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Volume regulation in pregnancy: hormonal modulation of the atrial volume receptor reflex

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

Pregnancy is characterized by a new steady state where blood volume expands without activation of homeostatic mechanisms. The atrial volume reflex, one of the mechanisms involved in volume homeostasis in the body, is suppressed during pregnancy. This contributes to expansion of the plasma volume, and the adaptation of the circulatory system to the changing needs of the fetus.

Many of the cardiovascular alterations with occur during pregnancy are thought to be the result of changing levels of steroid and peptide hormones. We hypothesized that the modulation of the reflex originates with altered sensory receptor function. Our experiments confirmed this, as pregnancy led to an inhibition in the response of the high-frequency subset of receptors to atrial distension.

We proposed that the alteration in atrial receptor reflex function in pregnant animals could be reproduced in rats administered the progesterone metabolite pregnanolone (chronic and acute), ADM, or NO. While the effects of ADM and NO may be confined to central modulation of the reflex, chronic administration of pregnanolone mimicked the effects of pregnancy on atrial receptor function.

In conclusion, the changing hormonal milieu of pregnancy contributes to the suppression of the atrial volume receptor reflex at both central and peripheral levels. This offers some explanation as to the mechanism by which blood volume is permitted to expand in pregnancy without initiation of the normal corrective homeostatic response.

TABLE OF CONTENTS PAGES

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

LIST OF ABBREVIATIONS

AII	Angiotensin-II
ACTH	Adrenocortico
ANF	Atrial natriuretic factor
AP	Action potential
AR	Atrial receptor
AVP	Vasopressin
AVR	Atrial volume receptor
cGMP	cyclic guanosine monophosphate
CNS	Central nervous system
CRF	Corticosteroid-releasing factor
CVLM	Caudal ventrolateral medulla
d	dorsal mediocellular
ECFV	Extracellular fluid volume
ECG	Electrocardiogram
GFR	Glomerular filtration rate
HF	High frequency
i.m.	intramuscular
Inactin	Ethyl-(1-methyl-propyl)-malonyl-thio-urea
i.p.	Intraperitoneal
i.v.	Intravenous
ivc	Inferior vena cava
K	Potassium
LAP	Left atrial pressure
LF	Low frequency
map	medial anterior parvocellular
mcp	medial caudal parvocellular
mlp	medial lateral parvocellular
mmp	medial medial parvocellular
Na	Sodium
NO	Nitric oxide
nNOS	neuronal nitric oxide synthase
NTS	Nucleus of the solitary tract
OT	oxytocin
p	posterior
P_{osm}	plasma osmolality
Pregnan	3α-OH-5β-pregnan-20-one (pregnenolone)
pv	Periventricular
PVN	Paraventricular nucleus
RA	Rapidly-adapting
RAAS	Renin-angiotensin-aldosterone system
RSNA	Renal sympathetic nerve activity
RVLM	Rostral ventrolateral medulla
SA	Slowly-adapting
s.c.	Subcutaneous

Somnotol	Sodium pentobarbital
SNAP	S-Nitroso-N-acetyl-D,L-penicillamine
SNS	Sympathetic nervous system
TRH	Thyrotropin-releasing hormone
VLM	Ventrolateral medulla

CHAPTER 1.
INTRODUCTION

1.1 THE ATRIAL VOLUME RECEPTOR REFLEX: AN OVERVIEW

The effective circulating volume- or the amount of blood which does the 'real work' of the circulation, carrying oxygen and nutrients to the cells and removing the byproducts of metabolism, is defined by the interplay between arterial pressure and plasma volume (58). These two cardiovascular parameters- pressure and volume- must therefore be kept within a tightly regulated range (18). The cardiovascular system has evolved a simple yet effective system of stretch-sensitive receptors to carry out this regulatory function (134,172). Depending on their location in the circulatory system, these mechanoreceptors sense changes in pressure (located in the high-pressure carotid artery) or volume (located in the low-pressure venoatrial junction). Through activation of specific reflex pathways, the variables of pressure and volume are kept within the range which promotes a maximally effective circulating volume (18,82,134).

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The atrial volume receptor reflex is one of many the mechanisms which together act to maintain volume homeostasis in the body (72,128) (Fig. 1). Initiated by the stimulation of receptors which sense an increased return of blood to the heart and processed by specific areas of the brainstem and hypothalamus, the product of reflex activation is neural and humoral output to the kidney which results in fluid loss and a return to volume homeostasis (72,74,128,134). Under normal conditions, blood volume must be continually adjusted to fill the changing vascular bed; however there are physiological conditions where expansion of the blood volume is a physiological necessity. In pregnancy, expansion of the circulating volume is essential for the development and delivery of a healthy fetus (18,50). There is evidence to suggest that the atrial volume

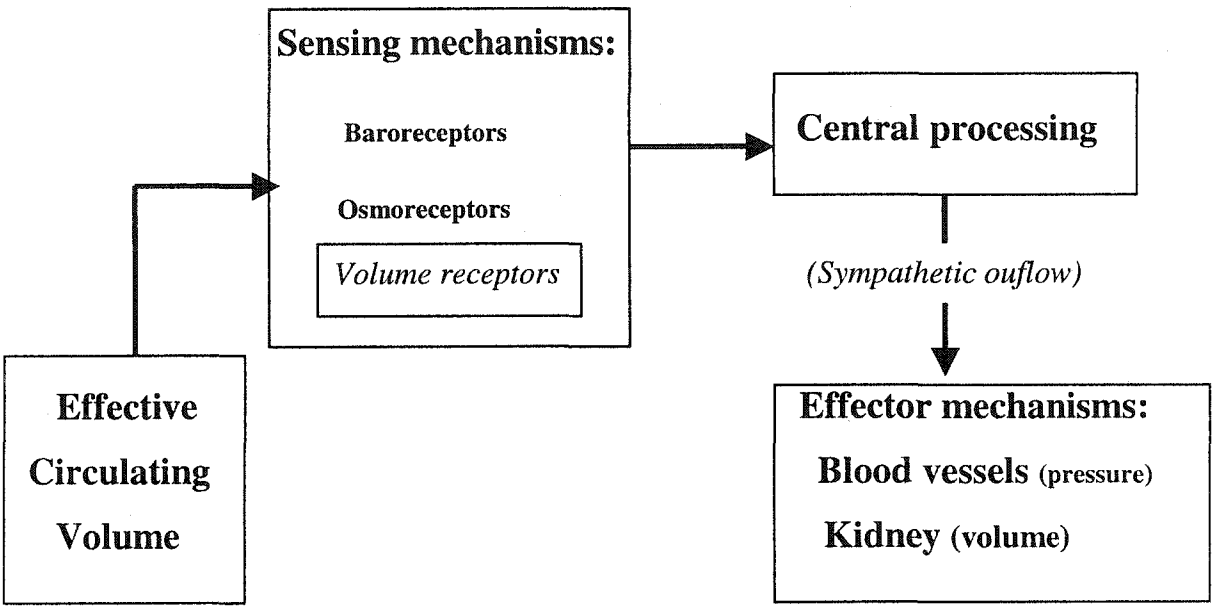


Figure 1. Summary of homeostatic mechanisms responsible for monitoring components of the effective circulating volume. Various receptor systems sense changes in effective circulating volume, which is subsequently communicated to areas of central processing and finally to two main effectors, blood vessels and kidney, which control pressure and volume respectively.

receptor reflex is attenuated in pregnancy to allow this expansion to occur (42,43,84,107,109). This discussion will relate to the function of the atrial volume receptor reflex in pregnancy and the role that hormones may play in this modulation.

1.1.1. Atrial receptor location and morphology

Over 100 years ago, Von Bezold and Hirt were among the first to suggest the presence of stretch-sensitive receptors in the heart which, by sensing changes in filling, could affect the volume of the circulatory system (203). The presence of these receptors was not confirmed until 50 years later, when Nonidez located receptor endings located at the venoatrial junctions of several species of animal (161). Subsequent histological research revealed two structures innervating the junctions of the great veins and atria: the complex unencapsulated endings, and the endocardial nerve network (154) (Fig.1).

The first detailed examination of complex unencapsulated endings was carried out by Paintal in 1953 (164). These receptor endings, also called 'flower sprays' due to their appearance, are spatially arborizing structures that vary greatly in form and size (22,128,141). Each nerve fiber is attached to between one and 11 endings in one region of the atrium, and each ending lies along the plane of the endocardium, though some are found over the epicardial surface (22,128,141). These receptor endings are attached to myelinated nerve fibers that conduct at velocities of 8-32 m/s, and mainly innervate the junctions of the great veins and atria, with roughly 150-300 receptors found in the heart (128).

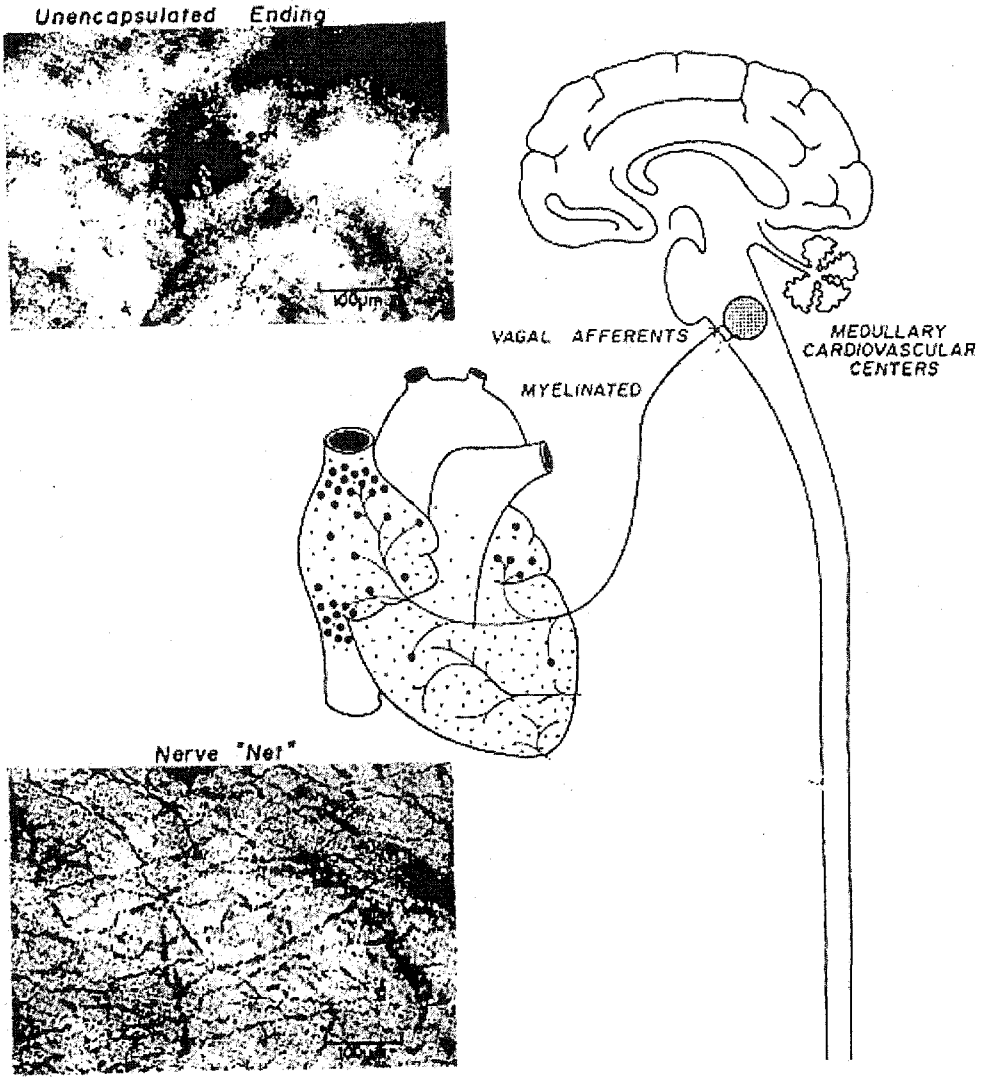


Figure 2. Atrial receptor morphology and central connections. Upper picture shows morphology of unencapsulated ending, lower shows that of unmyelinated nerve net. Drawing shows afferent connection with centers in brainstem. Illustration adapted from Shepherd and Vanhoute (1979) (191).

Jarisch and Zotterman first described innervation of the heart by nonmedullated fibers; this was localized to the atrium by Coleridge et al. twenty years later (26,95). Unlike the myelinated fibers, no receptor ending was associated with these thin fibers; rather, the collective structure was assigned the name the endocardial end-net. The end-net appeared, after supravital staining of the endocardium with methylene blue, as a branching, fine network of fibers interspersed with numerous nodal points (Fig. 1). This network could be seen over the entire endocardial surface of the heart, but its origin was uncertain: Shepherd surmised the end-net as subserved by nonmedullated afferent fibers that would conduct at velocities of 2.5 m/s or less (47). Most of these C-fibers appear to exit the heart at the junction of the superior caval vein and the right atrium, indicating that, though they lack the specific receptor ending associated with myelinated fibers, they innervate the same area of the atrium (201).

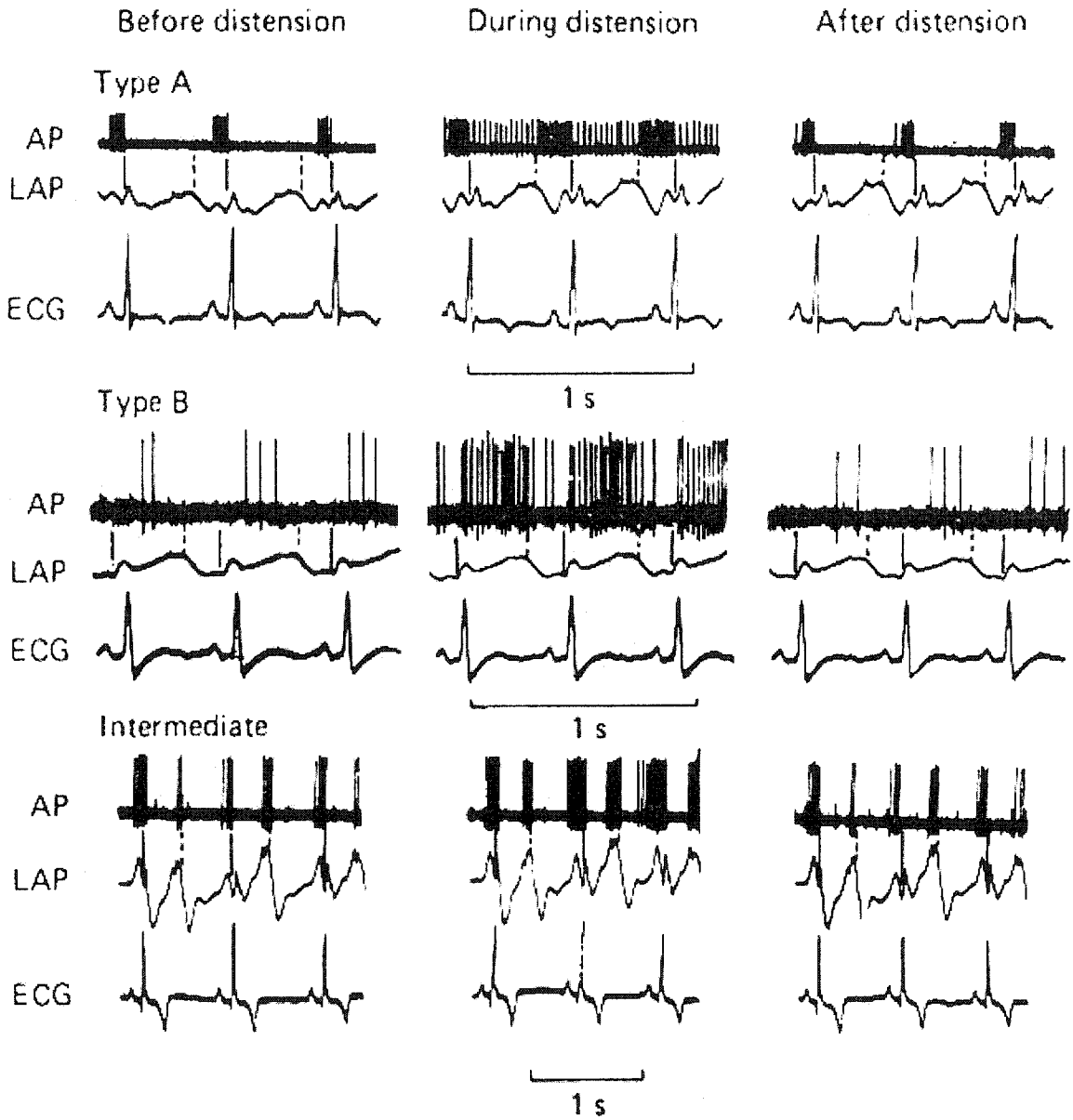


Figure 3. Discharge characteristics of the three Paintal types of myelinated atrial receptors in response to balloon distension at the pulmonary vein- left atrial junctions. Upper panel record of actions potential (AP), middle panel record of left atrial pressure (LAP), and bottom panel record of electrocardiogram (ECG). Continuous line indicates the a wave and the interrupted line the v wave of the atrial pressure pulse. Adapted from Hainsworth et al. (1987) (75).

1.1.2. Atrial receptor discharge characteristics

The first evidence that the complex unencapsulated endings found in the venoatrial junctions of large animals such as cats and dogs were in fact mechanoreceptors was presented by Coleridge et al. in 1957 (26). In this elegant study, histological and electrophysiological techniques were combined to show that the myelinated nerve endings which innervated the endocardium of the atrium, and the mechanically sensitive receptors which fired with an atrial discharge pattern in the vagus nerve, were one and the same (26). A few years earlier, Paintal (164) had characterized the discharge pattern of these receptors, now shown to be myelinated atrial receptors (Fig. 2). The receptors were characterized according to their pattern of discharge in relation to the atrial pressure wave: type A discharging with the 'a' wave of atrial systole, type B with the 'v' wave of atrial filling, and type AB (or intermediate) discharging with both atrial events (164). A knowledge of the basic principles of mechanosensitivity combined with further, detailed studies in dogs and cats examining receptor characteristics both in vivo and in vitro, allowed these receptors to be characterized as slowly adapting mechanoreceptors (6,103).

Characterization of the discharge properties of unmyelinated atrial receptors was carried out by Thoren (199,201) who showed that these slowly conducting fibers, unlike their myelinated counterparts, did not have a spontaneous discharge which coincided with parts of the atrial pressure wave: most were silent or had irregular discharge until stimulated by stretch. Stimulated discharge, though higher in frequency, was often irregular and of short duration. These receptors were also found to have higher thresholds and lower discharge frequencies than the myelinated endings (199). In larger

mammals such as cats and dogs, the unmyelinated fibers were frequently seen to fire with the v-wave of the atrial pressure pulse, indicating these receptors respond mainly to atrial distension and not to atrial contraction (201).

Thus in larger mammals, such as cats and dogs, two different atrial receptors, innervating the same area of the heart but distinguished by morphology and function, were characterized. Atrial receptors in smaller mammals were not studied until Thoren's work on anesthetized, ventilated rats in 1979 (199). The most striking result of this work was the observation that the rat heart possessed no receptors with myelinated afferents (199). All fibers localized to the atrium conducted at velocities of 2.5 m/s or less, yet there was a clear sub-grouping of receptors into two types: those with an activated maximal discharge of higher frequency (>25 Hz) and regular pattern that often persisted for the length of the stimulus, and those with an activated maximal discharge of lower frequency (<25 Hz) and irregular pattern that lasted for only a few seconds. The high frequency (HF) receptors had a lower response threshold (2.5-5mmHg), and had similar properties to the medullated fibers in larger mammals, while the low frequency (LF) receptors had a much higher response threshold (5-9mmHg) (199). This subdivision of nonmyelinated atrial receptors into two subtypes was consistent with studies on the properties of aortic baroreceptors in the rat which demonstrated that, again, though the majority of afferent output was carried by nonmyelinated fibers, similar low and high frequency receptors would be identified (199,200). This suggests that afferent output from mechanoreceptors in the cardiovascular system is unmyelinated in smaller mammals, perhaps because

signals need travel over shorter distances to reach their destination in the brain; some time delay is therefore more acceptable than it would be in larger mammals (199).

Though atrial receptors in both larger and smaller mammals could be clearly subdivided based on differing response thresholds and discharge properties, it was unclear whether subgroups of both myelinated and unmyelinated fibers actually arose from different receptors, or from one receptor with characteristics dependent on location and atrial mechanics (5,6,69,102). It was suggested that type A, B and AB discharge patterns recorded in myelinated fibers in larger mammals were the result of alternate locations of a single type of atrial receptor: the type A pattern arose from a receptor located in tissue that stretched in response to atrial contraction (i.e. within the atrium), the type B pattern arose from a receptor located in tissue that stretched in response to filling (i.e. at the venoatrial junction), and type AB at some location in between (6,102). This theory was supported by an experiment carried out by Arndt et al. in which sinusoidal length changes were applied to strips of cat atrial tissue containing stretch receptors (6). The response of a receptor to this controlled stretch was then compared to its response within the circulation: it was found the responses of type A and B receptors were identical in that their primary stimulus was change in length, and they showed the same degree of adaptation. Thus different discharge patterns in myelinated fibers innervating the atrium arose from one single receptor type (6). In addition, it was suggested that each discharge pattern arose from receptors operating in different parts of the stimulus-response curve: type A arose from receptors operating in the saturation range of the curve (high frequency bursts), type B arose from those operating in the linear part of the curve (activity varied

directly with degree of atrial distension) (6). This work was supported by later work conducted by Kappagoda et al., where it was found, through precise localization of different receptor types, that the type A discharge pattern arose from receptors located near the venoatrial junction (where torsion due to contraction is greatest), and type B from receptors located more distally in the lateral walls of the atrium and in the veins. Discharge patterns could be converted from one type to another by changing atrial dynamics (i.e. through hemorrhage or atrial infusion) (102).

Similar studies were carried out using segments of rat superior vena caval tissue to determine whether HF and LF responses of unmyelinated fibers were also the product of receptor properties or receptor location (152,153). Mifflin et al. exposed the segments of vessel to pulsatile pressure waves superimposed on lower frequency components designed to mimic changes in venous return (i.e. atrial filling) (152). The receptors maintained their unique properties in responding to the static stretch, yet now these different properties could be attributed to the rate of adaptation of the receptors to the stimulus. The high frequency receptors appeared to function as type B receptors in larger animals, in that there was a steady-state component to their discharge which was directly dependent on the mean pressure level. Due to their slow rate of adaptation to the stimulus, these receptors were classified as 'slowly-adapting'(SA). The low frequency receptors behaved very differently, as the steady state component of their discharge was very low frequency and was not dependent on mean pressure level. Rather, they responded to the dynamic component of the pressure wave. Thus these receptors responded to an increase in pressure with an irregular discharge that returned to the pre-

increase level within 60 seconds, indicating their function would be to transiently signal a change in mean pressure. Due to the rapid rate of adaptation to stimulus, these receptors have been classified as 'rapidly-adapting'(RA) (152).

