, it is possible that the presynaptic alpha$_2$-adrenoceptor is not linked to adenylate cyclase via a pertussis toxin sensitive inhibitory guanine nucleotide binding protein. Again, this would suggest that the

"effect" at the lowest dose of clonidine was artefact. However, this interpretation would agree with a recent study which suggested that pertussis toxin did not attenuate alpha$_2$-adrenoceptor-mediated inhibition of noradrenaline release in mouse atria (Musgrave et al, 1987). The third possibility is that the presynaptic alpha$_2$-adrenoceptor is linked to adenylate cyclase in such a way that pertussis toxin pretreatment abolishes the receptor-mediated inhibition and thus at low doses clonidine is unable to inhibit the release of NA. It is possible that at higher doses clonidine acts on presynaptic alpha$_1$-adrenoceptors on the sympathetic nerve terminal which also inhibit the release of NA but which are unaffected by pertussis toxin pretreatment. Evidence exists for the presence of inhibitory alpha$_1$-adrenoceptors on the cardiac sympathetic neurones in the rat (Docherty, 1984).

It is possible that a non-selective action at high doses of clonidine is masking the presynaptic effect of pertussis toxin pretreatment. Certainly, in non-peripheral tissue, such as hippocampal slices, pertussis toxin attenuated clonidine inhibition of stimulation-induced [^{3}H]-NA release (Allgaier et al, 1985).

Given that the evidence for an involvement of cAMP in inhibition of transmitter release was slight, the study examined the possible role of cGMP and its modulation of transmitter release. The role in nerves of guanylate cyclase and cGMP is complex and currently undefined (Drummond, 1984). One report provided electrophysiological evidence for a calcium-dependent prejunctional role for cGMP possibly linked to an alpha-adrenergic receptor (O'Dea & Zatz, 1976). In this study it was found that in the rat pineal gland in vitro, both exogenous NA and high potassium caused an accumulation of cGMP in the whole gland. The

effect was abolished by phenoxybenzamine or phentolamine and also by chronic sympathetic denervation. The authors suggested the effect may be associated with presynaptic alpha-adrenoceptor inhibition. A further transmitter release study in this preparation found that dibutyryl cGMP, a cell permeant derivative of cGMP, inhibited the potassium-evoked release of [^{3}H]-NA. In addition, a specific cGMP phosphodiesterase inhibitor also inhibited the potassium-evoked release of [^{3}H]-NA (Pelayo et al, 1978).

Contrary to the work cited above the present study found that sodium nitroprusside, which stimulates guanylate cyclase and cGMP formation (Rapaport & Murad, 1983) caused a dose-dependent potentiation of field stimulation-induced release of [^{3}H]-NA from the atria. In addition, it was also found that the cell permeant derivative of cGMP, 8-bromo-cGMP also potentiated the field stimulation induced release of [^{3}H]-NA overflow. These results apparently provide evidence implicating cGMP in the enhanced release of NA from sympathetic nerves in the atria.

If activation of guanylate cyclase promotes NA release, then the question arises as to where this enzyme is located. If this enzyme is in the sympathetic nerves it appears as if it might facilitate NA release. On the other hand, if the guanylate cyclase is located in the concomitantly-stimulated cholinergic nerves, then it could be involved in inhibiting ACh release, so that the restraining influence of ACh on NA release would be reduced and NA overflow would increase.

It is interesting to note that several recent studies (Loiacono et al, 1985; Johnston & Majewski, 1986; Johnston et al, 1987; Musgrave et al, 1987) that have examined the release of NA in the atria have failed to take into account the fact that field stimulation releases both NA

and ACh. These studies have formulated conclusions based on the principle that the drugs employed are acting solely on the adrenergic nerve terminals while ignoring the possible action of the drugs on the release of ACh.