Although the peripheral mechanoreceptors monitor changes in the physical status of the organ they innervate, the larger physiological purpose of atrial receptors is to inform the brain of an increase in circulatory volume through a stretch of tissue in response to increased venous return to the heart. *The most important component of atrial receptor signaling is the average number of spikes/sec communicated to the brain via the afferent fibers* (152). Thus the purpose of different types of atrial receptors, HF (or SA) and LF (or RA), is that there will be differences in the information each class of receptor sends to the brain (152).

1.1.3. Atrial receptor reflex: Afferent pathway and central processing

Nearly all sensory afferent fibers travel upwards to their connections in the central nervous system via the vagus nerve (126). The actual cell bodies of these neurons are positioned just outside the brainstem in the nodose ganglion (60,126). Through this pathway, atrial receptors convey information concerning the degree of atrial filling to sensory transmission neurons in the brainstem (193) (Fig.3)(14).

When the signal carried by afferent sensory fibers arrives in the central nervous system, it becomes part of what is most conveniently called the 'central autonomic network' (14). This is a group of areas in the brain which communicate closely with one another, and

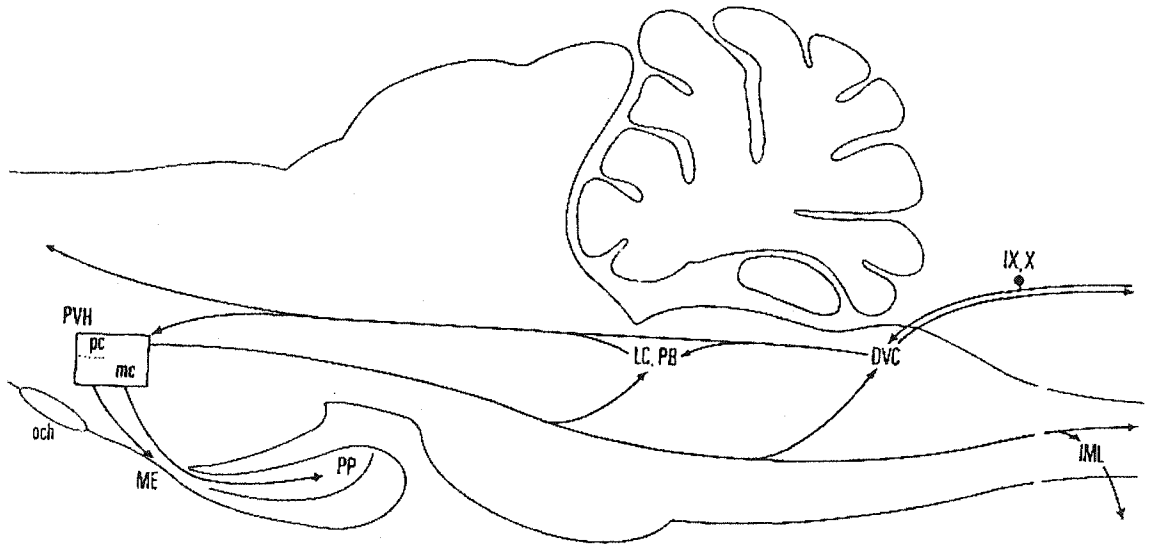
through cooperative action, coordinate reflex adjustments of autonomic responses (14). It is this community of nuclei in the brainstem, hypothalamus and other areas which is critical for the maintenance of homeostasis (14). The central autonomic network is most clearly represented as a series of individual, parallel reflex pathways. Due to the involvement of higher centers such as the hypothalamus, these pathways can be integrated into specific patterns of response which maintain homeostasis in the cardiovascular system despite changes in the internal environment (14).

Central autonomic network processing of afferent signals originating from the atrial mechanoreceptors, first occurs in the nucleus of the solitary tract (NTS) (36,187,192). This longitudinal nucleus is located in the dorsomedial medulla oblongata and receives information from nearly all the visceral afferents in the body, including those from arterial baroreceptors, chemoreceptors in the great vessels, and atrial volume receptors (192)(Fig. 3)(36,168). It has been shown that vagal afferents arriving in the NTS terminate in the intermediate and caudal regions of the nucleus (169). This is supported by studies showing the activity of neurons in these areas vary during atrial pulsation and blood volume infusion (20,169). Processing in the brainstem has two functions: 1) to initiate medullary reflexes, for second-to-second control of cardiovascular homeostasis, and 2) to provide sensory input to other, higher levels of the central autonomic network (36,63).

While the NTS is the nucleus in the brainstem which receives all of the cardiovascular afferent input, the ventrolateral medulla (VLM) is one of the areas responsible for

communicating the results of this input to the body (36). One area in particular, the rostral ventrolateral medulla (RVLM), contains a heterogenous mix of presympathetic neurons which communicate directly with preganglionic sympathetic neurons in the spinal cord (36,63). The RVLM is involved in controlling the autonomic outflow which is the end result of most cardiovascular reflexes (36,62). Using anatomical tracing methods, it has been shown that NTS neurons make both monosynaptic and multisynaptic connections with neurons in the ventrolateral regions of the medulla oblongata; thus there is a great deal of communication between the 'receiving station' (the NTS) and the 'departing station' (the RVLM) (98). The most clearly defined of these pathways involves another nucleus in the caudal part of the ventrolateral medulla (CVLM) (36). It has been shown that an inhibitory pathway from the NTS to the RVLM acts through neurons in the CVLM. The NTS activates the CVLM, which in turn inhibits presympathetic neurons in the RVLM via the release of the neurotransmitter GABA (63) (Fig. 3)(36). The involvement of this pathway in the atrial volume receptor reflex is further indicated by a study showing that activation of vagal afferents elicits long-lasting, late-onset inhibition of RVLM neurons (169). Thus, through this simple pathway in the brainstem, many cardiovascular reflexes are effectively processed and homeostasis is maintained. It is believed that baroreceptors from the arterial side of the circulation signaling changes in pressure, and mechanoreceptors from the venoatrial junctions signaling changes in circulatory volume, are both processed through similar pathways in the brainstem (187). Neurons of the NTS receive convergent information from aortic baroreceptors and vagal afferents, and this convergence has also been described in the RVLM (63,208)

A.



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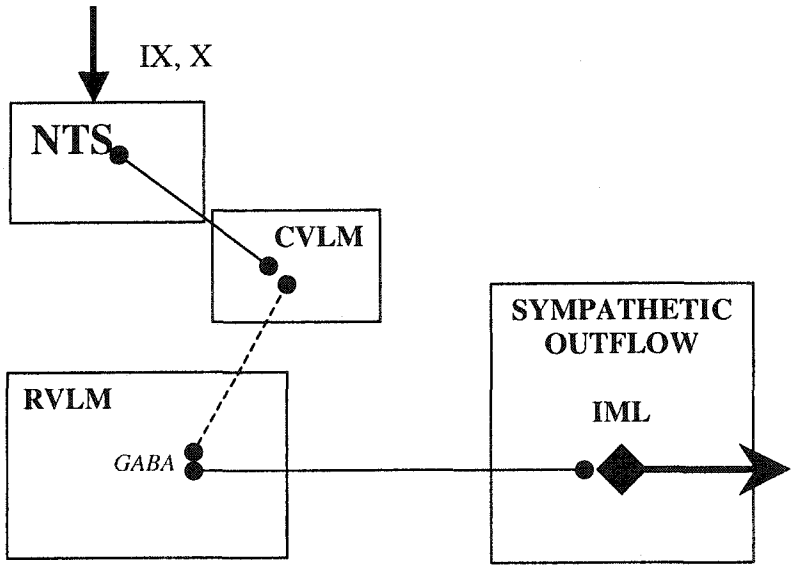


Figure 4. Connections of brainstem with higher (A) and lower (B) centers. (IX, X = sensory afferent nerves, DVC = dorsal vagal complex, LC = locus coeruleus, PB = parabrachial nucleus, pc, mc = parvocellular, magnocellular subdivisions). Illustration adapted from Swanson and Sawchenko (1980) (197).

It is important to note that, although central reflex pathways of these two afferent signals may closely overlap, the autonomic outflow they trigger may be quite different. This is reflected in the structure of the RVLM: distinct areas of presympathetic neurons are arranged topographically according to their functional effects (62,98,187).

Although the brainstem, mainly through the RVLM, can effectively carry out cardiovascular reflex processing, it is essential that higher centers in the brain are also involved (63,195). These higher centers integrate individual reflex responses into patterns of outflow that are appropriate to the conditions of the internal environment of the body (98). This allows cardiovascular reflex pathways to become more dynamic, changing as the needs of the body change.

The paraventricular nucleus (PVN) of the hypothalamus is one of the key areas involved in modulation of cardiovascular reflex processing (16,36). This nucleus is located in the lateral hypothalamus, bordering either side of the third ventricle (16). The PVN contains two main subgroups of specialized neurons: those which synthesize, secrete and release hormones into the circulation, and those which alter the activity of autonomic premotor nuclei in the brainstem (36,196). The PVN thus effectively coordinates central control of autonomic function (14,76,114,185). Within the nucleus there is a topographic segregation of pathways which have output to the median emininence, the posterior pituitary, autonomic relay centers in the brainstem, and to the spinal cord (14,114,196) (Fig. 3).

These topographically separate cell populations have been clearly demarcated and identified as subnuclei in a series of studies carried out within the last two decades (4,114,196). The PVN can be loosely subdivided into three zones on the basis of cell size and efferent projections: 1) the magnocellular zone, which consists of large cells which project to the posterior pituitary, 2) the parvocellular zone, which consists of small cells which project mainly to the median eminence and 3) the mediocellular zone, which consists of medium-sized cells which projection to spinal cord, and to autonomic relay centers in the brainstem (113,114). Each of these zones consists of subnuclei characterized by slight differences in projection pattern or neurochemical characteristics, though they are not exclusive; there is always some overlap between different subdivisions (114,196,197).

The magnocellular zone comprises roughly 20% of the cells of the entire nucleus (114). The neurons in this region are large, neurosecretory cells, each of which sends a single axon down into the neural lobe of the pituitary (124). The neurons secrete either oxytocin or vasopressin, and, though there are no clear subdivisions within the magnocellular region, it is patterned as a core of vasopressinergic neurons surrounded by oxytocinergic neurons (16,114). When neurons in the magnocellular region are stimulated, the cell membrane is depolarized, and the subsequent calcium influx through voltage-gated channels allows secretion of the peptide hormone from the nerve terminal (16,34).

The parvocellular region of the PVN comprises just over half the neurons in the nucleus (114). These are small neurons that mainly project to the external zone of the median eminence (114,196). A great number of these neurons synthesize corticotrophin-releasing factor (CRF), and others secrete thyrotropin-releasing factor (TRH) (35). On the basis of cell packing density, Kiss has further divided this region into five subdivisions: periventricular (pv), medial anterior parvocellular (map), medial medial parvocellular (mmp), medial lateral parvocellular (mlp) and medial caudal parvocellular (mcp) (114).

The remaining, medium-sized neurons of the PVN make up the mediocellular region (114). These neurons have often been classified together with the parvocellular region, but they may be clearly distinguished from the parvocellular area on the basis of their efferent projections (113,114). These neurons have been generally described as pre autonomic neurons: the dorsal (d) mediocellular region has direct projection to the spinal cord (terminating in a complex pattern in the intermediolateral cell column), while the posterior (p) region projects both to the spinal cord and to the brainstem (35,114).

The magnocellular, mediocellular and parvocellular regions of the PVN are quite clearly physically and functionally distinct, and the sensory information they receive and process remains within these topographically separate pathways (147). Afferent signals arising from the cardiovascular system travel upwards to the PVN via a complex system of noradrenergic fibers which mainly originate in the brainstem (16,177,185,197). The A1 noradrenergic cell group in the ventrolateral medulla, and the A2 cell group in the NTS

are major sources of afferent input to each region of the PVN (16,124). It has been shown that both A1 and A2 neurons project to nearly all regions of the parvocellular and mediocellular regions, with an emphasis on the medial and dorsal areas (197). A1 fibers synapse preferentially on vasopressinergic neurons in the magnocellular region (16,36). Thus only the ventrolateral medulla mediates cardiovascular influences upon vasopressin cells (124). The A1 region also projects heavily to the locus coeruleus (A6 cell group), which in turn has output to both the periventricular region of the PVN and to the spinal cord (33,185). It is significant to note that, as described above, there is a strong reciprocal connection between the 'receiving station' in the brainstem, and the PVN, which allows a bi-directional communication in the processing of cardiovascular afferent signals (177,197).

Activation of atrial receptors via stretch of the caval-atrial junction is transmitted, via the vagus nerve, to neurons of the caudal division of the NTS (185). The information is then communicated either directly or indirectly (via the PVN) to the RVLM, which in turn projects directly to the sympathetic preganglionic motoneurons of the thoraco-lumbar spinal cord (8,185). In the PVN, atrial receptors influence activation of parvocellular, mediocellular and magnocellular regions. In the magno division, there is a decrease in the activity of AVP neurons with no effect on OT neurons, an effect which is effectively eliminated by vagotomy (68). Thus atrial receptor activation leads to a suppression of the secretion of vasopressin from the posterior pituitary, which in turn allows water to be excreted from the kidney. Atrial stretch also elicits widespread activation in the parvocellular and mediocellular divisions in response to distension of the venoatrial

junction (42). Activation of the atrial receptors thus affects activity in autonomic centers in the brainstem, and consequently in the spinal cord, both directly and via modulation of parvocellular and mediocellular subdivision activity. The role of the mediocellular subdivision is further supported by evidence indicating non hypotensive hemorrhage alters the activity of spinally projecting neurons of the PVN (9). A link has also been established between the volume receptor reflex and the hypothalamo-pituitary-adrenal axis: distension of the venoatrial junction leads to an increase in ACTH levels, and this response can be blocked by vagal cooling (71,101).

As the result of reflex activation is a decrease in sympathetic outflow to the kidney, and subsequent water loss, it may be supposed that atrial receptor activation of central nuclei leads to an inhibition of sympathetic premotor neurons in the RVLM and spinal cord (129). While there is no direct evidence identifying the connections between these nuclei as excitatory or inhibitory, the baroreceptor reflex pathway may be safely taken as a model for atrial receptor reflex processing due to the similarity between the pathways (185). Activation of baroreceptors via an increase in arterial pressure stimulates neurons in the caudal region of the NTS (36,118). The signal is then sent to the CVLM, which in turn inhibits neuronal activity in the RVLM via an inhibitory, GABAergic projection (36). In addition, there is a direct projection from the NTS to the PVN (36). Neurons of the PVN are mainly inhibited by baroreceptor inputs, as both direct and indirect (via activation of the RVLM) sympathetic outflow is suppressed with baroreceptor activation (36,118,173). As the purpose of atrial receptor activation is similarly a decrease in sympathetic outflow (specifically to the kidney), it is reasonable to accept the

baroreceptor pattern of central processing as a working model for the atrial receptor reflex.

1.2 REGULATION OF BLOOD VOLUME

Regulation of fluid volume in the body is essential for survival (186). Roughly 60% of total body weight may be attributed to water, which translates in volume to 40L (58). This 40 L of water is distributed throughout different compartments of the body, and maintenance of specific volumes of fluid in these compartments is essential for the continued survival of the organism (59) (Fig. 4). Thus it is important to note that blood volume is not controlled solely by intake or excretion of fluid from the body, but by redistribution of fluid amongst these inner compartments (59).

1.2.1 Body fluid volume and distribution

Approximately 60% (or 24L) of the total body water is found inside the cells (intracellular), while 40% of this volume (16L) is outside of the cells (extracellular). Thus, the cells can be viewed as a single compartment which is separated from another compartment by a semipermeable membrane. Furthermore, the extracellular compartment can be subdivided into fluid found between the cells (interstitial fluid (ISF): 11.2L), and fluid found within the vascular system (plasma volume (PV): 3.2L). Thus the cells communicate directly with the ISF, which is separated from the plasma volume by vessel walls. The plasma volume (less than 10% total body water) can be further subdivided into reservoirs of fluid, such as that found in the splanchnic venous circulation and fluid which is actively circulated (67). This plasma, which circulates throughout the

body and distributes the materials necessary for survival to the cells, is therefore called the 'effective circulating volume' (59). Thus for maximal cardiovascular performance, it is this volume which must be actively monitored by a series of parallel regulatory systems (25) (Fig. 4)(31).

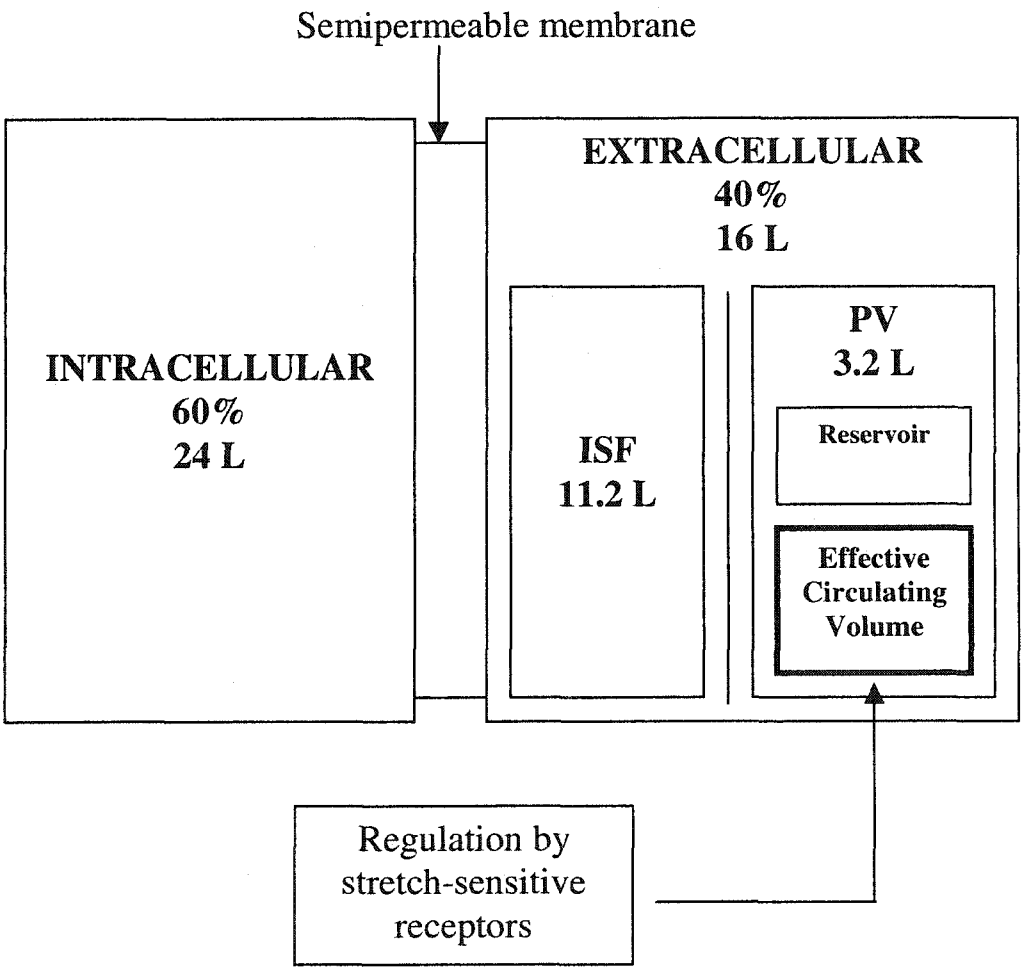


Figure 5. Distribution of body water between physiological compartments. Total body water is divided into intracellular and extracellular compartments, with the extracellular compartment further subdivided into the interstitial space and plasma volume. Plasma volume is defined by fluid held within the splanchnic reservoir, and the effective circulating volume that fills the vascular spaces.

1.2.2 Overview: Regulation of plasma volume

As fluid is in osmotic equilibrium between the two major extracellular and intracellular compartments, the extracellular fluid volume (ECFV) is fundamentally dependent on the relative quantities of Na in each area (25,31). Thus as Na is pumped out of the cells by the Na/K-ATPase in the cell membrane, water will passively follow, resulting in an expansion of the ECFV (25,31). The amount of fluid in each sub-compartment of the extracellular area is further controlled by a series of forces that act across the capillary wall. Fluid movement in/out of the vessels (and therefore the plasma volume) is primarily governed by hydrostatic and colloid osmotic forces. Should fluid in the vessels increase, for example, the hydrostatic pressure would increase while the colloid osmotic pressure decreases, allowing water to move passively out of the vessels into the interstitial space (59). Thus this shift in fluid between the two compartments of the extracellular space allows the vital effective circulating volume to be maintained at an optimal level while the ECFV remains constant (59). In this way, the fluid shift between compartments is the first line of defense in protection of the effective circulating volume (59). While this ability to shift fluid into/out of the vascular compartment corrects for disturbances in plasma volume to some extent, the ultimate responsibility for maintenance of fluid volume and composition lies with the kidney (66,186).

1.2.3 Regulation of kidney function

The kidney plays an essential role in regulating plasma volume in that it maintains constant volume and electrolyte composition of the circulating fluid in the face of hourly variations in salt and water consumption (1,66,70). The plasma is continually filtered by

the kidney; that which is needed by the body is reabsorbed and the remaining components are excreted (25). The neurohormonal regulatory mechanisms which monitor the plasma volume, ensuring it is kept at a level optimal for cardiovascular performance, determine what fraction of the plasma is filtered by the kidney, what fraction of the filtered material is reabsorbed and thus, what fraction is excreted (186).

1.2.4 Regulation of kidney function: filtration

Roughly 25% of cardiac output at any given time is directed towards the kidney (191). The renal artery enters the kidney and subdivides into a series of branches which ultimately supply the functional unit of the kidney: the nephron (25). At its smallest branching point the renal artery becomes an afferent arteriole which forms the capillary bed of the glomerulus, and then reconverges to form the efferent arteriole which plasma is cleared. The relative magnitudes of the four forces governing fluid movement across the glomerular capillary wall determine the filtration rate (GFR) (25). Two forces push/draw fluid out of the vessels (hydrostatic pressure in the capillary, osmotic pressure in the interstitium), and two forces push/draw fluid in (hydrostatic pressure in the interstitium, oncotic pressure in the capillary) (25). Hydrostatic pressure within the capillary, which provides the major force pushing fluid across the capillary wall and into the nephron, can be altered by varying plasma flow through the capillary bed (25). Neurohormonal mechanisms which affect the respective resistances of the afferent and efferent arterioles of the nephron can therefore indirectly control the amount of plasma filtered by the nephron, and ultimately the amount of fluid excreted from the body (31,64,66).

Activation of sympathetic nerves innervating the arterioles of the glomerulus can immediately alter blood flow, affecting filtration rates (25,45). Innervation of the afferent arteriole is comparatively greater than that of the efferent arteriole, thus activation of sympathetic outflow to the kidney leads to an increase in the resistance of the afferent vessel which exceeds that seen in the efferent (25). The result of this activation is a decrease in hydrostatic pressure within the glomerular capillaries, and a subsequent decrease in the amount of plasma filtered by the kidney (25). Activation of the sympathetic nervous system also stimulates the release of the proteolytic enzyme renin from the juxtaglomerular apparatus of the nephron (31,45). Renin participates in the formation of the vasoconstrictor angiotensin, which also acts to increase resistance of the afferent arteriole, though it has a more potent effect on resistance of the efferent arteriole- thus, the overall effect of RAAS activation is to decrease GFR, promoting fluid conservation (31,45,64). Another hormone which affects blood flow through the glomerulus is atrial natriuretic factor (ANF). This peptide hormone, released from atrial myocytes with increased stretch of the tissue, acts as a vasodilator, and has effects on renal plasma flow which are opposite to those of the SNS and of the renin-angiotensin-aldosterone system (RAAS) (31,64). ANF decreases afferent, and increases efferent, resistances, facilitating flow and therefore increasing filtration of the plasma. This leads to a subsequent excretion of salt and water from the body (31,64).

1.2.4 Regulation of kidney function: reabsorption

Once the plasma has been filtered at the glomerulus, the filtrate passes into a series of tubules which possess varying degrees of permeability to salt/water (25). Thus, as the filtrate passes through the nephron it undergoes a process of concentration or dilution, which depends on the osmolality of the surrounding interstitium (25). All material which leaves the tubule is restored to the general circulation by diffusion into the adjacent peritubular capillaries (66).

In the proximal tubule, salt is actively transported out of the tubule and into the interstitium, passively followed by water. Both activation of the sympathetic nerves supplying the kidney and the hormone angiotensin II stimulate the Na/K-ATPases in the basolateral membrane, increasing Na and therefore water reabsorption from the proximal tubule (25). In the loop of Henle, the contents of the tubule are first concentrated and then diluted with water first moving out of the tubule (tubule cells permeable to water), then remaining within the tubule (tubule cells impermeable to water), as salt is pumped into the interstitium. Fluid then enters the collecting duct, where Na is again actively removed, but reabsorption of water is governed by the peptide hormone vasopressin (AVP), which is released from the posterior pituitary in response to stimulation of central osmoreceptors (3,64). In the presence of AVP, this segment of the tubule becomes permeable to water, and thus the fluid in the lumen becomes more concentrated. In the absence of AVP, water is not permitted to cross the membrane, and the fluid in the tubule therefore becomes more dilute (3,64). The distal convoluted tubules from many adjacent nephrons empty into the common collecting duct which passes deeper in the medulla of

the kidney before the fluid is finally passed into the renal pelvis for removal via the ureters . Sodium transport in the collecting duct is primarily controlled by the mineralocorticoid hormone aldosterone, which is the end product of stimulation of the RAAS (64,186). Aldosterone stimulates the activity of the Na^+/K^+-ATPase, as well as increasing the number of Na channels in the luminal membrane (186). While aldosterone stimulates reabsorption of sodium, AVP allows water to follow sodium out of the tubule and back into the circulation. Thus the RAAS and AVP, which are often released concurrently when the effective circulating volume decreases, lead to increased reabsorption of salt and water, and a decrease in urine volume (25).

1.2.5 Response to increase in plasma volume

As described above, the sympathetic nervous system, the RAAS, AVP and ANF all work together to regulate kidney function. By altering the amount of fluid filtered, and the degree of reabsorption of fluid by the tubules, these mechanisms control the composition and volume of the ECFV, and indirectly, the plasma volume (64,186). The cardiovascular reflexes which function to keep pressure and volume of the circulation at an optimal level, in turn exert higher, central control over these regulatory mechanisms (64). This may be illustrated by describing what occurs when a volume load is administered, and the effective circulating volume increases.

In response to a large (hypertensive) increase in the circulating blood volume, stretch sensitive receptors in the atrium are activated (described in 1.1.2), as are those located in the high pressure vessels (carotid artery, aortic arch) (31,59). The atrial receptors send

afferent signals via the vagus nerve (baroreceptors via the glossopharyngeal and vagus nerves) to specific regulatory centers in the brainstem and hypothalamus (described in 1.1.3) (71,129,136). Central activation leads to inhibition of the release of AVP from the posterior pituitary, as well as to a decrease in sympathetic nerve outflow (65,111,129). Decreased renal sympathetic activity has three consequences: 1) to decrease resistance of the afferent arteriole, and thus increase GFR, 2) to decrease Na reabsorption from the proximal tubule of the nephron, and 3) to decrease RAAS activity (61,65,129). Concurrently, the absence of AVP prevents reabsorption of water from the distal convoluted tubule and collecting duct (25). In addition, stretch of the atrial tissue due to the increased circulating volume leads to increased secretion of ANF from the myocytes (78). ANF in turn increases filtration rate and decreases reabsorption at the collecting duct (41). Thus, activation of the cardiovascular reflexes, which regulate pressure and volume of the circulation, elicits responses in the regulatory mechanisms which directly control renal function (71,165). There is a highly integrated participation of every level of the reflex, from sensory to efferent output, that ensures disturbances in homeostasis are corrected (73,112). In this way, effective circulating volume is maintained within narrow limits which ensure cardiac performance and intravascular pressure is maintained (64).

1.3 REGULATION OF BLOOD VOLUME IN PREGNANCY

The previous section has established the importance of maintaining plasma volume at a constant level to ensure the needs to the tissues are constantly met, and that pressure and volume do not exceed what is required for normal cardiovascular function. However, there are specific physiological states, such as pregnancy, where cardiovascular

parameters such as pressure and volume are permitted to change (186). This change, pathological in the normal condition, is accepted and, indeed, required by the body (58,107,186).

1.3.3 Cardiovascular adaptation to pregnancy

In pregnancy, nearly every organ system undergoes some degree of change (57). Some of the most drastic alterations have been reported in the human cardiovascular system (2,57,162), beginning within the first 8 weeks of pregnancy, and continuing on until term (21). Some of the better documented changes are: the increase in cardiac output, due to an increase in both stroke volume (75% by end of first trimester) and heart rate (increase of 15 beats/min within first 4 weeks), general relaxation of peripheral vascular tone (within first trimester), the increase in red cell mass, and the progressive increase in plasma volume (average increase of 30-50%) (7,21,50,57). In humans, blood flow to the kidney is also significantly increased in pregnancy, with renal blood flow (and GFR) increasing by an average of 50% (39,58,204). Under normal conditions, when the filtered load is initiated by an increase in plasma volume, reabsorption is blunted and Na excretion increases (58,186). In pregnancy, however, this does not occur; despite the drastic increase in plasma volume, reabsorption does not decrease- rather it increases in parallel with the increase in filtration (38,58,186). This indicates the body does not recognize or respond to the increased plasma volume (2,7,107,186).

Changes in plasma osmolality are also seen: within the first 5 weeks of gestation, P_{osm} decreases by 8-10mosmol/kg below that seen prior to pregnancy (40,130). It is important

to note that, if such a change in P_{osm} occurred in the non pregnant state, AVP secretion would be suppressed and diuresis would immediately occur (25,130). This does not occur in the pregnant state, indicating the osmotic threshold for the release of AVP (and for thirst) are lowered in pregnancy in both humans and rats (49,130,131).

1.3.4 Plasma volume regulation in pregnancy

Lindheimer described the increase in plasma volume as 'one of the most striking adaptations to pregnancy' (130). This is illustrated by the observation that, of the 12.5 kg of weight gained in pregnancy, the increase in ECFV accounts for 6-8kg of this weight (11,58,92). Plasma volume, extracellular fluid volume and total body water all increase progressively in pregnancy, and peripheral edema is often observed (10,18). The increase in plasma volume is maximal by the ninth month of pregnancy (an average of 1230 ml above normal), but by the end of the second week following labour, plasma volume returns to normal (58,204). Previous sections of this discussion have shown that, normally, an increase in plasma volume of this magnitude would trigger activation of a series of homeostatic regulatory mechanisms which would collectively act to eliminate the excess fluid from the body (58). What happens to these regulatory mechanisms in pregnancy? Two theories have been proposed, each built upon the suggestion that the body's perception of fluid volume is altered in pregnancy (107,186).

In the *underfill hypothesis*, it is proposed that the expansion of the vascular bed, which occurs early in pregnancy, is the primary alteration which leads to fluid retention (109,186). However, the body, due to expansion of the vascular spaces, perceives itself

as *underfilled* (50,57). Homeostatic mechanisms which correct for a decrease in volume are thus activated; there is a reabsorption of salt and water in the kidney tubules, an increase in activity of the Na-retaining systems (i.e. RAAS), and inhibition of Na-losing systems (i.e. ANF) (109,160,186). The *overfill hypothesis*, in contrast, proposes renal retention of salt and water as the primary event. Subsequent changes occur as a reaction to the body's mistaken perception that it is *overfilled* (109). This theory really only explains the one detail disregarded by the underfill hypothesis, namely the marked increase in renal blood flow and filtration rate, which seem to indicate an attempt to eliminate excess fluid (109). There are flaws in both theories: if the underfill hypothesis is correct, why is it that comparable vascular expansion in non pregnant animals does not lead to the same changes in renal function? And if the overfill hypothesis is nearer the truth, how can it explain the increased reabsorption of Na/ water which parallels the increase in filtration (109)? Attempts to clarify these questions by administering stimuli of volume loads or saline loads to experimental animals has only added further complications, it being impossible to administer equipotent stimuli to pregnant animals with varying extracellular fluid volumes. Thus the atrial receptors of each animal would experience varying degrees of stretch based on the original plasma volume (107). In addition, the stimulus of a volume load would be impossible to localize to the atrium; the fluid may accumulate in an area remote from these receptors, or may stimulate other stretch-sensitive receptors throughout the cardiovascular system which would possibly obscure the atrial volume receptor reflex (71,107). Experiments were thus designed to examine this question (underfill vs. overfill) by measuring activation of the volume receptor reflex in pregnant vs. non pregnant animals, using the stimulus of balloon

inflation. Insertion of a small balloon in the external jugular of the rat ensures a direct, localized stimulus to the atrial receptors, which delivers a consistent stimulus to each animal regardless of their state of volume expansion (107). Equally as important, previous experiments carried out in male rats have confirmed that this stimulus evokes the reflex response consistent with atrial receptor activation: there is an increase in renal output and ANF secretion, and a decrease in fluid intake and plasma renin activity (105,106,111,111).

1.3.3. The AVR reflex in pregnancy

When a small balloon, positioned at the venoatrial junction of the heart is inflated in conscious virgin rats, the subsequent stimulation of the atrial receptors leads to an increase in urine volume and Na and K concentrations. When the same procedure is carried out in pregnant rats, the response to balloon inflation is abolished (108). The only explanation for the loss of renal response to atrial distension in pregnant animals is an inhibition of the atrial volume receptor reflex (108). As described in section 1.1, stimulation of the atrial receptors generates a signal, sent via the vagus nerve, processed by centers in the brain, and received by the main effector organ: the kidney. At any one of these processing stations (afferent, central, efferent) the reflex could be inhibited in pregnant animals (107).

To determine whether this inhibition was confined to efferent output, or was the result of inhibition of central processing, the central response to intracardiac balloon distension was compared in virgin and pregnant animals (42). In virgin rats, it was found that

stimulation of the atrial receptors by balloon inflation caused significant stimulation of the paraventricular nucleus of the hypothalamus, as shown by an increase in *c-fos* expression (a marker of neuronal activation). This confirmed that balloon inflation did stimulate the atrial receptors, and that this stimulation in turn activated neurons in the PVN. In 7 day pregnant rats, *c-fos* expression in the PVN in response to balloon inflation was decreased, and, by day 21, was abolished. (42). This attenuation of central response was also shown in the brainstem, in NTS neurons receiving afferent information from cardiac receptors. Tina Hines showed that the discharge frequency of these neurons following an atrial saline injection was the same in virgin and pregnant animals, despite a much higher atrial pressure in pregnant animals (84). These studies collectively establish that, in pregnancy, the central response to atrial distension- or an increase in blood volume- is inhibited, (42,84,107) i.e. there is an absence of the normal homeostatic mechanisms which hold fluid volume in check in pregnancy (42,107).

1.3.4 Pregnancy as a new steady state

As the above studies show, the atrial volume receptor reflex, which acts to keep plasma volume within narrowly defined limits, is suppressed during pregnancy i.e. the body does not respond to information indicating plasma volume has increased (42,84,107). These results seem to support the underfill hypothesis: that due to primary expansion of the vascular bed, the body perceives itself as underfilled (186). However, it may be that neither the underfill nor overfill hypotheses adequately explain altered volume homeostasis in pregnancy. When the vascular compartment is increased in non pregnant animals, corresponding physiological changes are not observed, indicating the underfill

hypothesis does not cover all the facts. Similarly, the overfill hypothesis only explains one component of renal function, leaving many pieces of the puzzle unaccounted for (109). It may be that best way to explore the changes in the cardiovascular system in pregnancy is to understand the reasons why they occur.

Physiological changes occur in pregnancy for one purpose: to fulfill the needs of the fetus (57,58,186). Thus plasma volume increases, not because of a response to perceived underfill or overfill, but because there is increased need for volume accumulation, as well as for vascular dilation, in fetal and maternal tissues (57,58,151,175). This is supported by several pieces of evidence indicating volume expansion is necessary for a healthy pregnancy: there is a highly significant correlation between plasma volume expansion and fetal growth, plasma volume expansion is less than normal in pregnancies complicated by intrauterine growth restriction, and a significant decrease in plasma volume precedes the development of the clinical syndrome of preeclampsia (57,58,186). It is also interesting to note that women in normal pregnancies with the greatest weight gain and plasma volume expansion have bigger babies with lower perinatal mortality rates than those with less fluid accumulation. Indeed, it is widely held that the amount of increase in plasma volume is strongly correlated with the health of the fetus post-delivery (18). Specifically, increased plasma volume in pregnancy fulfills several purposes: 1) provides extra blood flow to the uterus to meet the metabolic demands of the fetus, 2) provides increased perfusion of other organs, such as the kidneys, which also contribute to support of the fetus and 3) provides extra volume to compensate for blood loss during

delivery (58,175). Thus the changes in the circulatory system occur to support and sustain the fetus throughout pregnancy (160,162).

In this context, it may be easier to harmonize adaptations- which may be better called adjustments- in the cardiovascular system in pregnancy (91,92). To allow the increase in volume and decrease in pressure necessary for support of the fetus, cardiovascular reflexes are adjusted to different homeostatic thresholds (107). This is supported by evidence showing that, in pregnant women, an acute sodium load is handled appropriately for the prior sodium balance- thus pregnant women retain their new, decreased plasma osmolality within a narrow range (130). Thus water loading and fluid restriction leads to an appropriate concentration/ dilution of the urine (130).

1.4 REGULATION OF BLOOD VOLUME IN PREGNANCY: ROLE OF HORMONES

It has been established that pregnancy is defined by a series of changes in the normal physiology of the female body, all of which occur for the purpose of supporting and sustaining the life of the growing fetus (2,57,58). There has been some debate, however, as to the primary impetus to some of these changes; it has been suggested that, for the most part, they are a reaction to the 'stress' placed on the body by the demands of the conceptus (92). There is far greater support, however, for the idea that changes in the major organ systems- especially those which occur during the course of the first trimester- are the result of variations in levels of hormones in the circulation (21). The greatest support for this idea is found in the observation that many adjustments to

pregnancy, specifically those seen in the cardiovascular system and kidney, begin very early in pregnancy during the 'embryonic period', when it can hardly be said that the fetus is placing noticeable stress on the maternal system (21,57,58). Thus these changes must be ascribed to fluctuating hormone levels. It has been well-documented that levels of both peptide and steroid hormones begin to change within the first weeks of pregnancy (21,58). Additional support arises from the observation that there is a complete reversal of physiological adaptations to pregnancy very soon following delivery, often within the first two weeks (162). This process in fact coincides with the return of plasma levels of many hormones to the pre-pregnancy level (92).

Variations in levels of both peptide and steroid hormones are noted as pregnancy progresses. It is important to note that these two classes of hormone have very different mechanisms of action: peptides, which are too large to enter the cell, act on cell surface receptors and initiate a cascade of intracellular signals, while steroids traditionally diffuse easily from the blood into many types of cell, but only stimulate the select few that possess the corresponding intracellular receptor (132). These two groups of hormones are capable of causing widespread changes in cellular, and therefore organ, function.

1.4.1 Steroid hormones

The reproductive steroids are a group of hormones which play a very significant role in physiological adjustments to pregnancy (53). In humans, levels of both estrogen and progesterone increase with the advent of pregnancy, and are maintained significantly above normal throughout gestation, first by the corpus luteum, then by the developing

trophoblast of the implanted fetus, and finally by the fully developed placenta (132). Several studies have specifically examined the effect of ovarian sex steroids on fluid balance (12,37,94,202). It was found that high doses of estradiol alone, or in combination with progesterone, have little effect on basal P_{osm} or plasma AVP levels, while estradiol lowers the osmotic threshold of AVP secretion (12). Ueda et al. showed that estrogen administration in sheep leads to hemodynamic changes similar to those observed in pregnancy; namely, a decrease in MAP, an increase in HR, and an expansion of the blood volume (202). It has been suggested that the effects of estrogen on fluid balance are the indirect result of remodeling of the cardiovascular system, such that the heart, veins and arteries have a larger volume at normal physiological pressures (37). In this way, estrogen could contribute to the primary expansion of the vascular bed proposed as the trigger for volume accumulation in the 'underfill' hypothesis of cardiovascular alteration in pregnancy (see section 1.3.4) (37). While progesterone itself plays a significant role in remodeling tissues such as the uterine endometrium for support of the developing fetus, the metabolites of progesterone may play a unique role in establishing the new steady state of volume regulation in pregnancy (142).

The enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase metabolise progesterone to form pregnanolone (also called 3α-OH-5α-pregnan-20-one) (170). Given that pregnanolone (pregnan) is produced by the breakdown of progesterone, its levels in plasma temporally follow those of progesterone in pregnancy; as progesterone levels increase from 14 ng/ml to 80 ng/ml, pregnan levels rise from 4 ng/ml to 30-40 ng/ml (30,46,170). Pregnan is of interest in terms of its involvement in physiological

adaptations to pregnancy, not simply because its plasma levels increase progressively in pregnancy, but because it has also been found to exert unique effects on the central nervous system (13,53,170).

In the past decade it has been shown that some steroids are able to alter the excitability of neuronal cells in a matter of seconds; this is very different from the traditional, long-term effects of steroid hormones (13,170). These hormones were therefore called 'neurosteroids', and found to have several characteristics in common: 1) they altered neuronal excitability without recourse to genomic action, but solely through binding of cell-surface receptors and 2) they could be synthesized de novo in the central nervous system from sterol precursors, completely independent of any peripheral sources (15,170,180). Pregnan was classified as a neurosteroid when it was found that administration of the hormone caused anxiolytic and hypnotic effects within a matter of seconds, indicating the hormone was exerting rapid effects on CNS function (13,170). Pregnan's classification as a neurosteroid is also supported by observations of its presence in the brain: it has been shown that, in female rats, levels of pregnan are consistently higher than seen in the plasma. Moreover, in pregnancy, there is an increase of roughly 208% (up to 100nmol/g) by day 19, and a return to control levels on day 21 (27,30).

Pregnan exerts its effects in the brain by affecting the function of the $GABA_A$-receptor. This receptor, which is found ubiquitously in the CNS, is a member of a superfamily of ligand-gated channels and is composed of 5 subunits that form an integral chloride

channel (54,149). Thus when the neurotransmitter GABA binds the receptor, the chloride channel opens and the cell is consequently hyperpolarized by the inward flux of negative charge (170,180). In 1986 pregnan was found to be one of the most potent positive allosteric modulators of GABA function: at low concentrations, it increases frequency and/or duration of chloride channel openings in response to ligand binding, and a higher (micromolar) levels it directly opens the channel (79,122).

Thus pregnan can potentiate the effects of the inhibitory neurotransmitter GABA and, potentially, suppress processing of specific central pathways (27,56). Inhibition of central processing of the atrial volume receptor reflex in pregnancy, as described previously, led to the hypothesis that pregnan, with levels drastically increased in the brain in pregnancy, could be involved (43). It was found that pregnan (administered chronically to virgin rats at concentrations designed to reproduce mid-pregnancy levels in the plasma) increased plasma volume 13% above baseline, the same as that observed in mid-pregnant rats (133). Subsequently pregnan was shown to abolish the central response to the stimulus of atrial distension provided by balloon inflation (43). These results, indicating that pregnan plays an important role in the pregnancy-related suppression of cardiovascular reflexes, is consistent with the work of Laiprasert et al. showing brainstem processing of baroreceptor reflex afferent input to be decreased in the presence of pregnan (122).

1.4.2 Adrenomedullin

While many of the physiological adjustments to pregnancy may be attributed to changing levels of steroids and neurosteroids, other hormones- specifically, peptide hormones- are also involved in this process. The hallmark of such participation is generally an increase in plasma levels. Plasma levels of the recently discovered hormone adrenomedullin (ADM), a 52 amino acid peptide, with plasma levels which increase as pregnancy progresses (roughly 5-fold from first to third trimester) (86,115,138). Significantly, in rats it is possible to mimic the effects of pregnancy on plasma ADM levels by the administration of pregnan (97). Thus it would appear that pregnan, with a clearly defined role in pregnancy-related modulation of cardiovascular reflex function- is closely linked to ADM.

ADM, which is generated by the cleavage of preproADM into proADM N-terminal 20 peptide (PAMP) and ADM, is synthesized in a large number of tissues, from the adrenal medulla and cardiac atrium to the kidney and thyroid gland (77,178). The most abundant transcription of the ADM gene is found in the endothelial cells of the vasculature; indeed, it has been suggested that increased circulating levels of ADM in pregnancy are, in part, the result of increased production by the vascular system of the newly-formed placenta, though this is still under debate (86). ADM has been classified as a 'vasoactive peptide', and its effects on both pressure and volume of the circulatory system are well documented (166,182). Exogenous ADM has a potent hypotensive effect in both animals and humans; physiologically, it is believed to act in a local paracrine or autocrine manner in regulating blood flow to specific tissues (86,93,120). ADM increases renal blood

flow, urine flow and sodium excretion in a dose-related manner that is unaccompanied by changes in heart rate or mean arterial pressure, indicating the hormone acts directly on the vasculature of the kidney and on the renal tubules (77,87,99). ADM has also been shown to influence hormone secretion from the pituitary and the adrenal gland (86,144). It is found in both the posterior and anterior pituitary, and inhibits release of AVP in response to hypovolemic stimuli, and decreases plasma levels of ACTH (86,167). In the adrenal gland, ADM inhibits the release of aldosterone in response to both AII and potassium (143). Centrally administered ADM has been shown to inhibit drinking and salt appetite, and, in contrast to its effects in the periphery, to increase peripheral blood pressure (77,181). In reviewing these effects of ADM, both centrally and systemically, Samson described ADM as a 'candidate for physiologic regulator of vascular volume and electrolyte homeostasis' (181).

1.4.3 Nitric oxide

Nitric oxide is another factor which increases in pregnancy, and has been shown to play an even greater role in cardiovascular homeostasis than that of steroids or peptides (176). Pregnancy-related hormones, such as estradiol, have been shown to increase NO biosynthesis (205). Work by Conrad et al. has shown urinary excretion of cGMP (a by-product of NO activation) is increased in pregnancy (29). In addition, urinary nitrate levels are elevated in the pregnant rats. Levels of NO in the brain have also been shown to increase in pregnancy (85). Pregnan also increases NO biosynthesis in virgin female rats, possibly through increased ADM levels (133).