The ability of drugs which mimic cGMP or prevent the breakdown of cGMP to enhance NA release has been reported previously (Cubeddu et al, 1975). In this study 8-bromo-cGMP promoted the release of [^{3}H]-NA from cat spleen. In addition 8-bromo-cGMP produced a concentration-dependent inhibition of phosphodiesterase activity in the whole preparation. It has been suggested therefore that 8-bromo-cGMP by inhibiting phosphodiesterase, prevented the breakdown of neural cAMP thus promoting transmitter release. However, since nerve terminal phosphodiesterase probably represents a very small fraction of the total phosphodiesterase present in the spleen, correlation between enhanced exocytosis and enzyme inhibition must be interpreted with caution (Cubeddu et al, 1975).

This is particularly true when it is possible that the drug reputedly inhibiting phosphodiesterase in the adrenergic nerve may have a completely different site of action. Thus, in the recent study by Johnston et al (1987) it was report that 8-bromo-cGMP and a selective cGMP phosphodiesterase inhibitor (M + B 22948) both increased the field stimulation-induced overflow of radioactivity from atria incubated in [^{3}H]-NA. The explanation for such an effect was again that these drugs may act to inhibit cAMP dependent phosphodiesterase. This, undoubtedly, is a possibility, however, the report ignored the possibility that these drugs may act on the cholinergic nerve terminal to inhibit the release of ACh.

Indirect evidence was obtained in the present study that suggested SNP was not acting directly on the adrenergic nerve and indeed was acting in some way through ACh to promote the field stimulated-induced release of [^{3}H]-NA. Thus, SNP was able to reverse the inhibitory effects of the alpha$_2$-adrenoceptor agonist clonidine but not the inhibitory effects of exogenous ACh on [^{3}H]-NA release. If SNP inhibits ACh release, then such an effect would not interfere with the inhibitory effects of exogenous ACh on [^{3}H]-NA overflow from the sympathetic nerves. In addition, the study examined the ability of SNP to potentiate field stimulation-induced [^{3}H]-NA release in the presence of atropine, which, if used in a high enough concentration should remove the cholinergic-mediated restraint on NA release. SNP was still able to a potentiate [^{3}H]-NA release in the presence of a sub-maximal dose of atropine, however, at a high dose of atropine SNP was unable to further potentiate this response. This indicates that the net effects of SNP and atropine are similar, with atropine blocking the effects of ACh and SNP perhaps blocking the release of ACh. It also indicates that SNP and by extrapolation guanylate cyclase and cGMP have no direct influence on the release of NA from the sympathetic nerve terminals in the rat atria.

Direct evidence for an inhibitory effect of SNP on ACh release from cholinergic nerves was obtained by examining the effects of SNP on the field stimulation-induced release of [^{14}C] from atria previously in [^{14}C]-choline. The results show that SNP inhibited the release of [^{14}C] from field stimulated atria. However, in this experiment it is possible that the inhibitory effect was indirectly due to an increased release of endogenous NA resulting in an inhibition of ACh release due to increased activation of the presynaptic alpha-adrenoceptor on the cholinergic nerve terminal. Therefore, the ability of SNP to inhibit

$[^{14}C]$ overflow was examined again, this time in the absence of noradrenergic influence.

Thus, in the presence of the alpha-adrenoceptor antagonists, prazosin and yohimbine, which potentiated $[^{14}C]$ release, SNP retained its ability to inhibit the release of $[^{14}C]$. This indicates that SNP, presumably by activating soluble guanylate cyclase and increasing intraneuronal cGMP levels inhibits the release of ACh from cholinergic nerve terminals. Normally, activation of guanylate cyclase may occur as a result of muscarinic cholinoceptor-mediated auto-inhibition by ACh, which elevates cGMP in nerves (Kebabian et al, 1975; Drummond, 1984). It is not inconceivable that the presynaptic inhibitory muscarinic cholinoceptor situated in the cholinergic nerve terminal described in this study and by Wetzel et al (1985) is also linked to guanylate cyclase such that activation of the receptor results in activation of the enzyme and increased cGMP levels leading to inhibition of ACh release.