Nitric oxide synthases (enzymes found in neurons, endothelial cells and a host of other tissues) convert L-arginine and molecular oxygen to L-citrulline and NO (171,176). In endothelial cells and neurons, NOS activation is dependent on an increase in intracellular calcium; thus an influx of Ca into the cell stimulates production of NO (176). As NO is a highly reactive, aqueous soluble gas, it easily diffuses into cells, and is thought to act as an autocrine/paracrine factor in regulating the function of surrounding cells (117,176). Once NO has entered the cell, it activates the enzyme guanylyl cyclase which, through production of cGMP, effects changes in cell function through phosphorylation of specific cellular proteins (176).

It has been stated that NO is 'to some extent involved in the function of virtually every organ system' (176). It has been shown to have specific effects on the function of cardiovascular reflexes. A great deal of research has been conducted examining the effect of NO on nearly every level of the baroreceptor reflex (23,89). NO-producing neurons are concentrated in areas of the brain which process afferent information from cardiovascular reflexes, mainly the PVN and SON of the hypothalamus (116). Neurons of both the magnocellular and parvocellular divisions of the PVN have been found to contain nNOS (116). In the parvocellular part of the nucleus, groups of neurons projecting to autonomic centers in the brainstem and spinal cord, as well as those projecting to the median eminence, were found to contain nNOS, generally in company with a combination of other neurotransmitters (117). Areas of the brainstem, most specifically the NTS and VLM, contain a high number of NO-producing neurons; many of these axon terminals arise from the nodose ganglion through which most

cardiovascular afferent signals pass (117). Although NO is thought to have many central effects, one of its main effects is an inhibition of sympathetic outflow (116,117,146). Evidence indicates that, overall, the NO system in the brain is activated during homeostatic imbalance, and that 'the role of NO in regulating sympathetic outflow may be a function of the balance between its inhibitory and excitatory effects in different autonomic centers' (117). As both hemorrhage and a decrease in blood pressure triggers widespread activation of NO-producing neurons in the nuclei described above, it is possible to conclude that NO may be involved in the reflex sympathoinhibition mediation by the alteration of activity of receptors in the arteries and in the heart (116,117).

NO has also been shown to have an effect on the baroreceptor reflex at the peripheral level: recent work by Matsuda et al. has shown that NO decreases the discharge frequency of baroreceptor fibers (145). NO acts in the spinal cord (inhibition of sympathetic outflow) and at the blood vessels themselves (vasodilation); thus it is truly possible to say the NO acts to suppress nearly every level of the baroreceptor reflex (23,89,117). Thus it has been established that NO biosynthesis is increased in pregnancy, yet whether the role of NO is limited to general vasodilation and attenuated pressor responses, or whether it involves more widespread modulation of cardiovascular homeostasis, remains to be determined.

1.5 EXPERIMENTAL PREPARATIONS AND HYPOTHESES

We utilized a series of experimental preparations during our investigations, which were:

1. *in vivo* experiments using anesthetized rats in which atrial receptor afferent activity in response to inflation of the intracardiac balloon was measured, together with hemodynamic variables (MAP, ECG). Recordings were carried out in: 1) pregnant vs. non pregnant animals, 2) animals treated chronically with either a vehicle or the steroid hormone metabolite pregnan, 3) animals acutely treated with pregnan, ADM or SNAP (to increase NO).

2. *in vivo* experiments using conscious unrestrained animals implanted with: 1) the intracardiac balloon and an inferior vena caval cannula, 2) the intracardiac balloon and a blood pressure transmitter and 3) a jugular venous cannula and an inferior vena caval cannula. In each of these experimental preparations, ADM was administered and: 1) neuronal activation in the PVN was quantified, 2) blood pressure was measured and 3) plasma volume was determined.

The first series of *in vivo* experiments examined the effect of pregnancy on atrial receptor afferent discharge. Based on evidence indicating that pregnancy suppresses function of the volume receptor reflex (42,108), we proposed that:

pregnancy would suppress atrial volume receptor discharge in response to atrial distension.

As pregnan has both chronic and acute effects, and research has indicated that pregnan can act acutely to blunt central processing of both the volume receptor and baroreceptor reflexes (43,122), we proposed that:

acute pregnan would suppress atrial volume receptor discharge in response to atrial distension.

The second series of in vivo experiments investigated the effect of chronically-administered pregnan on atrial receptor afferent discharge. Based on evidence indicating chronic pregnan increases plasma volume (133), we proposed that:

chronic pregnan would suppress atrial volume receptor discharge in response to atrial distension.

Plasma levels of ADM have been shown to increase progressively in pregnancy, and ADM is released in response to exogenous pregnan administration (97). Given that ADM plays an important role in volume regulation we hypothesized that:

ADM would decrease the central response to atrial distension.

In light of evidence that ADM has potent natriuretic and diuretic effects in the periphery, we sought to establish whether circulating levels of ADM in pregnancy could contribute to the marked blood volume expansion in pregnancy (181). We hypothesized that:

ADM would increase plasma volume in virgin female rats.

While ADM may affect central processing of the volume receptor reflex, it is also possible that it alters sensory reception and signaling of reflex activation. Our hypothesis stated that:

ADM would suppress atrial volume receptor discharge in response to atrial distension.

Nitric oxide has been shown to play an important role in cardiovascular homeostasis (23,117). Previous work examining its effect on the baroreceptor reflex revealed that NO could suppress both central processing and sensory receptor signaling of reflex activation (117,145). Given that NO biosynthesis increases in pregnancy (133), we hypothesized that:

NO would suppress atrial volume receptor discharge in response to atrial distension.

CHAPTER 2.

MATERIALS AND METHODS

The experimental procedures in the following studies were approved by the local Animal Welfare Committee in accordance with the guidelines issued by the Canada Council on Animal care. At the completion of an experiment, all animals were euthanized with an anesthetic overdose (0.3 ml Euthanyl; intravenous, iv; 240 mg/ml sodium pentobarbital; MTC Pharmaceuticals, Cambridge, Ontario, Canada).

Animal model: Female Long Evans rats (250-350 g) were obtained from Charles River, (St. Foy, Quebec, Canada) and housed in a temperature- and humidity-controlled animal facility with a 12:12 hr light-dark cycle (light 0700-1900) for at least one week before the experiments. They were maintained on a 0.3% sodium diet and water ad libitum.

EXPERIMENT A: EFFECT OF PREGNANCY ON ATRIAL RECEPTOR AFFERENT DISCHARGE.

Surgery (Expt A): Animals were anesthetized with pentobarbital sodium (60 mg/kg body wt.) plus atropine (0.1 ml, 0.4 mg/ml), followed by a supplementary dose of Inactin [ethyl-(1-methylpropyl)-malonyl-thio-urea; 60 mg SC] to maintain a level plane of anesthesia. During surgery, the rats were placed on a Deltaphase isothermic heating pad (Braintree Scientific, Inc., Mass., USA) which maintained body temp at ~37°C.

Femoral Cannulae: A 1.5 cm long medial incision was made along the upper left leg, and the fatty tissue and adductor longus muscles were bluntly dissected to expose the femoral

artery and vein. The two vessels were separated from each other. The femoral vein was cannulated using silastic tubing (0.51 mm i.d., 0.94 mm o.d.; Dow Corning, USA). The femoral artery was cannulated with PE-50 tubing (0.58 mm i.d., 0.965 mm, o.d.; Intramedic, USA). Mean arterial pressure was monitored via the femoral artery, and Somnotol or drugs were administered through the femoral vein. The venous line was also used to infuse isotonic saline (3 ml hr^{-1}) to maintain adequate hydration of the animal throughout the duration of the experiment.

Intracardiac balloon implantation: A small inflatable balloon cannula was inserted about 2 cm into the right jugular vein, and loosely sutured to maintain its position. Animals then underwent tracheotomy and were subsequently ventilated at a rate of 60 strokes/min. Using a small rotating saw, a midline incision was made in the sternum and the chest cavity was held open with chest retractors. Once the venoatrial junction was clearly visible, the balloon cannula was passed carefully down the jugular vein and positioned with its tip at the venoatrial junction. Distension of the area of the venoatrial junction with inflation of the balloon was then visually confirmed, and the balloon was secured at the clavicle. The chest wall was then closed and spontaneous breathing was resumed.

Vagus nerve dissection: The sternocleidomastoideus muscle in the neck of the rat was cut and retracted to expose the right carotid artery. A pool was then constructed using the tissue at the edges of the wound. The cervical vagus was carefully dissected free of the carotid artery sheath and mounted on a stainless steel platform with a polished reflective surface, and the pool was filled with warm mineral oil. The vagus was isolated, stripped

of surrounding connective tissue, and desheathed. Bundles of fibers were pulled away from the nerve and split sequentially until a very small bundle could be isolated.

Electrocardiogram (Expt A): Three metal leads were placed in the rat, one in the right shoulder, one in the left and one at the bottom of the ribcage. The ECG waveform was then amplified (ECG/Biotach amplifier 13-4615-65, Gould Electronics, Cleveland OH) and displayed on the computer monitor (Windaq, Dataq Instruments, Akron OH).

Nerve recordings (Expt A): Once fiber bundles were dissected down to a single fiber or to a small bundle of 2-3 fibers, they were placed on a bipolar silver electrode. The nerve signal was amplified and filtered between 100 and 1000 Hz (Leaf Electronics Ltd. QT-5B; World Precision Instruments LPF-30, Sarasota FL). Output from the amplifier was then fed to an oscilloscope (Tektronix 7613, Wilsonville OR) and loudspeaker, and was displayed on a PC (10 kHz sampling rate, Windaq, Dataq Instruments, Akron OH.) along with blood pressure and ECG waveforms.

Atrial receptor identification (Exp. A): Single fibers or small filaments containing 3 to fibers were screened by an initial balloon inflation of 25 μL.

Baseline recording (Expt. A): Once a response had been established, fibers were stimulated with graded inflation of the balloon (15, 25, 35 and 50 μL) with periods varying from 10 to 60 seconds for each increment, depending on the condition of the

fiber. Baseline arterial pressure (AP), ECG and atrial receptor (AR) afferent discharge were recorded for 20 seconds before and 20 seconds after balloon inflation.

Receptor localization (Expt A): Following completion of the experimental protocol for each fiber, the chest was re-opened and the mechanosensitive receptive field was confirmed by probing the venoatrial junction. The site which, when probed, produced a high frequency discharge was accepted as the receptor location.

Protocol (Expt A): Rats were randomly allocated to one of two groups: 1) pregnant (n=8) or 2) age-matched virgin (n=8). The rats in group 1 were subjected to vaginal smear and mated. The success of pregnancy was estimated by the increase in body weight 7 days later. On day 20 of gestation the experiment was performed. To confirm viability of pregnancy, fetuses were counted at the end of each experiment.

Statistical analysis (Expt. A): Mean values of arterial pressure and heart rate were calculated from the 20 second baseline period preceding inflation, the 10-60 second balloon inflation period and the 20 second recovery period. The analysis of the nerve discharge was based on average discharge rate (spikes/sec). The mean discharge frequency for 2 seconds following atrial distension was calculated and compared with the mean discharge frequency in the 2 second period immediately preceding inflation. Receptor response was delayed for roughly 1 to 2 seconds as this was the time required to inflate the balloon to the correct volume. Hemodynamic measurement and nerve discharge frequency were compared between pregnant and non pregnant groups by unpaired t-tests. Slopes of receptor stimulus-response curves were determined by linear

analysis, and compared between groups by ANOVA. Values were presented as mean ± S.E., and a P value < 0.05 was considered significant.

EXPERIMENT B: EFFECT OF ACUTE PREGNAN ON ATRIAL RECEPTOR AFFERENT DISCHARGE

Surgery (Expt B): As described in experiment A, with one additional cannulation.

External jugular cannula: Following insertion of the intracardiac balloon, a cannula (polyethylene (PE 10), 0.28 mm ID x 0.61 mm OD) was inserted into the jugular vein for administration of the hormone. The cannula was secured in a position adjacent to the balloon, with its tip ~2 mm above the site of balloon distension in the venoatrial junction. Following completion of the experiment, the exact position of the cannula in relation to the site of balloon distension was confirmed.

Electrocardiogram (Expt B): As described in experiment A.

Nerve recordings (Expt B): As described in experiment A.

Atrial receptor identification (Expt B): As described in experiment A.

Drug preparation (Expt B): 5α-Pregnan-3α-ol-20-one (Sigma Chemical, Oakville, ON, Canada) was dissolved in 20% 2-hydroxypropyl-β-cyclodextrin (Sigma) in sterile distilled

water. 0.2 ml of an acute dose of pregnan (2.6 μg/kg i.v.) or vehicle (20% β-cyclodextrin) was prepared for injection via the jugular cannula.

Baseline recording (Expt B): Once an atrial receptor had been identified by increased afferent discharge in response to a 25 μl balloon inflation, fibers were stimulated with graded inflation of the balloon (25, 35 and 50 μL) with periods varying from 10 to 60 seconds for each increment, depending on the condition of the fiber. In these experiments the 15 μl recording was omitted to reduce recording time, as signal amplitude normally began to decay after 10-15 minutes and fibers were required to last for up to 15 minutes. Baseline AP, ECG and atrial receptor afferent discharge were recorded for 20 seconds before and 20 seconds after balloon inflation.

Receptor localization (Expt B): Following completion of the experimental protocol for each fiber, the chest was re-opened and the mechanosensitive receptive field was confirmed by probing the venoatrial junction. The site which, when probed, produced a high frequency discharge was accepted as the receptor location.

Protocol (Expt B): Once an atrial receptor had been identified by increased afferent discharge in response to 25 μl balloon inflation, 5α-Pregnan-3α-ol-20-one (n=8) or vehicle (n=7) was injected into the cannula positioned in the external jugular. Three recording sessions were carried out: 3, 5 and 10 minutes following drug/vehicle injection.

Statistical analysis (Expt B): Mean values of arterial pressure and heart rate were calculated from the 20 second baseline period preceding inflation, the 10-60 second balloon inflation period and the 20 second recovery period. The analysis of the nerve discharge was based on average discharge rate (spikes/sec). The mean discharge frequency for 2 seconds following atrial distension was calculated and compared with the mean discharge frequency in the 2 second period immediately preceding inflation. Receptor response was delayed for roughly 1 to 2 seconds as this was the time required to inflate the balloon to the correct volume. Hemodynamic measurement and nerve discharge frequency were compared between acute pregnan and vehicle-treated groups by unpaired t-tests. Slopes of receptor stimulus-response curves were determined by linear analysis, and compared between groups by ANOVA. Values were presented as mean ± S.E., and a P value < 0.05 was considered significant.

EXPERIMENT C: EFFECT OF CHRONIC PREGNAN ON ATRIAL RECEPTOR AFFERENT DISCHARGE

Surgery (Expt C): As described in experiment A.

Electrocardiogram (Expt C): As described in experiment A.

Nerve recordings (Expt C): As described in experiment A.

Atrial receptor identification (Expt C): As described in experiment A.

Drug preparation (Expt C): 5α-Pregnan-3α-ol-20-one (Sigma Chemical, Oakville, ON, Canada) was dissolved in 20% 2-hydroxypropyl-β-cyclodextrin (Sigma) in sterile distilled water. Each treatment consisted of 500 μg pregnan in 400 μl cyclodextrin plus 200 μl sunflower oil, or vehicle alone (cyclodextrin and oil), given subcutaneously. Animals were given the treatment at 8 A.M. for 2 consecutive days.

Baseline recording (Expt C): Once an atrial receptor had been identified by increased afferent discharge in response to a 25 μl balloon inflation, fibers were stimulated with graded inflation of the balloon (15, 25, 35 and 50 μL) with periods varying from 10 to 60 seconds for each increment, depending on the condition of the fiber. Baseline AP, ECG and AR afferent discharge were recorded for 20 seconds before and 20 seconds after balloon inflation.

Receptor localization (Expt C): Following completion of the experimental protocol for each fiber, the chest was re-opened and the mechanosensitive receptive field was confirmed by probing the venoatrial junction. The site which, when probed, produced a high frequency discharge was accepted as the receptor location.

Protocol (Expt C): Rats were randomly chosen to receive either pregnan (n=6) or vehicle (n=7) injections at 8 A.M. for 2 consecutive days. Experiments were then carried out (in the afternoon of the second day of injection) as described in experiment A.

Statistical analysis (Expt C): Mean values of arterial pressure and heart rate were calculated from the 20 second baseline period preceding inflation, the 10-60 second

balloon inflation period and the 20 second recovery period. The analysis of the nerve discharge was based on average discharge rate (spikes/sec). The mean discharge frequency for 2 seconds following atrial distension was calculated and compared with the mean discharge frequency in the 2 second period immediately preceding inflation. Receptor response was delayed for roughly 1 to 2 seconds as this was the time required to inflate the balloon to the correct volume. Hemodynamic measurement and nerve discharge frequency were compared between chronic pregnan and vehicle-treated groups by unpaired t-tests. Slopes of receptor stimulus-response curves were determined by linear analysis, and compared between groups by ANOVA. Values were presented as mean ± S.E., and a P value < 0.05 was considered significant.

EXPERIMENT D: EFFECT OF ADRENOMEDULLIN ON C-FOS EXPRESSION IN THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS.

Surgery (Expt D: Part I): Animals were anesthetized with sodium pentobarbital (62 mg per kg body weight; i.p.). This was followed by penicillin (0.1 ml i.m. Ethacillin Rogas/STB Inc, London, Ont. Canada) and atropine (0.1 ml, 0.4 mg/ml). Buprenorphine (0.01mg/kg) was given after surgery was completed. Throughout the surgical procedures, rats were maintained on a Deltaphase isothermic heating pad (Braintree Scientific, Inc., Mass,. USA) which maintained body temp at ~37°C. Silastic cannulae (0.020 in. ID, 0.037 in. OD; Dow Corning) were implanted nonocclusively into the

inferior vena cava. A small inflatable balloon cannula was passed down the right jugular vein and secured to the clavicle such that the tip of the balloon lay at the venoatrial junction (105). After the initial surgery, the rats were allowed to recover to their pre-operative weights, which in this study took about one week.

Protocol (Expt D: Part I): Seven days after surgery, the rats were randomly allocated to the following groups: 1) rats receiving the bolus dose of vehicle (0.5 ml saline) without atrial distension (AD) (n=6), 2) rats receiving vehicle with AD (n=6), 3) rats receiving the bolus dose of ADM (15 ng in 0.5mL saline) without AD (n=7), 4) rats receiving the bolus dose of ADM with AD (n=5), and 5) rats receiving 10-fold the bolus dose of ADM (150 ng in 0.5 ml saline) with AD (n=6). Two rats were also assigned to confirm the effects of the 10-fold dose of ADM alone on *c-fos* expression. All the rats were placed in metabolism cages the day before testing, for ease of accessing the cannulas. The next day, the rats were infused with saline for one hour via the inferior vena cava cannula (3 ml/hr). The intracardiac balloons in groups 2, 4 and 5 were then inflated with 50 μL saline for one hour; the balloons in the control group were not inflated. The bolus dose of ADM or vehicle was administered simultaneously with inflation. Following the one hour inflation period, the rats were then deeply anesthetized with sodium pentobarbital, and perfused intracardially with 4% paraformaldehyde. The fixed brains were processed for visualization and quantitation of *c-fos* expression.

Immunocytochemistry (Expt D: Part I): Coronal sections (50 μm) were incubated overnight in cold anti-*fos* antiserum [2 μg/ml in 0.3% Triton X-100 in PBS, AB-2, polyclonal rabbit immunoglobulin (Ig)G, no. PC05, Oncogene Science]. Tissues were

sequentially incubated for 1 h with anti-rabbit IgG (1:200 in PBS, biotinylated antibody, Vectastain, Vector Laboratories, Burlingame, CA), followed by ABC reagent for another hour (1:100 in PBS). To visualize *fos*, sections were treated with 0.05% diaminobenzidine (Sigma) solution containing 0.01% hydrogen peroxide in PBS for 5-10 min, until they turned brown.

Quantitative analysis (Expt D: Part I): Cell nuclei in subdivisions of the PVN were examined. The number of *fos*-labeled cells was counted in every other section throughout the whole PVN (5-6 sections). The total number of neurons expressing *c-fos* was recorded.

Statistical analysis (Expt D: Part I): Values were expressed as mean number ± SE of mean per nucleus. Statistical multiple comparisons between the groups within one nucleus were evaluated using one-way analysis of variance (ANOVA). Where the ANOVA revealed significance between the groups, the Fisher LSD method was used to determine which group(s) contributed to these differences. Significance was accepted with $P<0.05$.

Surgery (Expt D: Part II): Rats were prepared as described in Part I, but with the addition of a blood pressure recording device (Physiotel PA-C40 blood pressure transmitter, Data Sciences International, St. Paul, Minnesota) implanted in the peritoneal cavity with the sensing catheter inserted into the abdominal aorta (mid-way between the branch to the left of the renal artery and the bifurcation to the femoral arteries). This

device allowed for continuous measurement of MAP. Rats were again allowed to recover their preoperative weights before the experimental protocol was carried out.

Protocol (Expt D: Part II): To ensure that changes in *c-fos* expression in the nuclei of the lateral hypothalamus would not be due to the effects of ADM and/or balloon inflation on mean arterial blood pressure, blood pressure was monitored in a group of virgin female rats through implanted recording devices while the intracardiac balloon was inflated and/or the bolus dose of ADM was given. Seven days following the surgery, rats were randomly allocated to the same groups as those described in protocol A. Following a one hour stabilisation period, the identical protocol was carried out (no AD+vehicle (n=4), AD+vehicle (n=4), AD+ADM (n=4), no AD+ADM (n=4), no AD+ADM (X10) (n=3)) while blood pressure was recorded during the hour long period.

Measurement of heart rate (Expt D: Part II): The heart rate (beats/min) was calculated by analysis of the MAP data using Windaq software (Windaq, DATAQ Instruments Inc., Akron, Ohio). Heart rate was determined by counting the number of spikes in the MAP recordings from data collected for two 5 min periods: one before, and one after drug administration.

Statistical analysis (Expt D: Part II): Results were recorded as average blood pressure $\pm$ SE of mean per rat. Statistical multiple comparisons between the groups were evaluated using one-way analysis of variance (ANOVA). Significance was accepted with $P<0.05$.

EXPERIMENT E: EFFECT OF ADRENOMEDULLIN ON PLASMA VOLUME.

Surgery (Expt E): The rats were prepared under pentobarbital sodium anesthesia (0.65 mg/kg body wt; MTC Pharmaceuticals) with Silastic cannulas (0.05 cm ID x 0.09 cm OD; Dow Corning, Midland, MI) implanted nonocclusively into the inferior vena cava (IVC) and the jugular vein. The cannulas were exteriorized by tunneling them subcutaneously to the back of the neck and connecting them to stainless steel tubing secured to the interscapular region.

Plasma and blood volume determination (Expt E): Plasma volume was determined using the Evan's Blue dye dilution method. Initial blood samples (0.25 ml) were taken. Evans blue dye (Fisher Scientific, Whitby, ON, Canada) was injected into the jugular cannula (0.3 ml of a 0.5% wt/vol solution in sterile 0.9% sodium saline), and the line was flushed with 0.2 ml saline. Blood samples (0.15 ml) were taken from the IVC line at 10, 20, 30, 40, and 60 min after the dye was injected. Blood was transferred to Caraway Micro Blood Collecting tubes (Fisher) and centrifuged at 11,700 rpm (Clay Adams Micro-Hematocrit II). The tubes were cut, and plasma (50 μl) was diluted in 950 μl of 0.9% sodium saline. Dye concentration was read at 605 nm (LKB Novaspec). The readings were compared with standards obtained by adding 0, 1, 2 µl of the 0.5% Evan's Blue solution to 50 µl initial plasma plus 950 µl saline. Plasma and blood volume were determined by comparing the degree of dilution of dye from time 0.

Experimental protocol (Expt E): Rats were placed in Nalgene metabolism cages after preoperative weight was reached. To ensure adequate and standardized hydration levels before dye injection and blood sampling for each experiment, food and water were removed and animals were infused with 0.9% sodium saline through the IVC cannula, over a period of 60 min at a rate of 3 ml/h using an LKB (Bromma) Micro Perpex pump. Following this stabilization period, the animal was administered a bolus loading dose of ADM (15 ng/0.25 ml saline, n=4) via the IVC cannula, followed by a 2 hr infusion of ADM (11.25 ng/3 ml saline per hour). For the group administered the higher dose of ADM, the loading dose was 150 ng/0.25 ml saline, and the infused dose 112.5 ng/saline/hr (n=4). For the control group (n=4), saline was administered at the same volumes and infusion rates. Once drug infusion was completed, the plasma volume measurement was carried out as described above.

Statistical analysis (Expt E): ANOVA was used to determine the significance of changes in plasma volume and blood volume between groups.

EXPERIMENT F: EFFECT OF ADM ON ATRIAL RECEPTOR AFFERENT DISCHARGE

Surgery (Expt. F): As described in experiment B.

Electrocardiogram (Expt F): As described in experiment A.

Nerve recordings (Expt F): As described in experiment A.

Atrial receptor identification (Expt. F): As described in experiment A.

Drug preparation (Expt F): 100 ng of ADM (Phoenix Pharmaceuticals, etc.) was prepared in 190 µl of saline and diluted to 0.2 ml (saline) for injection via the jugular cannula.

Baseline recording (Expt F): Once an atrial receptor had been identified by increased afferent discharge in response to a 25 µl balloon inflation, fibers were stimulated with graded inflation of the balloon (15, 25, 35 and 50 µL) with periods varying from 10 to 60 seconds for each increment, depending on the condition of the fiber. Baseline AP, ECG and AR afferent discharge were recorded for 20 seconds before and 20 seconds after balloon inflation.

Receptor localization (Expt F): Following completion of the experimental protocol for each fiber, the chest was re-opened and the mechanosensitive receptive field was confirmed by probing the venoatrial junction. The site which, when probed, produced a high frequency discharge was accepted as the receptor location.

Protocol (Expt F): Following establishment of a baseline recording, ADM (n=5) or vehicle (0.2 ml saline) (n=5) was slowly injected into the cannula positioned in the

external jugular. Three recording sessions were carried out: 1, 3 and 5 minutes following drug/vehicle injection.

Statistical analysis (Expt F): Mean values of arterial pressure and heart rate were calculated from the 20 second baseline period preceding inflation, the 10-60 second balloon inflation period and the 20 second recovery period. The analysis of the nerve discharge was based on average discharge rate (spikes/sec). The mean discharge frequency for 1 or 2 seconds following atrial distension was calculated and compared with the mean discharge frequency in the period preceding inflation. Receptor response was delayed for roughly 1 to 2 seconds as this was the time required to inflate the balloon to the correct volume. Hemodynamic measurement and nerve discharge frequency were compared between ADM and vehicle-treated groups by paired t-tests. Slopes of receptor stimulus-response curves were determined by linear analysis, and compared between groups by ANOVA. Values were presented as mean ± S.E., and a P value < 0.05 was considered significant.

EXPERIMENT G: EFFECT OF S-NITROSO-N-ACETYL-D,L-PENICILLAMINE ON ATRIAL RECEPTOR AFFERENT DISCHARGE

Surgery (Expt. G): As described in experiment A.

Electrocardiogram (Expt G): As described in experiment A.

Nerve recordings (Expt G): As described in experiment A.

Atrial receptor identification (Expt. G): As described in experiment A.

Drug preparation (Expt G): The NO donor (SNAP, Sigma, Canada) was prepared in distilled water for infusion at a rate of 10 μg/50 μl/min. Alternatively, vehicle (saline) was infused at a rate of 50 μl/min.

Baseline recording (Expt G): Once an atrial receptor had been identified by increased afferent discharge in response to a 25 μl balloon inflation, infusion of either SNAP or saline into the femoral vein was initiated. Three recording sessions were carried out: 1, 3 and 5 minutes following the commencement of drug/saline infusion. In the course of one recording session, fibers were stimulated with inflation of the balloon (25 and 50 μl) with a period of 10 seconds for each increment. Baseline AP, ECG and AR afferent discharge was recorded for 10 seconds before and 20 seconds after balloon inflation.

Receptor localization (Expt G): Following completion of the experimental protocol for each fiber, the chest was re-opened and the mechanosensitive receptive field was confirmed by probing the venoatrial junction. The site which, when probed, produced a high frequency discharge was accepted as the receptor location.

Protocol (Expt G): Once an atrial receptor had been identified by increased afferent discharge in response to 15 μl balloon inflation, SNAP (n=5) or vehicle (0.2 ml saline) (n=5) was infused into the cannula positioned in the external jugular. Three recording sessions were carried out: 1, 3 and 5 minutes following drug/vehicle injection.

Statistical analysis (Expt G): Mean values of arterial pressure and heart rate were calculated from the 10 second baseline period preceding inflation, the 10 second balloon inflation period and the 20 second recovery period. The analysis of the nerve discharge was based on average discharge rate (spikes/sec). The mean discharge frequency for 1 or 2 seconds following atrial distension was calculated and compared with the mean discharge frequency in the period preceding inflation. Receptor response was delayed for roughly 1 to 2 seconds as this was the time required to inflate the balloon to the correct volume. Hemodynamic measurement and nerve discharge frequency were compared between groups by unpaired t-test. Values were presented as mean ± S.E., and a P value < 0.05 was considered significant.

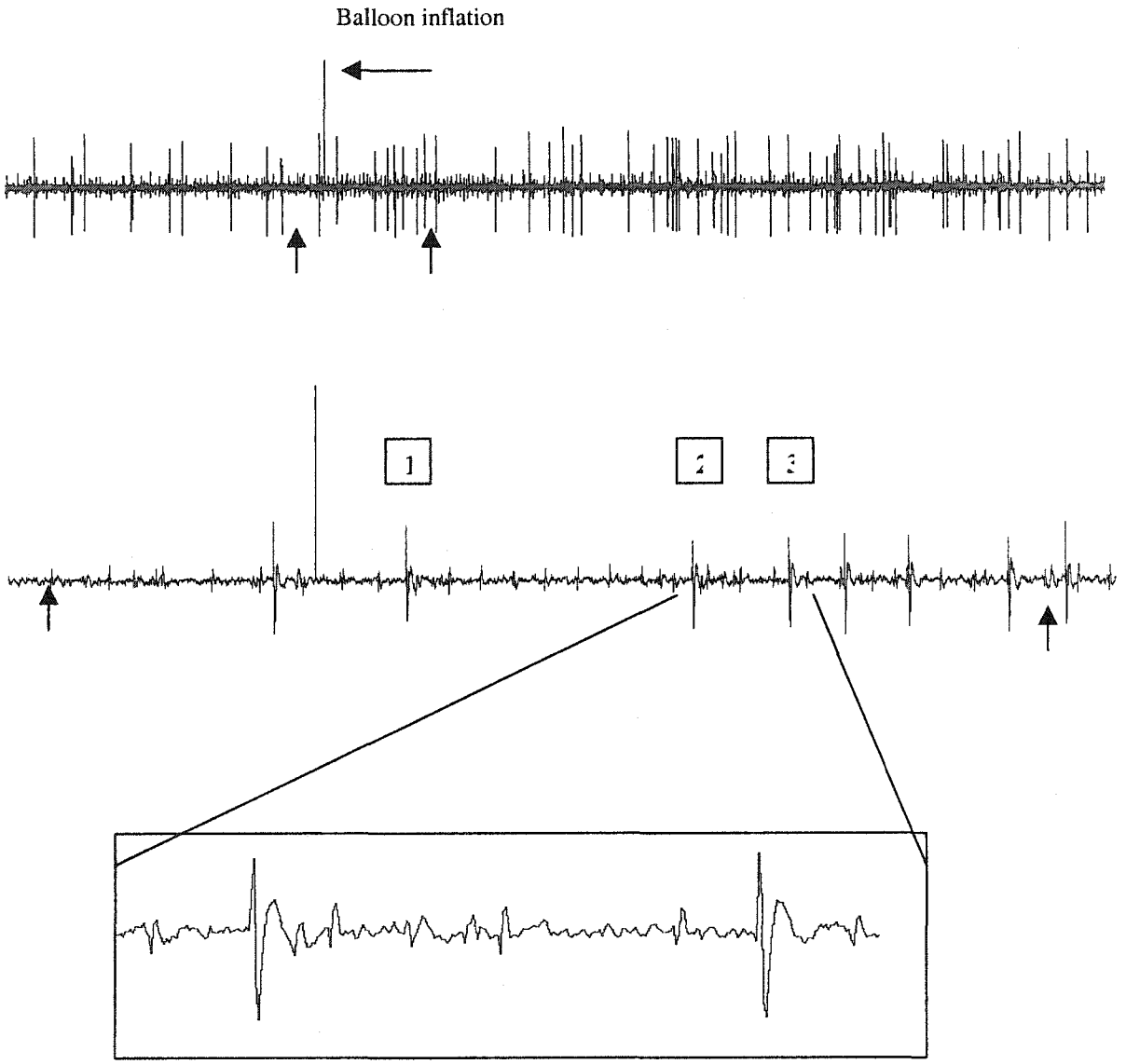


Figure 6. Example of procedure used in quantifying atrial receptor response to balloon distension. First tracing shows response of LF receptor to 25μl balloon distension (compression 30X), and second, the individual action potentials of LF response (compression 5X). Final trace shows close-up of individual action potentials. Selected on the basis of increase in discharge frequency of 20% or more.

CHAPTER 3.

RESULTS

EXPERIMENT A: EFFECT OF PREGNANCY ON ATRIAL RECEPTOR AFFERENT DISCHARGE.

Mean arterial pressure (MAP) was lower in pregnant (76.9±4.4 mmHg, n=8) compared with nonpregnant rats (98.6±3.6 mmHg, n=8, $P<0.05$). Baseline MAP in both pregnant and nonpregnant rats was unaffected by balloon inflation (pre-inflation: 70.2±4.1 mmHg (pregnant), 94.6± 2.5 mm Hg (nonpregnant), post-inflation: 78.1±3.0 mmHg (pregnant), 90.7± 2.2 mm Hg (nonpregnant)).

Thirty-one functional single fibers were dissected in 8 pregnant and 8 virgin rats. Raw data tracings showing the changes in atrial receptor afferent discharge and arterial blood pressure in response to graded balloon inflation in a virgin (A) and a pregnant (B) rat are shown in Figure 5. Each receptor responded to balloon inflation with an increase in discharge (Fig. 6). Roughly 47% of atrial receptors localized in virgin animals were classified as HF receptors based on response to balloon inflation. In addition, mean spontaneous discharge of LF receptors (6.9±1.8 Hz) was significantly lower than that of HF receptors (21.4 ±3.0 Hz. $P<0.05$) in virgin rats.

In contrast to the two populations of receptors localized in virgin rats, all receptors localized in pregnant rats discharged in the low-frequency range (Fig. 7). Mean spontaneous discharge of receptors in pregnant rats (4.8±0.8 Hz) did not differ from that of low-frequency receptors in virgin rats.

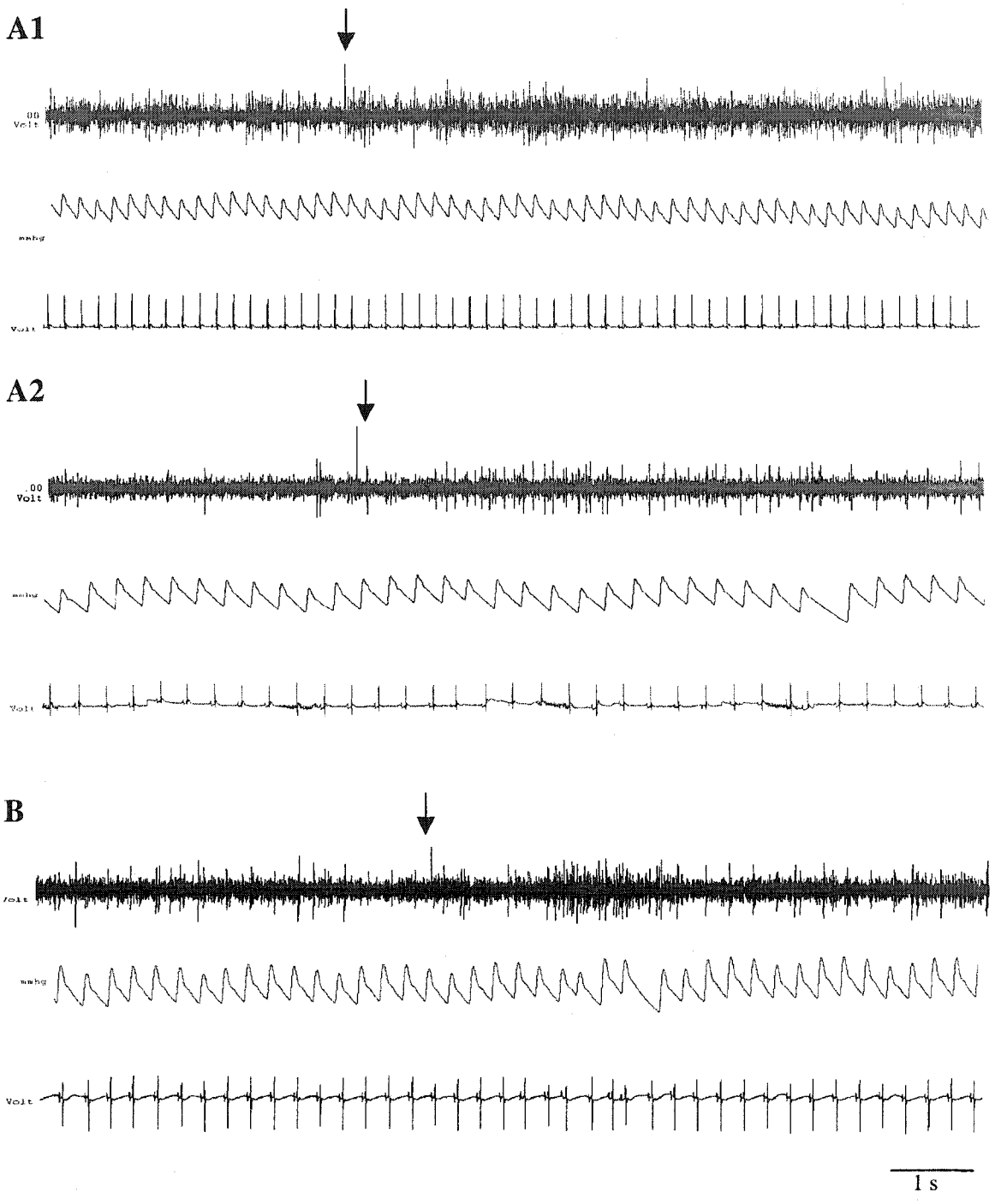


Figure 7. Raw data traces of effects of 25 μl balloon inflation (arrow) on atrial receptor afferent (trace 1), arterial blood pressure (trace 2), and ECG (trace 3) in nonpregnant (A) and pregnant (B) rats. A1 from nonpregnant animal classified as HF, A2 as LF.

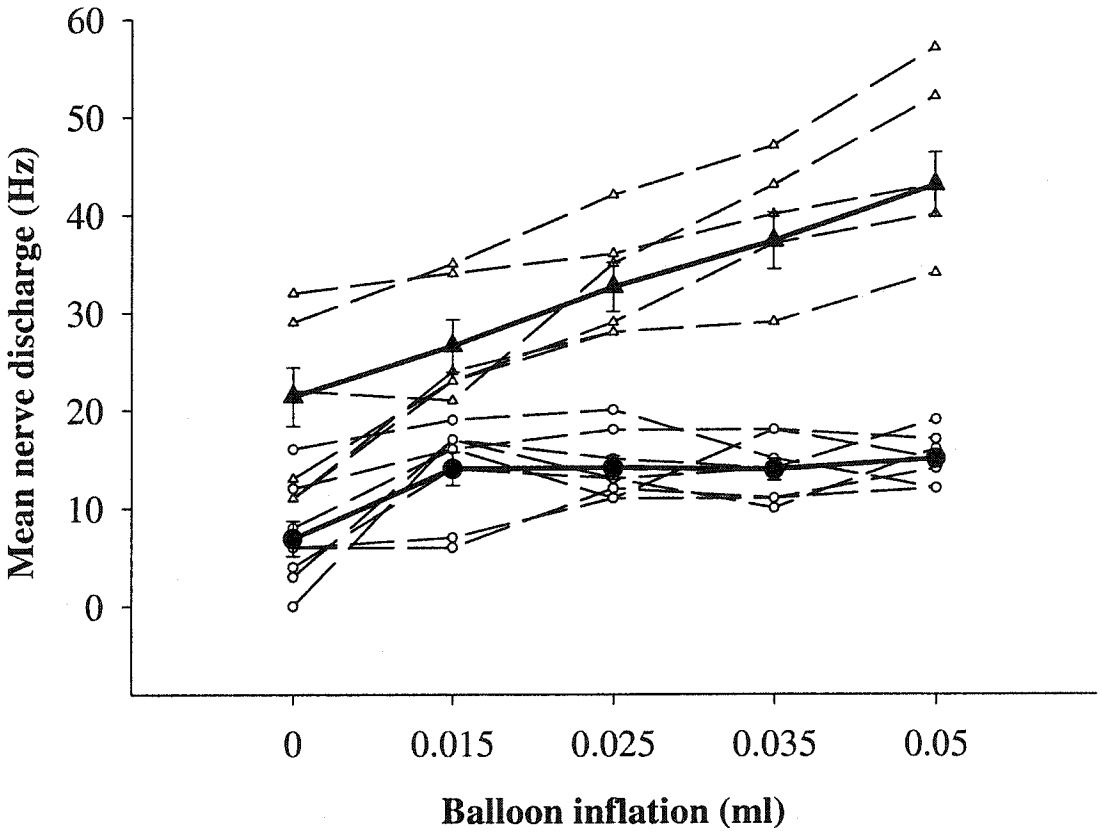


Figure 8. Low and high frequency atrial receptor discharge in nonpregnant rats (n=8). Individual low (○) and high (Δ) frequency receptors represented by interrupted lines, mean fiber discharge (●, ▲) represented by solid lines with vertical lines delineating SE of mean.

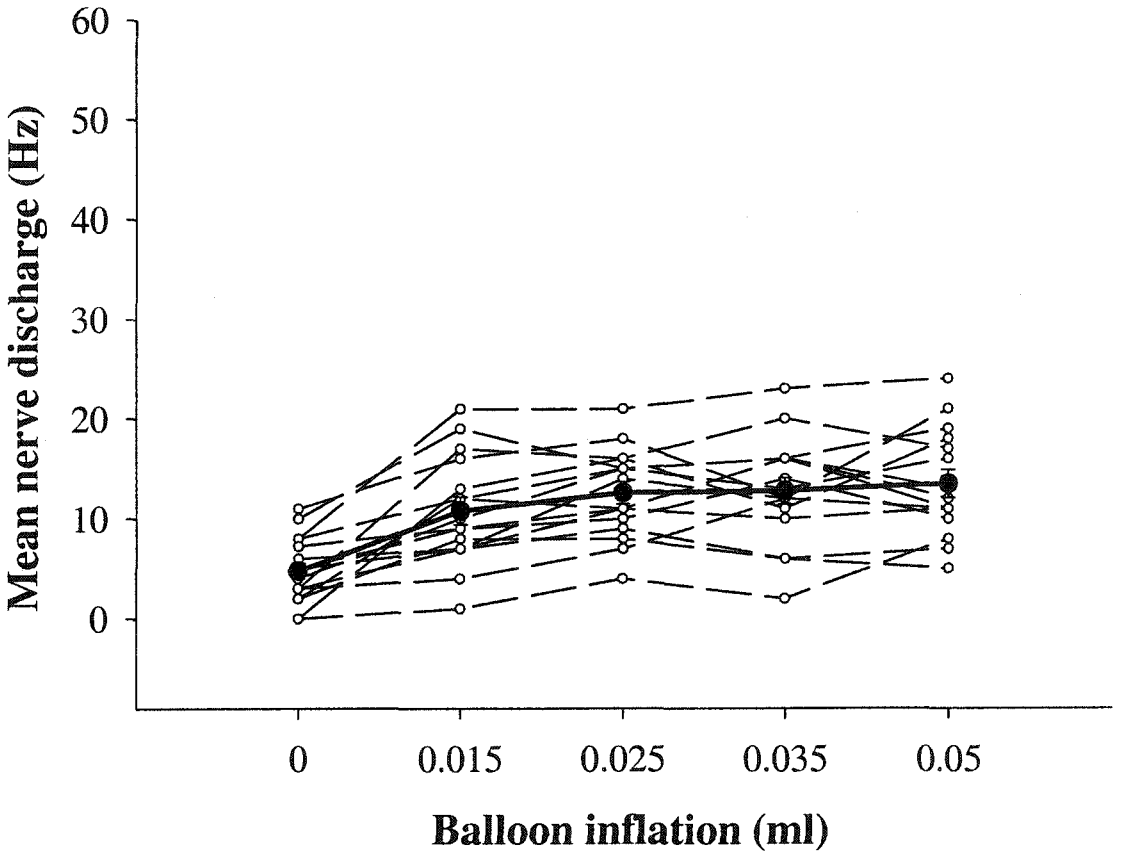
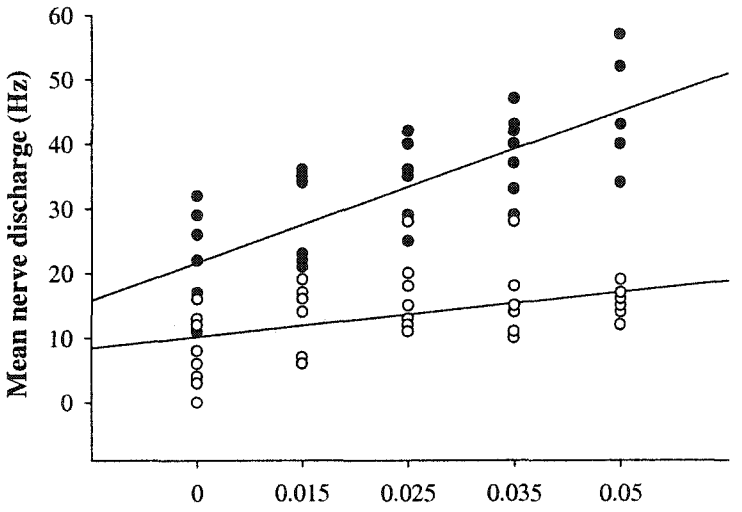


Figure 9. Low frequency atrial receptor discharge in pregnant rats (n=8). Individual low (○) frequency receptors represented by interrupted lines, mean fiber discharge (●) represented by solid lines with vertical lines delineating SE of mean.