Since the field stimulation-induced release of $[^{14}C]$ was assumed to reflect that of ACh and since field stimulation in atria releases both ACh and NA simultaneously, a response to vagal stimulation, in the whole animal was sought and the ability of SNP to inhibit this chronotropic response was investigated. Stimulation of the vagus nerve resulted in a decrease in heart rate in both pithed and anaesthetised animals. Stimulation-induced bradycardia was greater in anaesthetised animals in which there was a higher basal heart rate due to the existence of a basal sympathetic tone. In pithed animals the cardio-accelerator nerves at C7-T1 in the spinal column could be stimulated selectively by means of a spinal electrode. Such

stimulation produced an increase in heart rate. As before, the effect of SNP on this, sympathetic, response was examined.

In anaesthetised animals stimulation of the vagus nerve produced a decrease in heart rate and this response was unaffected by intravenous (i.v.) administration of SNP. Administration of the alpha-adrenoceptor antagonists prazosin and yohimbine potentiated the vagally-induced bradycardia. This was presumably due to blockade of the alpha-adrenoceptors on the cholinergic nerve terminals that were tonically activated by the existing sympathetic tone. Under these circumstances SNP did inhibit the vagally-induced bradycardia. Thus, in anaesthetised rats SNP could inhibit the response to vagal stimulation in the presence of alpha-adrenoceptor antagonists. The effect was believed to be presynaptic since SNP did not affect the bradycardia which was induced by i.v. administration of ACh either in the absence or presence of prazosin and yohimbine.

The ability of SNP to inhibit the response to vagal stimulation in the presence of the alpha-adrenoceptor antagonists suggests that this effect of SNP is normally masked by the tonic sympathetic inhibitory influence on ACh release. It is perhaps possible that presynaptic alpha-adrenoceptor activation on the cholinergic nerve terminal and SNP affect the same intracellular pathway governing the release of ACh.

If it were true that SNP could inhibit vagal stimulation in absence of sympathetic influence on the release of ACh then in pithed rats, in which there exists no sympathetic tone, SNP should be able to inhibit the response to vagal stimulation in the absence of alpha-adrenoceptor antagonists. The study has shown that, in the pithed rat,

administration of SNP inhibited the response to vagal stimulation at low frequencies of stimulation. In pithed rats, at higher frequencies of stimulation of the vagus nerve, a biphasic effect on heart rate occurred. This response consisted of a decrease in heart rate which was transient and soon developed into an increase in heart rate. The negative component of this response was blocked by atropine and the positive component was blocked by propranolol, suggesting that at this frequency of stimulation of the vagus excitation, of both cholinergic and adrenergic fibres occurred. When such a response occurred SNP only slightly inhibited the cholinergic component but markedly potentiated and prolonged the second sympathetic component. It appeared that this effect of SNP on the adrenergic component was indirect and, like the effect of SNP in isolated atria, was due to inhibition of ACh release. In the pithed rat i.v. administration of NA produced an increase in heart rate and this response was unaffected by SNP. This suggests that SNP did not potentiate the adrenergic component of the response to vagal stimulation by blocking NA uptake or by any post-synaptic effect.

Stimulation of the cardio-accelerator nerves in the pithed rat also resulted in tachycardia. This response was blocked by propranolol, inhibited by clonidine and potentiated by yohimbine. SNP, however, did not affect the response of the heart following stimulation from the spinal column of the cardio-accelerator nerves. Again, this result suggests that SNP was not acting directly on the sympathetic nerves to potentiate the sympathetic component following stimulation of the vagus. Rather, it seems that concomitant stimulation of cholinergic fibres is required before a potentiated sympathetic component can be observed.

This response was therefore, similar to the effect of SNP on [^{3}H]-NA overflow whereby [^{3}H]-NA release was potentiated due to inhibition of ACh release rather than a direct effect of SNP on sympathetic nerves. In the anaesthetised rat it appears that SNP can inhibit the response to vagal stimulation possibly by acting on the cholinergic nerves to inhibit the release of ACh. The effect does not seem to be post-synaptically mediated since the response to exogenous ACh was unaffected by SNP. However, the inhibitory effect of SNP following vagal stimulation in the anaesthetised rat appears to masked by the presynaptic inhibitory effect on ACh release exerted by an existing sympathetic tone. In the absence of such tone in the pithed rat the response to vagal stimulation, at a similar frequency, is inhibited by SNP. At a higher frequency of stimulation of the vagus in the pithed rat it appears that excitation of both cholinergic and adrenergic fibres occurs and under such conditions the sympathetic component is potentiated by SNP. The ability of SNP to potentiate the sympathetic component requires the simultaneous stimulation of cholinergic nerves.