Stimulated discharge of LF receptors in nonpregnant rats was unrelated to the degree of atrial distension caused by balloon inflation. This is shown by the weak relationship between afferent discharge of receptors and atrial stretch (i.e. balloon inflation) in nonpregnant rats (slope=0.20±0.04, $P>0.05$) (Fig. 8A). In contrast, discharge of HF receptors in nonpregnant rats had a significantly greater degree of relation to balloon distension (slope=0.55±0.02, $P<0.05$) (Fig. 8A). In the pregnant rats the LF receptors responded to atrial distension with discharge that showed a very weak relation to the degree of stretch of the atrium (slope=1.9±0.03, $P>0.05$) (Fig. 8B). The response of the LF receptors in both the nonpregnant and pregnant rats plateaued at an inflation of 25 µl. None of the receptors crossed over from a low to a high frequency discharge pattern: receptors with low or high spontaneous discharge retained these discharge properties with application of the stimulus of atrial stretch.

A.



B.

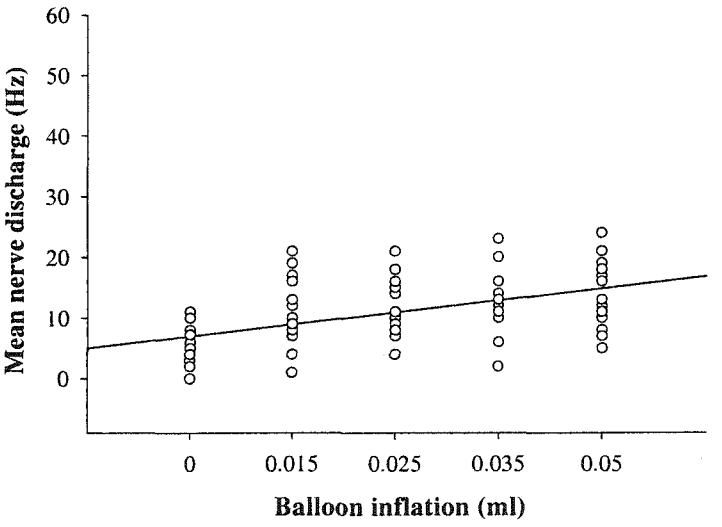


Figure 10. Scatter plot of relationship between balloon inflation and discharge frequency of high (●) and low (o) frequency receptors from nonpregnant (n=8, A) and pregnant (n=8, B) rats.

EXPERIMENT B: EFFECT OF ACUTE PREGNAN ON ATRIAL RECEPTOR AFFERENT DISCHARGE.

MAP of rats given an intravenous injection of pregnan (95.2±4.4 mmHg, n=8) was comparable to that seen in baseline recordings (100.8±7.5 mmHg) and following injection of vehicle (110±2.3 mmHg, n=8). As in experiment A, balloon inflation had no effect on MAP in any animal (pre-inflation: 111.3±3.1 mmHg, post-inflation: 103.9±3.6 mmHg).

Seventeen functional single fibers were dissected in a total of 16 rats, with nine of the receptors stimulated in the presence of drug, and eight in the presence of vehicle. Each receptor responded to graded balloon inflation with an increase in discharge frequency and was localized to the right venoatrial junction (Fig.5). Forty-one percent of the receptors localized to the venoatrial junction were classified as HF receptors. The mean spontaneous discharge of LF receptors (4.9±1.0 Hz) was significantly lower than that of HF receptors (19.7±2.4 Hz) ($P<0.05$).

Infusion of pregnan or vehicle into the femoral vein had no effect on spontaneous discharge of low (pregnan: 6.5±1 Hz, vehicle: 5.4±2.1 Hz) or high (pregnan: 18.8±2.3 Hz, vehicle: 19.0±5.0 Hz) frequency receptors in comparison with baseline recordings (LF: 4.9±1.0 Hz, HF: 19.7±2.4 Hz) (Fig 9).

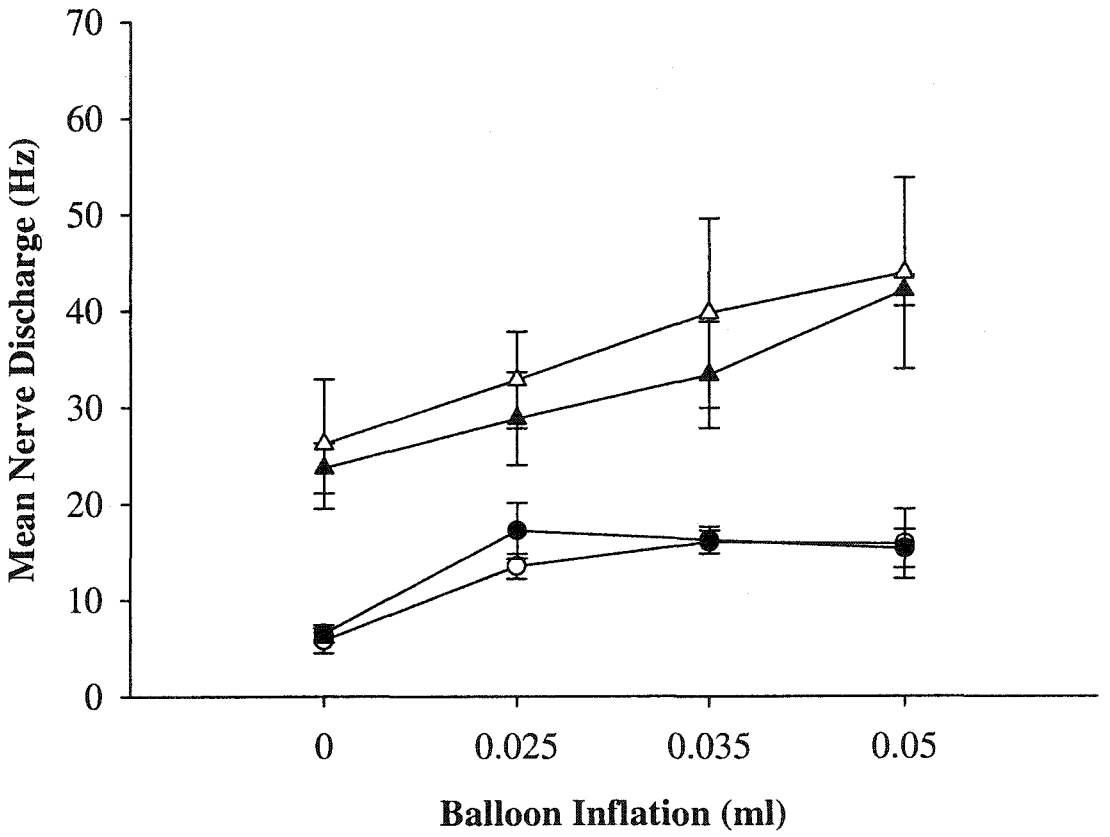
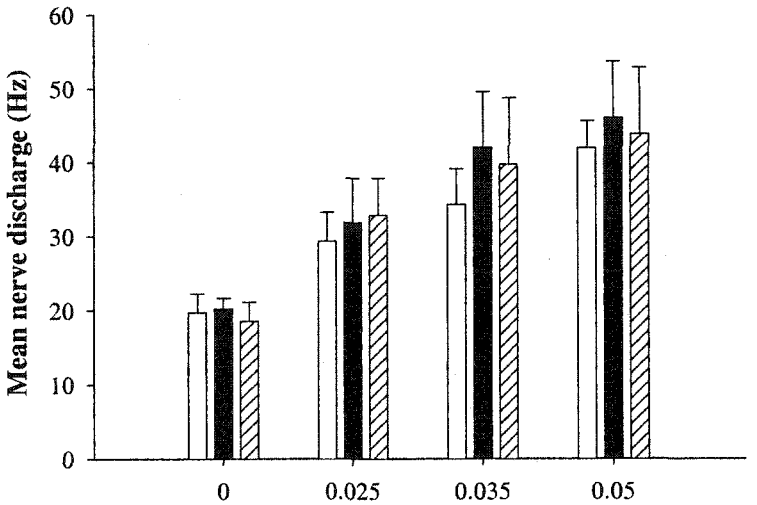


Figure 11. Mean discharge of low (circle) and high (triangle) frequency atrial receptors in animals infused i.v. with 0.2 ml of 2.6 μg/kg i.v. 5α-Pregnan-3α-ol-20-one (black, n=9) or 0.2 ml 20% 2-hydroxypropyl-β-cyclodextrin (white, n=8). Values shown are average discharge at time points 3, 5 and 10 minutes following drug/vehicle injection. Values presented as mean ± S.E., and a P value < 0.05 was considered significant.

As was found in experiment A, stimulated discharge of receptors with a low spontaneous discharge frequency was unrelated to the degree of atrial stretch due to balloon inflation (slope=0.19±0.03, $P>0.05$). This response was unaltered by i.v. infusion of pregnan (slope=0.19±0.05, $P>0.05$) or vehicle (slope=0.16±0.03, $P>0.05$) (Fig 10). Similar to the results of experiment A in nonpregnant rats, receptors with a high spontaneous discharge had a stimulated discharge more significantly related to the degree of atrial distension than was observed for LF receptors (slope=0.44±0.10, $P<0.05$). As was observed in LF receptors, infusion of pregnan (slope=0.62±0.1) or vehicle (slope=0.46±0.1) had no effect on this discharge ($P>0.05$).

A.



B.

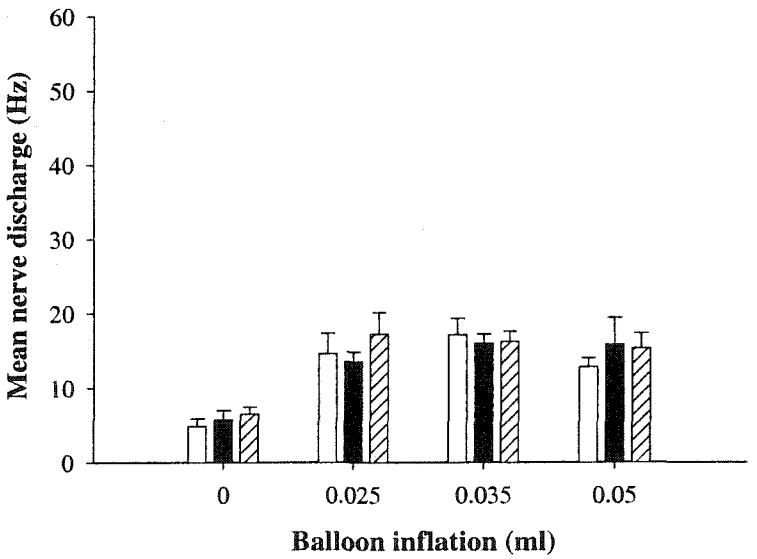


Figure 12. Mean nerve discharge of high (A) and low (B) frequency atrial receptors during baseline (n=17, white bars) and following acute administration of 20% 2-hydroxypropyl-β-cyclodextrin (n=8, black bars) or 5α-Pregnan-3α-ol-20-one (n=9, hatched bars). Values presented as mean ± S.E., and a P value < 0.05 was considered significant.

EXPERIMENT C: EFFECT OF CHRONIC PREGNAN ON ATRIAL RECEPTOR AFFERENT DISCHARGE.

MAP of rats chronically treated with pregnan (94.3±2.6 mmHg, n=6) was not different from that of rats treated with vehicle (88.1±4.8 mmHg, n=7). As shown in experiment A, balloon inflation had no effect on MAP in either pregnan or vehicle-treated animals (pre-inflation: 95.4±3.5 mmHg, post-inflation: 92.7±3.2 mmHg).

Twenty-two functional single fibers were dissected in 6 pregnan-treated and 7 vehicle-treated rats. Each receptor localized in the experiment responded to graded balloon inflation with an increase in afferent discharge (Fig. 11). Roughly 36% of receptors localized to the venoatrial junction discharged with an average spontaneous frequency of 18.8±7.2 Hz, while the remainder discharged with a significantly lower spontaneous frequency of 6.4±1.2 Hz ($P<0.05$).

In contrast to the two populations of receptors localized to the venoatrial junction in vehicle-treated rats, nearly all receptors localized in pregnan-treated rats discharged in the low frequency range; only one fiber was found with a spontaneous discharge of >20 Hz (Fig. 12). The mean spontaneous discharge of these receptors (7.9±1.6 Hz) did not differ from that of low-frequency receptors in vehicle-treated rats ($P>0.05$).

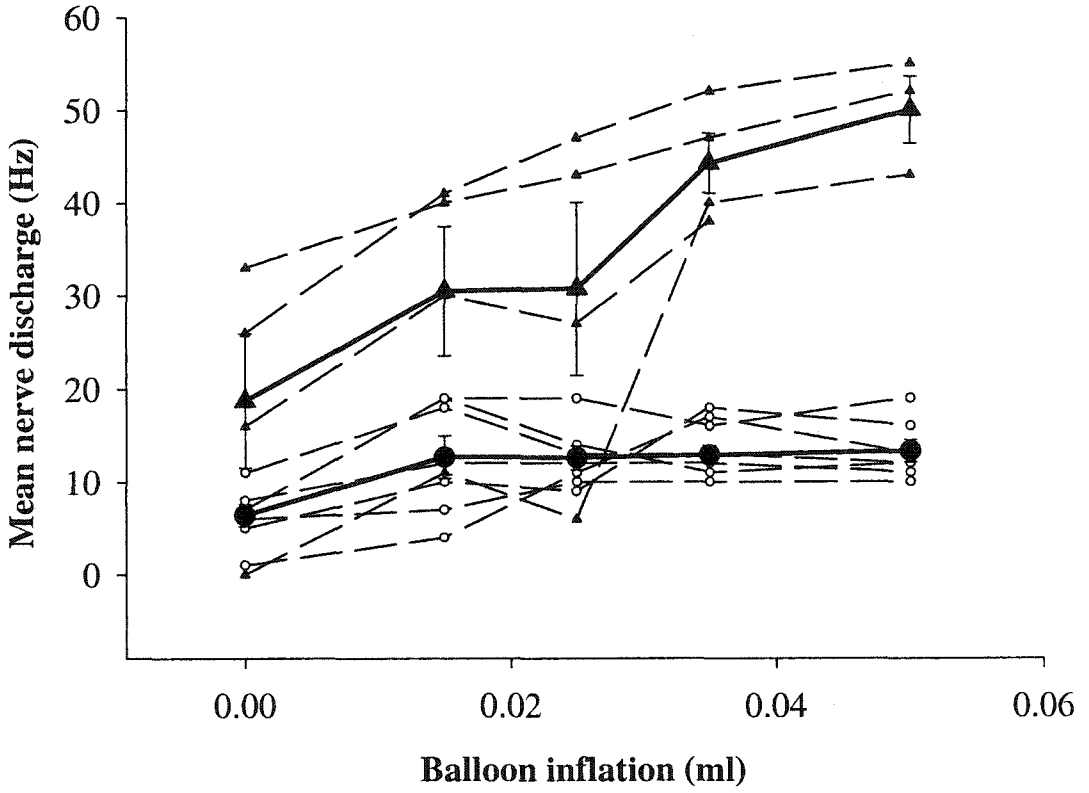


Figure 13. Low and high frequency atrial receptor discharge in vehicle-treated (400 μl 20% 2-hydroxypropyl-β-cyclodextrin s.c. for two days, n=8) rats. Individual low (●) and high (▲) frequency receptors represented by small symbols, mean fiber discharge represented by larger symbols with vertical lines delineating SE of mean.

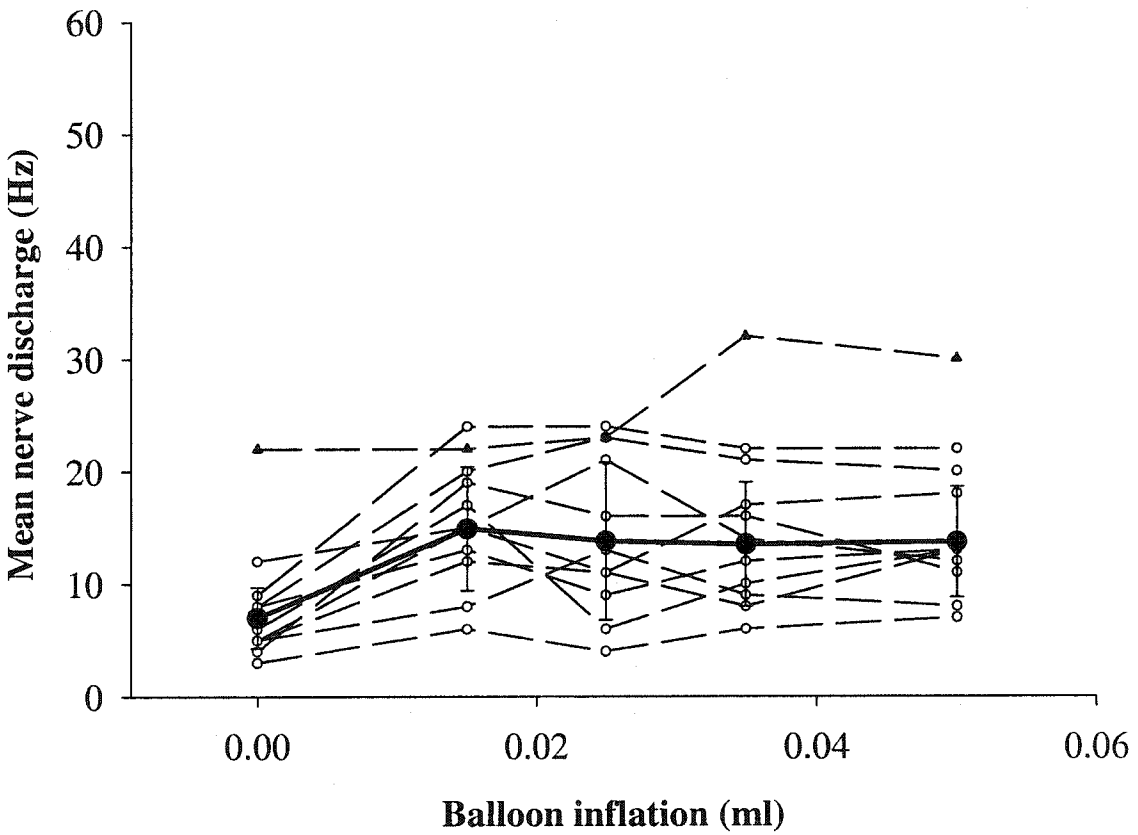
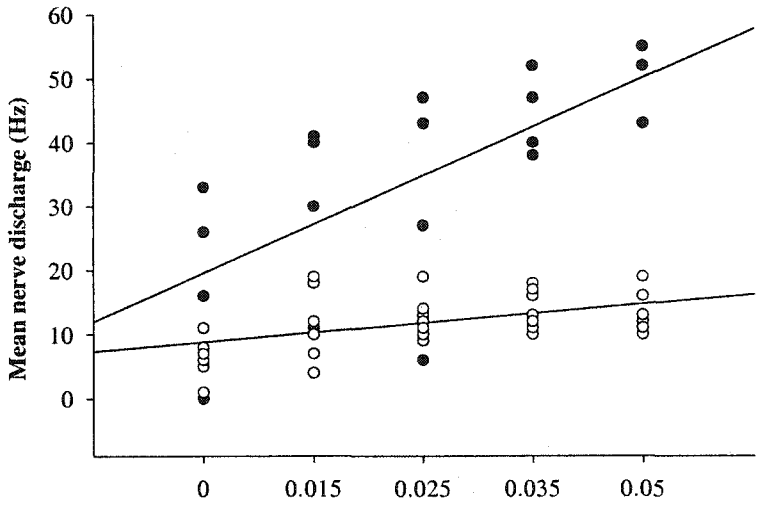


Figure 14. Atrial receptor discharge in animals chronically treated with pregnan (500 μg in 400 μl cyclodextrin s.c. for two days, n=9). Individual low (●) and high (▲) frequency receptors represented by small symbols, and mean nerve discharge represented by larger symbols with vertical lines delineating SE of mean.

Consistent with the observations of experiments A and B, receptors with a low spontaneous frequency in vehicle-treated rats responded to balloon inflation with discharge unrelated to the degree of atrial distension (slope=0.13±0.04, P>0.05) (Fig. 13A). Receptors in pregnan-treated rats also displayed a similarly weak relationship between atrial distension and mean nerve discharge (slope=0.11±0.03, P>0.05) (Fig. 13B). In addition, both LF receptors in vehicle-treated rats and receptors in pregnan-treated rats had a stimulated discharge which plateaued following the first volume of inflation (0.025 ml), as was shown in experiments A and B. Receptors with a high-frequency spontaneous discharge in vehicle-treated animals were found to have the same properties as those described in experiments A and B; there was a significantly stronger relationship between atrial distension and receptor discharge than was found in LF receptors (slope=0.62±0.1, P<0.001) (Fig. 7A). The single receptor with a spontaneous frequency greater than 10 Hz localized to the right venoatrial junction of a pregnan-treated rat responded to graded atrial distension with discharge unrelated to the degree of stimulus (slope=0.21, P>0.05), unlike all other receptors in the HF subgroup.

A.



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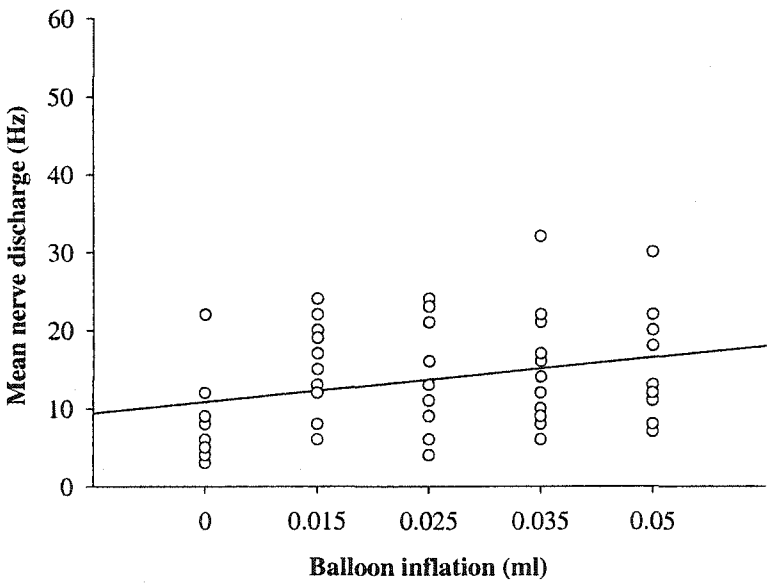


Figure 15. Scatter plot of relationship between balloon inflation and discharge frequency of high (●) and low (○) frequency atrial receptors from vehicle (400 µl 20% 2-hydroxypropyl-β-cyclodextrin s.c. for two days, n=8, A) and pregnan-treated rats (500 µg in 400 µl cyclodextrin s.c. for two days, n=9, B).

EXPERIMENT D: EFFECT OF ADRENOMEDULLIN ON *C-FOS* EXPRESSION IN THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS.

MAP was unaffected by balloon inflation (pre: 90.3±6.4 mmHg, post: 89.6±6.7 mmHg), as was heart rate (pre: 380.6±14.1 b/min, post: 389.7±13.8 b/min). Infusion of neither the low nor the high dose of ADM had any effect on MAP, either overall or in the 5 minute time period immediately following drug administration (low: 103.2±4.9 mmHg, high: 99.6±2 mmHg) (Fig. 14A). In addition, there was no significant change in heart rate in the 5 minute period following administration of the bolus (high) dose of ADM (pre-admin.: 367.5±12.4 b/min, post-admin.: 379.8±9.4 b/min) (Fig. 14B).

Inflation of the intracardiac balloon increased *c-fos* expression in the PVN (189±14) to a level significantly greater than that seen in control animals (11±1, $P<0.05$) (Fig. 15). As shown in Figure 16, this increase was concentrated in the medial part (m) of the parvocellular division (98±8), though expression was also seen in the pv (15±3), d (13±1) and p (29±2) parvocellular subdivisions.

Relative to the control group (AD+vehicle), increased *c-fos* expression in the PVN in response to balloon inflation was reduced in animals that received the bolus dose of ADM (179±13), and this reduction reached significance in animals that received the 10-fold higher dose of ADM (126±10, $P<0.05$). This decrease in *c-fos* expression in response to ADM administration was localized to the mp, pv and p subdivisions of the

parvocellular division of the nucleus (Fig. 16). A similar trend was observed in the dorsal subdivision with the higher dose of ADM, but this did not reach significance. In addition, ADM alone increased c-fos expression in the PVN to a level nearly greater than that seen when only the stimulus of atrial stretch was given (229±1). The effect of ADM alone was again concentrated in the mp subdivision (99±3), with increases also observed in the p subdivisions (27±2).

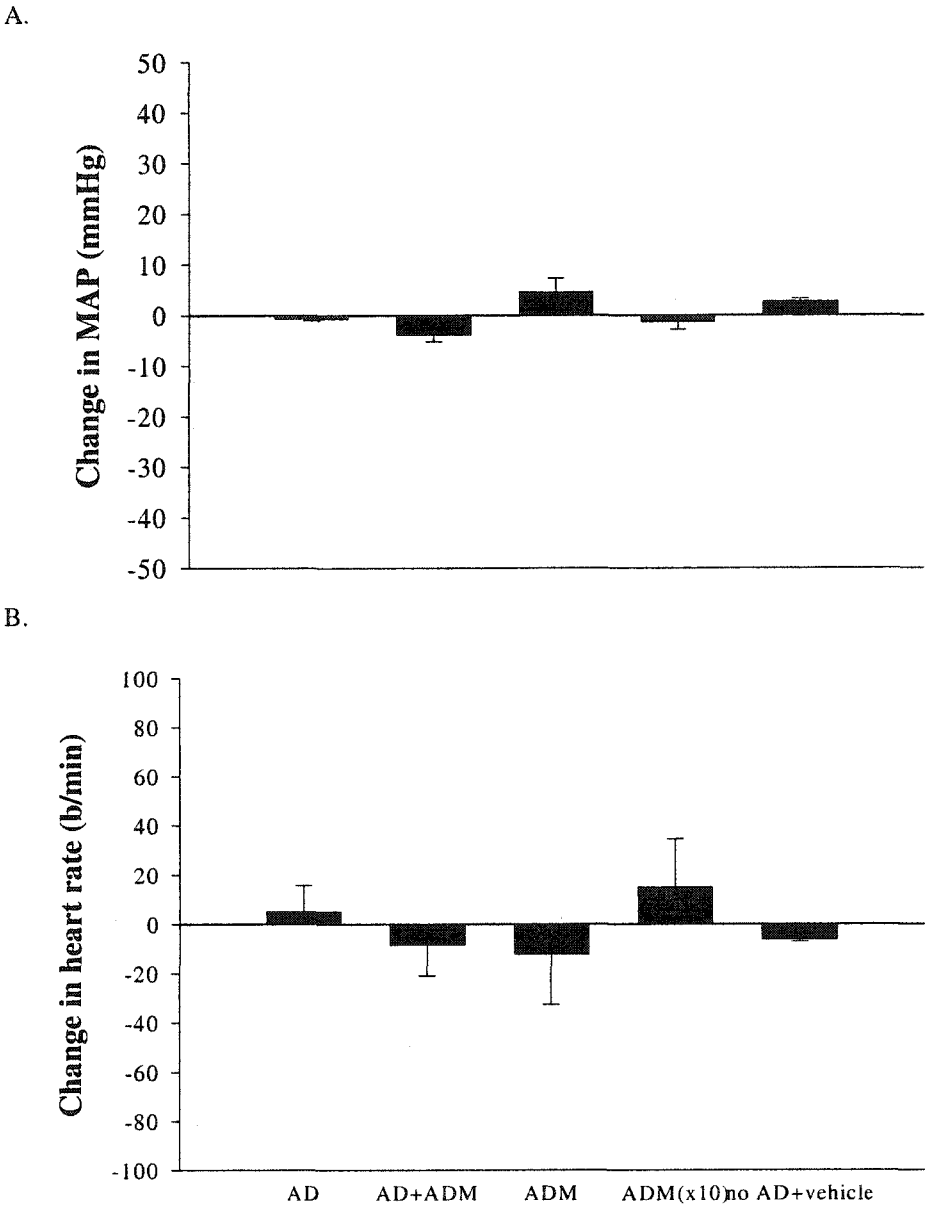


Figure 16. Effect of atrial distension with ADM (15 ng/0.2 ml)/ ADM (x10) (150 ng/0.2 ml)/ vehicle (0.2 ml saline) infusion on mean arterial pressure (A) and heart rate (B) in female virgin rats implanted with indwelling intracardiac balloons. Vertical bars delineate SE.

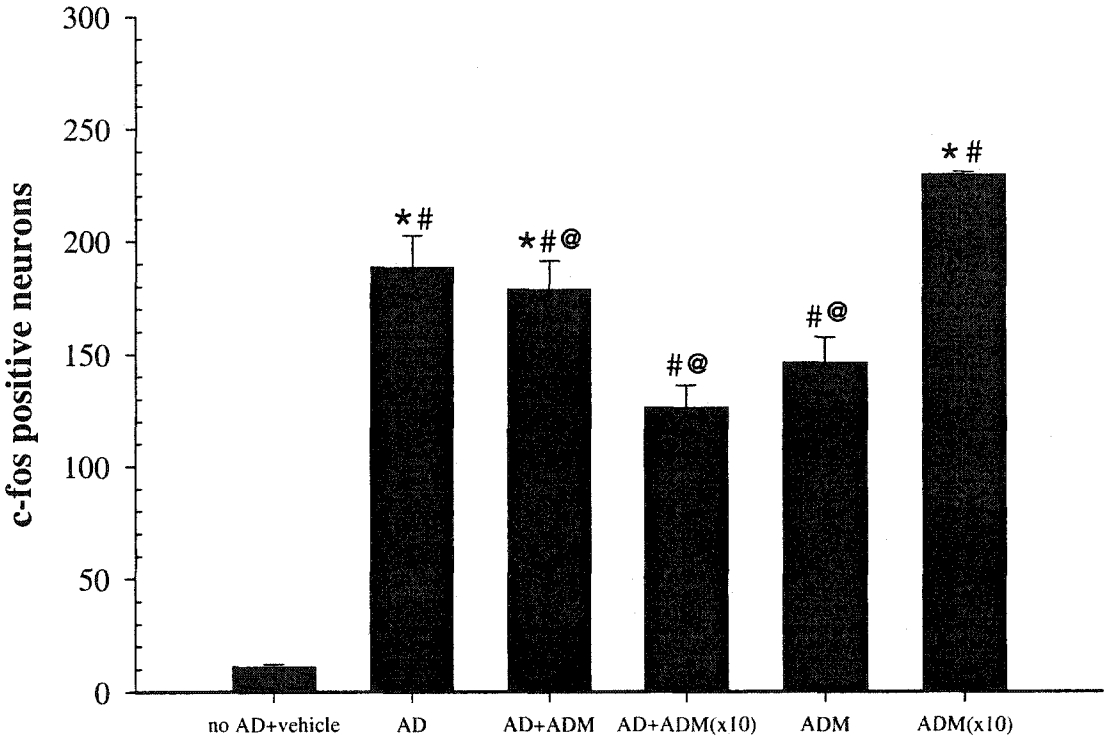


Figure 17. Effect of atrial distension with infusion of ADM (15 ng/0.2 ml)/ ADM (x10) (150 ng/0.2 ml)/ vehicle (0.2 ml saline) on *c-fos* expression in the PVN of virgin female rats implanted with indwelling intracardiac balloons. Vertical lines delineate SE of mean. *, Significant difference in *c-fos* expression compared with group AD+ADM (x10); #, compared with group no AD+vehicle, and @, compared with group ADM (x10). One-way ANOVA plus Fisher LSD for multiple comparisons.

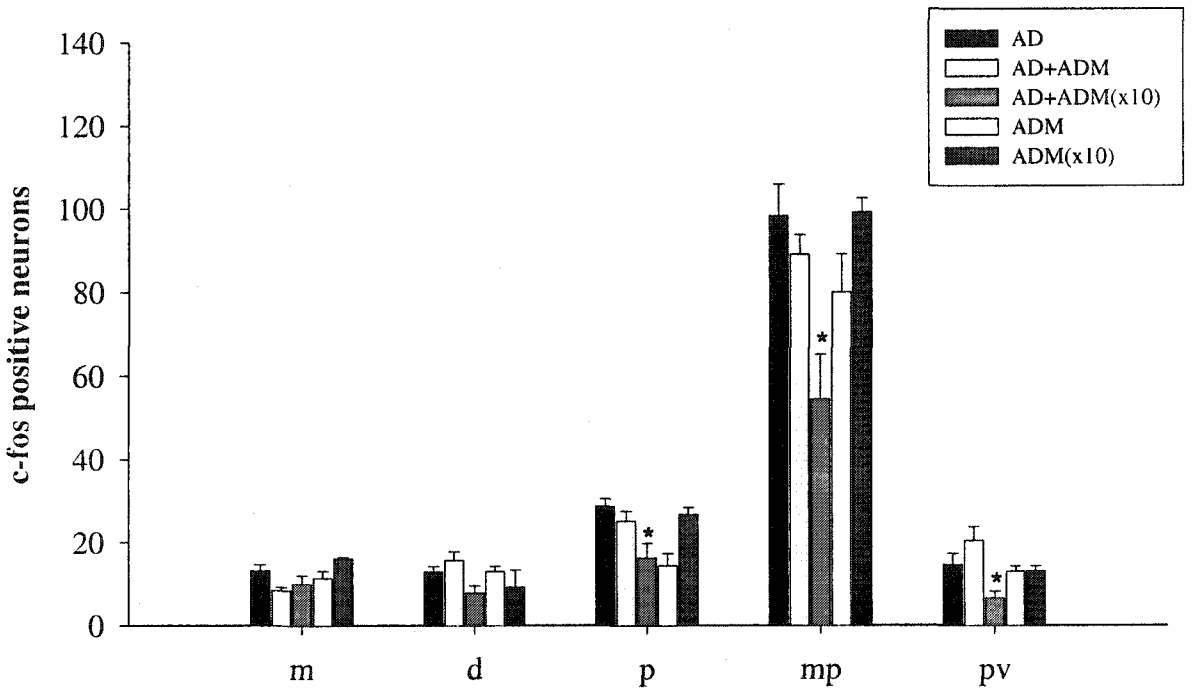


Figure 18. Effect of atrial distension with/without ADM infusion on *c-fos* expression in specific subdivisions of the PVN (m= magnocellular, d= dorsal, p= posterior, mp= medial parvocellular, pv= periventricular) of female virgin rats implanted with indwelling intracardiac balloons. Vertical bars delineate SE of mean. *, Significant difference in *c-fos* expression compared with group AD+vehicle. One-way ANOVA plus Fisher LSD for multiple comparison.

EXPERIMENT E: EFFECT OF ADRENOMEDULLIN ON PLASMA VOLUME.

Mean plasma volume of animals infused with either the high (4.7±0.3 ml/100g b. wt.) or the low (4.2±0.2 ml/100g b. wt.) dose of ADM tended to be higher that that of animals infused with saline, (4.0±0.2 ml/100g b. wt.), but was not significantly different (P>0.05). In addition there was no difference in mean blood volume for rats treated with saline (19.6±0.8 ml) in comparison with those treated with either the low (20.2±0.7 ml) or the high (23.1±1.5 ml) doses of ADM (P>0.05) (Fig. 17).

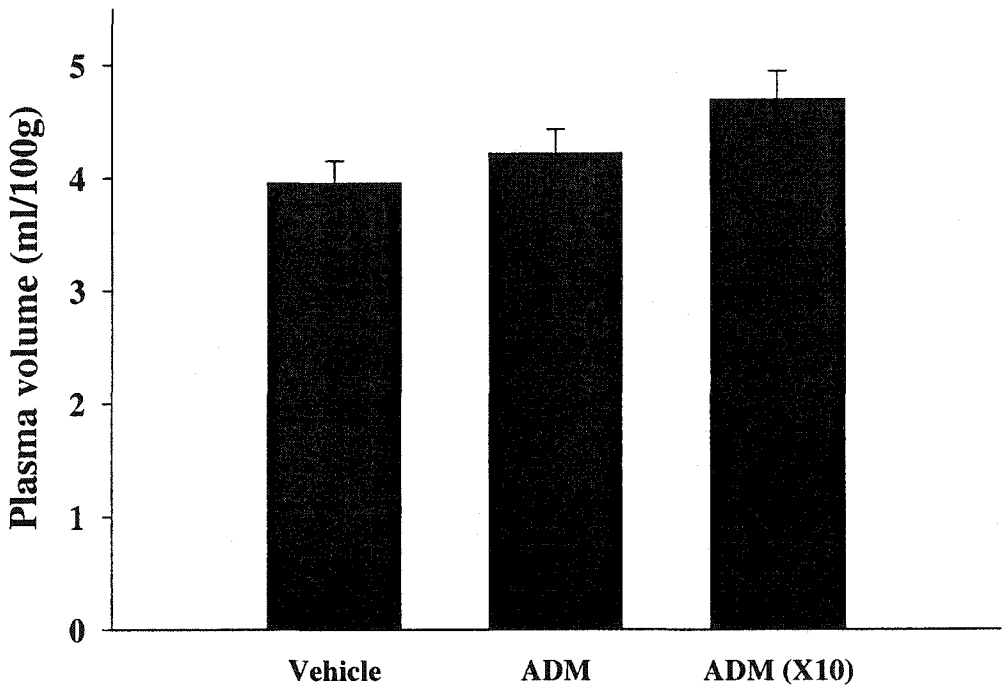


Figure 19. Mean plasma volume of animals infused with either ADM (11.25 ng/3 ml/hr, n=6), ADM X10 (112.5 ng/3 ml/hr, n=4) or vehicle (3 ml saline/hr, n=5). Vertical bars delineate SE of the mean. One-way ANOVA for comparison between groups.

EXPERIMENT F: EFFECT OF ADRENOMEDULLIN ON ATRIAL RECEPTOR AFFERENT DISCHARGE.

Baseline MAP was unaffected by balloon inflation (pre-inflation: 96.2±5.6 mmHg. post-inflation: 87.2±5.9 mmHg, n=6). With intra-atrial injection of ADM, there was a tendency for a decrease in MAP from the baseline value (89.1±5.2 mmHg) to 78.8±6.6 mmHg, which did not reach statistical significance. MAP had fully recovered by the fifth minute following administration of the bolus (87.9±3.7 mmHg, n=5).

Eight functional single fibers were dissected in a total of 6 rats, with 5 fibers stimulated in the presence of both vehicle and drug, one in the presence of vehicle alone, and 2 in the presence of drug alone. Each receptor responded to graded balloon inflation with an increase in discharge frequency and was localized to the right venoatrial junction. Sixty-three percent of the receptors localized to the venoatrial junction were classified as HF receptors. The mean spontaneous discharge of LF receptors (9.0±2.1 Hz) was significantly lower than that of HF receptors (18.6±2.0 Hz) ($P>0.05$).

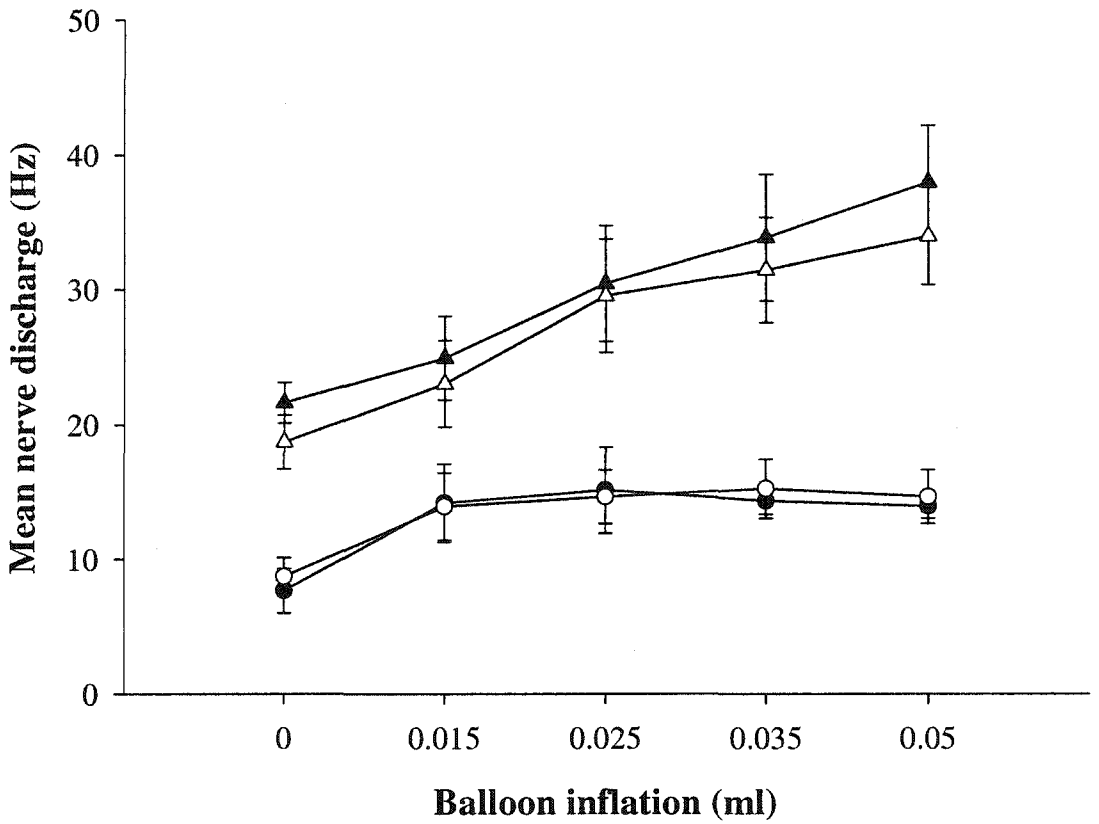
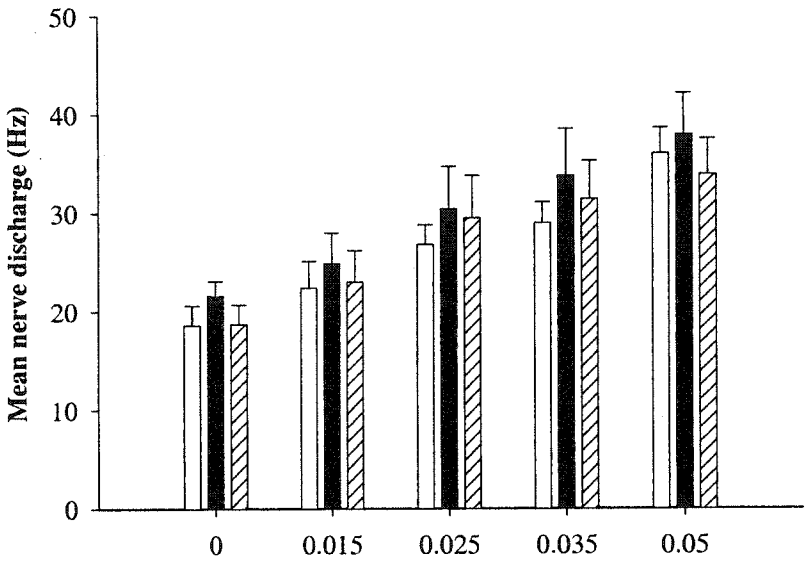


Figure 20. Mean discharge of low (circle) and high (triangle) frequency atrial receptors in animals infused with ADM (100 ng/0.2 ml, n=7, black) or vehicle (0.2 ml saline, n=6, white).

Infusion of ADM or vehicle into the external jugular had no effect on spontaneous discharge of low (vehicle: 10.3±1.0 Hz, ADM: 10.5±2.5 Hz) or high (pregnan: 21.7±1.5 Hz, vehicle: 18.7±2.0 Hz) frequency receptors in comparison with baseline recordings (Fig. 18).

As was found in previous experiments, stimulated discharge of receptors with a low spontaneous discharge frequency was unrelated to the degree of atrial stretch due to balloon inflation (slope=0.12±0.03). This response was unaltered by intra-atrial infusion of ADM (slope=0.11±0.03, $P>0.05$) or vehicle (slope=0.11±0.05, $P>0.05$) (Fig. 19B). Receptors with a high spontaneous discharge had a stimulated discharge more significantly related to the degree of atrial distension than was observed for LF receptors (slope=0.35±0.05, $P<0.05$) (Fig. 19A). As was observed in LF receptors, infusion of ADM (slope=0.32±0.06) or vehicle (slope=0.35±0.06) had no effect on this discharge ($P<0.05$).

A.



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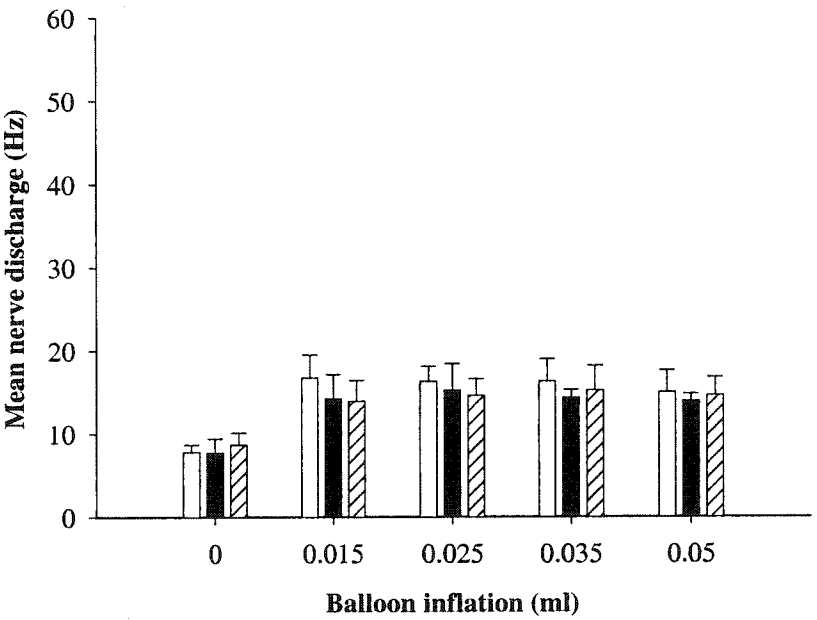


Figure 21. Mean nerve discharge of high (A) and low (B) frequency atrial receptors during baseline (n=8, white bars) and following administration of vehicle (0.2 ml saline, n=6, black bars) or ADM (100 ng/0.2 ml saline, n=7, hatched bars).