This latter effect of SNP can be repeated by performing experiments on the field-stimulated, spontaneously beating paired rat atria. SNP potentiated the positive, adrenergically-mediated inotropic field stimulation-induced response. Administration of atropine, and consequently the removal of the cholinergic influence, resulted in the a loss of ability of SNP to potentiate the positive inotropic post-stimulus response of the atria. SNP did not affect the basal rate or force of contraction of the spontaneously beating atria. Thus, once more, the ability of SNP to potentiate a sympathetically-mediated response requires a cholinergic influence. It appears that SNP itself acts to remove the cholinergic influence by inhibiting the release of ACh.

The present study therefore, has provided evidence, which suggests that cAMP is involved in promoting the release of NA from adrenergic nerves. The results support the hypothesis that SNP inhibits release of ACh from cholinergic nerves and only indirectly potentiates NA release from sympathetic nerves due to ACh-mediated neuromodulation of NA release. This demonstrates the importance of considering the interactions that occur between the adrenergic and cholinergic nerves in the atria particularly when examining the effect of drugs that influence the release of the transmitters.

THYROXINE-INDUCED CHANGES IN SENSITIVITY

Thyroid hormones are known to influence the sensitivity of sympathetically-innervated tissues to agonists. Whereas most studies investigating the effects of these hormones have examined their effects on postsynaptic receptors, this study sought to determine whether or not chronic T_4 pretreatment affected the sensitivity of presynaptic receptors. The study also re-examined the influence of these hormones on post-synaptic receptor sensitivity.

T_4 administration raised the levels of free T_4 in the plasma and produced changes in the responsiveness of the rat atria to drugs acting both pre-and post-synaptically. This pretreatment raised the resting rate of beating of the isolated atria by approximately 50% and increased the sensitivity of the atria to the beta-adrenoceptor agonist isoprenaline. This supersensitivity was characterised by a potentiation of the maximum response to isoprenaline with no shift in the position of the percentage/dose response curve. The parameter used to determine changes in sensitivity was the rate of beating which is potentiated by the pretreatment. This may explain why at

relatively low doses isoprenaline produced little change in the rate of beating in atria removed from T_4 pretreated animals in comparison with controls.

The study also examined the effects of T_4 pretreatment on the sensitivity of the spontaneously beating paired atria of the rat to the muscarinic cholinoceptor agonist carbachol. The results from this part of the study are complex. Pretreatment with T_4 produced no displacement in the percentage/dose response curve for carbachol. When the results are expressed as the change in rate ($\triangle$B.P.M.) produced by the drug it appears that T_4 pretreatment increased the maximum response to carbachol in atria from pretreated rats. This, however, is misleading since the inherent rate of beating in atria from T_4 pretreated rats is higher than in control atria. It appears that the highest dose of carbachol (10^{-5} M) is sufficient to cause maximum inhibition of the rate of beating of the atria despite this inherent higher rate of beating in the atria from pretreated rats. Unlike the situation with isoprenaline where the increased inherent rate of beating appeared not to mask any changes in sensitivity particularly at high doses, in this case, with carbachol it is difficult to interpret the effect of T_4 pretreatment on the sensitivity of the post-synaptic muscarinic receptor.

One possible means of overcoming this problem would be to examine the inotropic effects of agonists on paced left atria. This method was employed to determine the effect of T_4 pretreatment on the sensitivity of the post-synaptic alpha$_1$-adrenoceptor in the atria. Chronic pretreatment with T_4 resulted in a decreased sensitivity of the atria to the alpha$_1$-adrenoceptor agonist phenylephrine. The inotropic response of the paced atria to phenylephrine could be inhibited by the alpha$_1$-

adrenoceptor antagonist prazosin. The results of this study confirm that hyperthyroidism increases the sensitivity of the heart to beta-adrenoceptor agonists and decreases the sensitivity to alpha-adrenoceptor agonists as has been shown previously (Kunos, 1977; Gibson, 1981; McNeill, 1987). The observation that T_4 pretreatment potentiated the inherent rate of beating of the atria suggest the actions of the thyroid hormones are complex and may exert intracellular effects in addition to those at the level of the receptor (Tse et al, 1980; McNeill, 1987).

The study also compared responses to field stimulation in control atria and atria from pretreated rats. T_4 pretreatment potentiated the positive noradrenergic component of the inotropic post-stimulus response to field stimulation in atria from pretreated rats. In addition, the negative cholinergic component of the inotropic post-stimulus response was inhibited by T_4-pretreatment. These effects may be due to supersensitivity of the post-synaptic beta-adrenoceptors and/or subsensitivity of the post-synaptic muscarinic cholinoceptors. It is also possible, however, that enhanced effectiveness of the positive inotropic response is due to a subsensitivity of presynaptic alpha-adrenoceptors on the adrenergic nerve terminal which would result in a decreased ability to inhibit NA release. The study has shown that T_4 pretreatment reduced the ability of clonidine to inhibit the positive inotropic post stimulus response in the presence of atropine and also reduced the ability of clonidine to inhibit the overflow of $[^3H]$ in atria previously incubated in $[^3H]$-NA. T_4 pretreatment did not, however, affect the ability of ACh to inhibit the field-stimulated-induced overflow of $[^3H]$ from atria incubated in $[^3H]$-NA.

The possibility remains that T_4 pretreatment exerted a toxic effect on nerve terminals by removing the inhibitory capacity of presynaptic receptors, this is an unlikely explanation of the results, since the ability of clonidine to inhibit responses was affected while that of ACh was not. A non-specific effect on nerve-endings would have been more likely to affect the inhibitory capacity of both drugs similarly. T_4 pretreatment did not appear to significantly affect the ability of either clonidine or ACh to inhibit the field-stimulated induced release of [^{14}C] from atria previously incubated in [^{14}C]-choline. Although further investigation is merited it is possible that the effects of T_4 are confined not only to the sympathetic nerves but perhaps also only the inhibitory adrenoceptors. Speculation aside, the results of the study confirm those of previous work in the mouse vas deferens where T_4 pretreatment also resulted in a subsensitivity to clonidine (Forsyth et al, 1986).

The results also cast some doubt on the hypothesis of Kunos (1977) which suggests that there exists a single adrenoceptor that can interconvert to the alpha or beta state according to the circulating levels of T_4. The results of this study suggest that hyperthyroidism not only affects postsynaptic receptors but perhaps also presynaptic receptors. Thus, the tachycardia seen in hyperthyroidism may be due not only to post-synaptic supersensitivity of beta$_1$-adrenoceptors but also to subsensitivity of presynaptic alpha$_2$-adrenoceptors, the latter resulting in a diminished restraint on NA release. However, the possibility remains that interconversion between alpha- and beta-adrenoceptors may occur not only post-synaptically but also presynaptically on the nerve terminal.

In conclusion, the results of the study have confirmed that complex presynaptic interactions exist between the sympathetic and cholinergic nerves in the heart. The speculative hypothesis of Rand et al (1975, 1980) whereby ACh and NA exert autoinhibition and mutual cross inhibition of transmitter release is confirmed. The study has also implicated cAMP and cGMP in modulation of the release of NA and ACh. In addition, it has demonstrated the necessity to consider the complex interactions between the two sets of nerves when examining the role of cyclic nucleotides in transmitter release. Finally, the effect of chronic T_4 pretreatment on the response of the heart to pre-and post-synaptic agonists were examined. The study provided evidence that hyperthyroidism produced sensitivity changes not only post-synaptically but also pre-synaptically.

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