EXPERIMENT G: EFFECT OF SNAP ON ATRIAL RECEPTOR AFFERENT DISCHARGE.

Baseline MAP was unaffected by balloon inflation (pre-inflation: 89.2±2.2 mmHg vs. post-inflation: 86.9±2.7 mmHg, n=8). With infusion of SNAP, MAP dropped to 64.12±4.1 mmHg and recovered after the infusion was completed (n=6).

Ten functional single fibers were dissected from the right vagus in a total of 8 rats. Five receptors were studied during SNAP infusion, four during saline infusion, and one with both vehicle and SNAP. Each receptor responded to graded balloon inflation with an increase in discharge frequency and was localized to the right venoatrial junction. Twenty-five percent of the receptors localized to the venoatrial junction were classified as HF receptors. The mean spontaneous discharge of LF receptors (3.1±0.5 Hz) was significantly lower than that of HF receptors (12.0±5.0 Hz) ($P<0.05$).

Infusion of SNAP or vehicle into the femoral vein had no effect on spontaneous discharge of low (vehicle: 4.6±2.7 Hz, SNAP: 5.9±1.9 Hz) or high (vehicle: 12 Hz, SNAP: 11.1±2.4 Hz) frequency receptors in comparison with baseline recordings shown above (Fig. 20).

Receptors with a low spontaneous discharge frequency responded to graded balloon inflation with a discharge unrelated to the degree of stimulus (slope=0.13±0.04), while high frequency receptors showed a response with a greater degree of relation to the

stimulus (slope=0.31±0.10). Discharge of LF receptors at 25 μl volume inflation (11.2±0.8 Hz) was unaffected by infusion of either vehicle (10.1±0.7 Hz) or SNAP (11.4±1.0 Hz) into the femoral vein (Fig. 21B). An increase in inflation volume to 50μl did not significantly increase mean nerve discharge (9.6±0.9 Hz), and infusion of vehicle (8.7±0.8 Hz) and of SNAP (11.2±1.2 Hz) had no effect. Similar results were seen at 25 μl inflation for receptors with high spontaneous discharge frequency: discharge at baseline (18.5±2.5 Hz) was unaffected by infusion of vehicle (15 Hz) or SNAP (18.5±0.5 Hz) (Fig. 21A). A further increase in discharge frequency was seen with additional inflation to a volume of 50μl (24.5±1.5 Hz), but again, this was unaffected by vehicle (20 Hz) or SNAP (23.1±0.5 Hz) infusion.

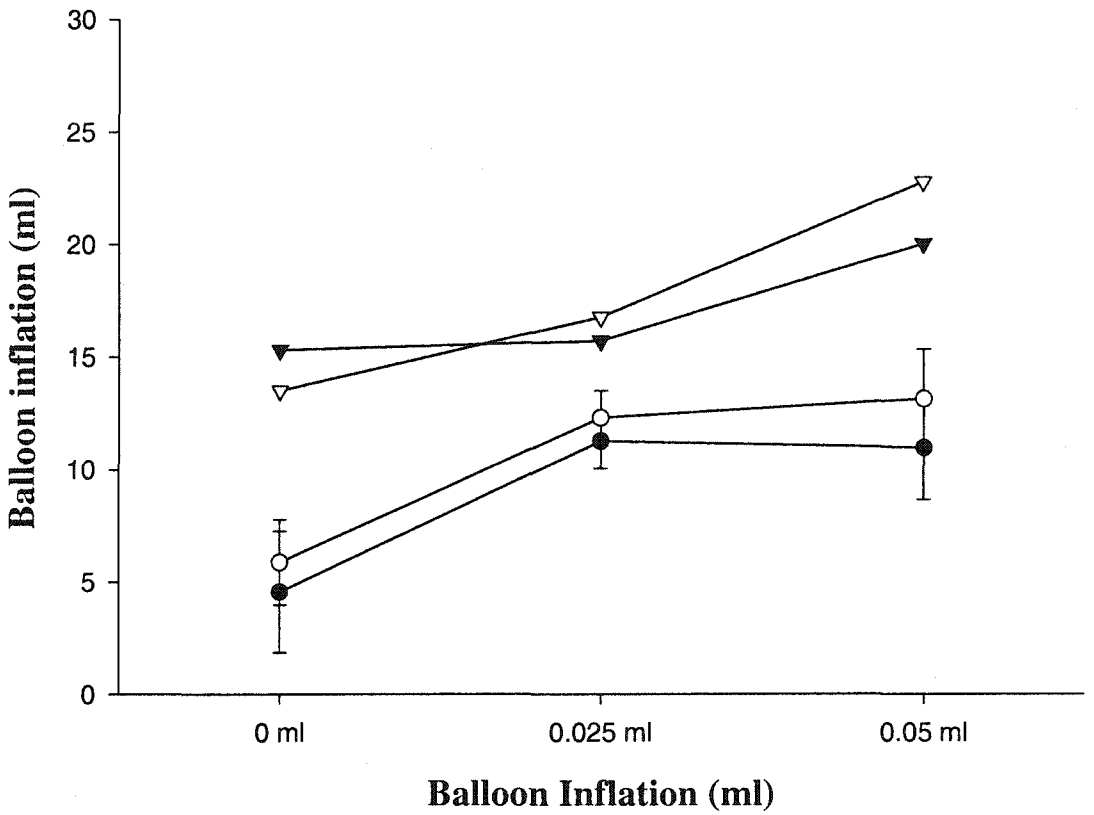
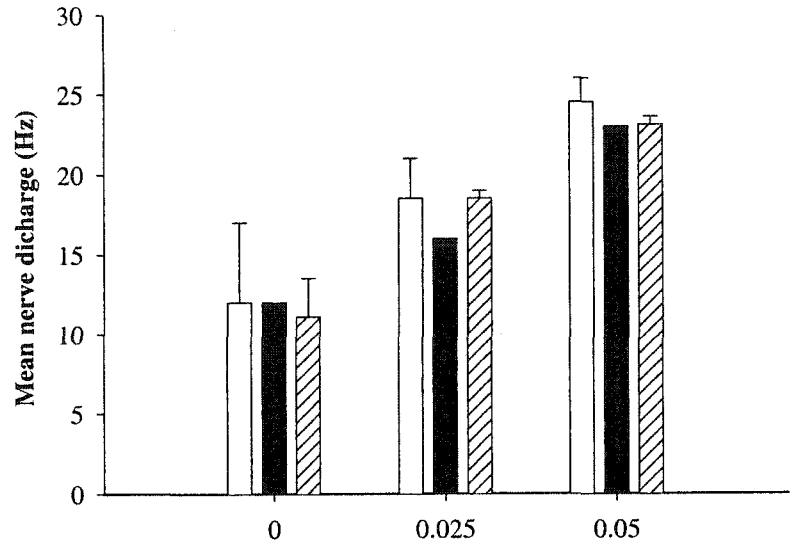


Figure 22. Mean discharge of low (circle) and high (triangle) frequency atrial receptors in animals infused with SNAP (10 μg/50 μl/min, n=6, black) or saline (50 μl/min, n=5, white).

A.



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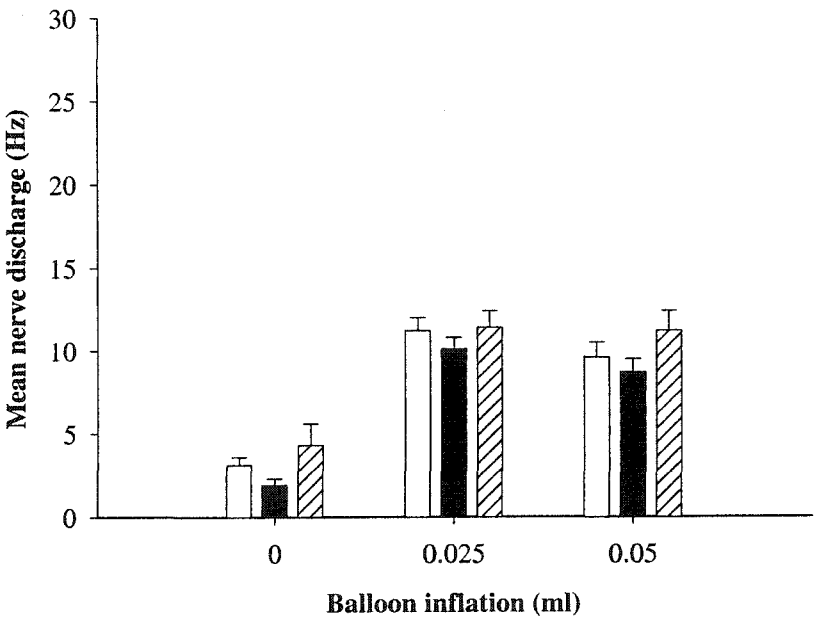

Figure 23. Mean nerve discharge of high (A) and low (B) frequency atrial receptors during baseline (n=8, white bars) and following infusion of vehicle (50 μl/min, n=5, black bars) or SNAP (10 μg/50 μl/min, n=6, hatched bars).

CHAPTER 4.

DISCUSSION

EXPERIMENT A: EFFECT OF PREGNANCY ON ATRIAL RECEPTOR AFFERENT DISCHARGE.

These *in vivo* experiments were designed to investigate whether the afferent limb of the atrial volume receptor reflex is altered in pregnancy. Previous work has shown that central processing of the atrial volume receptor reflex is attenuated in pregnancy: activation of neurons in the hypothalamus in response to stimulation of the atrial receptors is reduced in pregnant rats compared with nonpregnant rats (42). This experiment was designed to investigate whether the loss of central processing is due to an absence of afferent input from sensory receptors.

Atrial receptor responses have varied in different studies depending on the stimulus used to activate them (71). All previous research characterizing atrial receptor discharge *in vivo* has measured receptor response to an increase in atrial pressure created by altering blood flow through the heart, or by administering a bolus of fluid to the atrium (71,84,199). While these techniques stimulate atrial receptors, they also stimulate other stretch-sensitive receptors in the heart, and, when not in conjunction with receptor localization, make it difficult to determine which receptor group has responded (71). Use of the technique of intracardiac balloon inflation, as described in the present study, ensures a localized, discrete stimulus to receptors located at the venoatrial junction (105). Other hemodynamic variables are not affected by this localized distension of the junction between the superior vena cava and the atrium; with a slow rate of inflation, there is no subsequent change in heart rate or arterial blood pressure (105). It has been noted that rapid inflation can lead to a transient depressor response, though this is more likely due to

misplacement of the balloon during inflation than to any reflex response (104). Perhaps most importantly, use of balloon inflation ensures the same stimulus is administered to every animal. While previous studies examining receptor response to an increase in atrial pressure in pregnant animals have been complicated by the higher basal atrial pressure in these animals, the technique of balloon inflation allows receptor responses to be examined independent of the preexisting state of volume expansion of each animal (107).

Use of the balloon to stimulate atrial receptors does however complicate measurement of conduction velocity of afferent fibers, due to the difficulty of estimating the exact time atrial stretch begins. However, given the findings of Thorén and Hines that there are no myelinated cardiovascular afferent fibers in the rat, it is reasonable to assume that the fibers localized in this study are nonmyelinated, slowly-conducting C-fibers (84,199). It is interesting to note that the spontaneous discharge of both LF and HF receptors was significantly greater than that shown in the study by Thoren (199). In the present study, spontaneous discharge of LF receptors generally fell just below 10 Hz, while that of HF receptors was above this frequency. This could be explained by the volume loading of the animals through a continuous infusion of saline at a rate of 3 ml/hr during the course of the experiment, leading to a greater resting discharge of both LF and HF receptors (with a more significant increase in that of HF receptors, due to a lower threshold of activation). Differences from previous studies were also noted in receptor response to the stimulus of atrial stretch. Specifically, the high frequency receptor response to balloon inflation in this study differed from the response of HF fibers to an increase in atrial pressure in the study by Thorén (199). While Thorén describes a cardiac cycle-dependent

discharge pattern in these receptors, only a very small number of HF fibers in this study fired in sequence with the electrical activity of the heart (199). This difference in receptor response is again most likely due to the difference in stimulus used. Whereas the stimulus of atrial stretch in Thorén's study was provided by increasing atrial pressure via occlusion of the ascending aorta, the intra-cardiac balloon provides a direct, static stretch to the atrium, which would tend to dissociate the receptors from the cyclic activity of the heart. This emphasizes the dependence of atrial receptors on their position in the atrial tissue, i.e. response patterns are a direct result of where in the venoatrial junction the receptor is located (71).

In this study, atrial receptors in nonpregnant rats were divided into low and high-frequency subsets based on their response to atrial distension (Fig. 1, 2). In pregnant rats, only one receptor type could be identified, the discharge characteristics of which were identical to receptors of the LF subset in nonpregnant rats. No receptors responded to intracardiac balloon inflation with high frequency discharge in pregnant rats (Fig. 3). All receptors were localized to the venoatrial junction based on their response to atrial distension. We conclude that the high-frequency response to atrial distension is suppressed in pregnancy, while that of low-frequency receptors is preserved.

Atrial receptors in rats were first subdivided into two separate populations by Thorén, in a study characterizing the discharge pattern of the receptors in response to an increase in atrial pressure through occlusion of the ascending aorta (199). In that study, HF receptors were characterized as having a stimulated discharge of greater than 25 Hz. Discharge

would increase with increasing pressure in the atrium (i.e. stretch of the atrial tissue). These receptors were often observed to fire in sequence with the cardiac cycle, and they were described by Thorén as 'very much (like) the medullated atrial receptors' in larger mammals, such as the cat, despite the fact that all fibers studied conducted in the slow, C-fiber range (2-2.5 m/s) (199). HF receptors had a long-lasting response time (>1 min), which seemed to signal changes in the sustained pressure level in the atrium. In contrast, LF receptors had a stimulated discharge with far lower frequency than that observed for HF receptors. In addition, there was no relationship between receptor discharge and intra-atrial pressure; discharge was irregular and brief (generally <10 sec), seeming to respond to the change in pressure, but not to the sustained pressure level. It was also observed that LF receptors had a higher pressure threshold than HF receptors and that the conduction velocity of all fibers was in the C-fiber range (2-2.5 m/s) (199). The discharge patterns of high and low-frequency receptors were further characterized in later work on isolated segments of superior vena cava by Mifflin and Kunze, in which the receptors were classified according to their rates of adaptation (152). High frequency receptors were described as 'slowly-adapting' (SA), as they responded to the static component of the pressure wave with a sustained discharge. LF receptors were described as 'rapidly adapting' (RA), as they responded to the dynamic component of the pressure wave, signaling an acute change in pressure but adapting very quickly to a sustained increase in pressure. Thus it was concluded that HF (SA) receptors send information to the brain concerning the total fluid volume in the body, while LF (RA) receptors communicate acute changes in volume (152).

In the present study, atrial receptors in nonpregnant animals could be classified by the criteria for HF and LF receptors established by Thorén and Mifflin (152,199). Two types of receptors could be clearly differentiated: one which responded to atrial distension with a sustained discharge that increased with an increase in stimulus (HF/ SA receptors), and another which responded with an irregular, brief discharge that was unrelated to the degree of atrial stretch (LF/RA). In the pregnant animals, however, only receptors with characteristics of the latter group could be identified. These results are supported by a recent study by Hines et al., which showed that only LF-type receptors responded to a bolus of fluid injected into the heart (84). The present result, through use of a stimulus localized to the venoatrial junction and post-recording receptor localization, has confirmed that these LF receptors are in fact atrial receptors, which communicate changes in cardiac filling to autonomic centers in the brain.

This study has shown that, in pregnancy, discharge of HF atrial receptors in response to distension of the venoatrial junction is suppressed, while that of LF receptors is preserved. It has been suggested that LF receptors signal the brain regarding an acute change in volume, while HF receptors signal absolute volume (152). Therefore we propose that, while the ability to sense acute volume loads is retained, the sensation of absolute fluid volume is prevented in pregnancy. In Experiment B, the mechanism by which this occurs in the pregnant organism was investigated.

EXPERIMENT B: EFFECT OF ACUTE PREGNAN ON ATRIAL RECEPTOR AFFERENT DISCHARGE.

As shown in experiment A, two receptor populations could be identified in all virgin animals included in these studies, based on their spontaneous discharge and on their response to balloon inflation. Receptors with a high spontaneous frequency responded to balloon inflation with a high frequency, sustained discharge that increased with an increase in stimulus intensity. Receptors with a lower frequency spontaneous discharge responded to balloon distension with an irregular, brief discharge that, unlike the response of HF receptors, was unrelated to stimulus intensity. Acute intravenous administration of pregnan or vehicle had no effect on either spontaneous or stimulated discharge of HF or LF receptors.

Pregnan is one of the ring A-reduced metabolites of progesterone. Its levels in the plasma temporally follow those of progesterone: there is a dramatic increase in the pregnant rat from 10 ng/ml to 40 ng/ml in response to increased progesterone secretion from the feto-placental unit (90,170,174). Pregnan has been extensively studied with respect to its modulation of centrally-processed reflex pathways, it being a member of a family of neurosteroids (170). These steroid hormones are able to rapidly alter neuronal excitability through interaction with specific neurotransmitter receptors (150). Given that this process requires milliseconds to seconds to take effect, it is very different from the traditionally defined, genomic action of steroid hormones, which is limited by the rate of protein production (15,170,180). Pregnan binds to a unique neurosteroid recognition site on the $GABA_A$ receptor complex, and is able to allosterically modulate receptor function.

(122). The $GABA_A$ receptor is a pentameric structure composed of subunits which form a chloride channel (149). With the binding of the ligand GABA, the chloride channel opens, and negative charge flows in, hyperpolarizing the cell (125). When pregnan is bound to the receptor complex, frequency and/or duration of Cl channel opening in response to ligand binding is increased (123,150). At higher levels (micromolar range), pregnan exerts an intrinsic agonistic activity in the absence of other ligands (180). Acutely administered pregnan has been shown to affect arterial baroreceptor reflex function (81,122). Following intravenous administration of pregnan at levels estimated to reproduce plasma levels seen in pregnancy, the baroreceptor reflex function curve is shifted so that the renal sympathoexcitation normally associated with a given decrease in blood pressure was attenuated (81). In addition, arterial pressure sensitivity of neurons in the RVLM (the brainstem nucleus that processes signals from the arterial baroreceptors) is decreased in virgin rats following bolus administration of pregnan (122). The authors of this study suggested these results may be best explained by a potentiated responsiveness to endogenously released GABA in the presence of pregnan (122). Pregnan has also been shown to modulate processing of the atrial volume receptor reflex via this fast-acting nongenomic pathway (43). Intravenous infusion of pregnan reduces the central response to atrial distension, indicating pregnan is able to mimic the effects of pregnancy on the reflex (43). These studies collectively indicate acute administration of pregnan attenuates central processing of cardiovascular reflexes by enhancing GABA-mediated inhibition of central nuclei involved in the reflex pathways. Yet the question still remains as to whether reflex inhibition is confined to the brain, or whether it also involves an alteration at the sensory receptor level. This study was carried out to

determine whether atrial receptor discharge is altered in response to acute administration of pregnan.

The results of experiment A confirmed that atrial receptors in the rat heart may be subdivided into LF and HF subgroups, and that only LF receptor discharge is present in pregnant rats. Therefore, the more specific goal of this experiment was to determine whether intravenous administration of acute pregnan could inhibit the response of HF receptors to inflation of the intracardiac balloon.

Acute pregnan had no effect on the discharge of HF or LF atrial receptors of rats. Administration of pregnan through the femoral vein at a dose calculated to reproduce plasma levels seen in pregnancy did not alter the response to balloon inflation seen in either baseline or recordings following administration of vehicle. In addition, neither pregnan nor vehicle had any effect on spontaneous discharge of HF or LF receptors. While the dose of hormone used in this experiment is estimated to reflect plasma levels at mid-pregnancy, it may be that a higher dose is needed to effect acute changes in peripheral receptor function. However, as this dose inhibited *c-fos* expression in the PVN in response to balloon inflation, it is possible to consider that the acute effects of pregnan on the atrial volume receptor reflex may be confined to modulation of processing in central nuclei of the brainstem and/ or the hypothalamus. These results are supported by recent work showing acute pregnan has no effect on afferent baroreceptor discharge, indicating the effects of pregnan on the arterial baroreceptor reflex are also confined to modulation of central processing (121).

EXPERIMENT C: EFFECT OF CHRONIC PREGNAN ON ATRIAL RECEPTOR AFFERENT DISCHARGE.

The results of experiment B have shown that acutely administered pregnan has no effect on afferent discharge of atrial receptors. Thus the effects of acute pregnan are limited to the central component of the reflex (43). Previous studies from our lab have shown that chronically-administered pregnan may also alter volume homeostasis in rats by affecting other components of the regulatory mechanism (43).

Pregnan has been studied predominantly as a neurosteroid which acts rapidly in the central nervous system to modulate neuronal excitability (28,150). It has been shown, however, that pregnan may also act as a traditional steroid, effecting long-term changes in cellular behavior via the intracellular progesterone receptor (180). After pregnan has been internalized by the cell, it can be oxidized to 5α-pregnane, which binds the progesterone receptor (180). The ligand-receptor complex then binds DNA and activates transcription of specific proteins (133,170,180). Thus pregnan may act at peripheral sensory nerve endings to influence mechanosensitivity through altering expression patterns of ion channels. It is also possible pregnan alters atrial receptor activity indirectly by increasing levels of other factors, such as peptide hormones or nitric oxide, which could alter peripheral nerve function (97,133).

Pregnan administered subcutaneously for a period of two days causes an increase in plasma volume of 13.2% from baseline in female rats, a similar value to that seen in midpregnancy (day 9 = 14%) (133). Pregnan has been shown to act long-term to increase

synthesis of factors which play a role in volume regulation in the body: chronic administration of pregnan causes an increase in plasma levels of adrenomedullin (ADM) and an increase in nitric oxide (NO) biosynthesis to levels close to those seen in mid-pregnancy in the rat (97,133). Since ADM acts, in part, through NO, it has been postulated that the increase in NO levels is a result of the increase in ADM (86). NO has been shown to increase plasma volume (55,209). Thus the results of these studies indicate pregnan may affect volume regulation via rapid actions in the brain, but may also have more long-term effects as a traditional steroid, altering synthesis of factors that affect volume homeostasis. The experiments in this section were designed to investigate whether chronically administered pregnan could mimic the effects of pregnancy on receptor activity.

As in experiments A and B, atrial receptors in vehicle-treated rats could be differentiated into low and high-frequency subsets, with discharge characteristics identical to those described previously. In contrast, only receptors with low spontaneous discharge frequency were identified in pregnan-treated animals. Like the LF receptors in vehicle-treated animals, these receptors responded to atrial stretch with a discharge unrelated to the degree of balloon inflation. In addition, both LF receptors from vehicle-treated animals and receptors from pregnan-treated animals had a transient response that plateaued at 25 μl volume inflation. Only one receptor with spontaneous discharge greater than 20 Hz was localized to the venoatrial junction of a pregnan-treated rat. This receptor responded to balloon inflation with a slight increase in discharge frequency at a volume of 50 μl which did not increase further with an increase in inflation volume. As

receptors were identified and localized on the basis of their response to balloon inflation, it is not possible to conclude that spontaneous discharge frequency of HF receptors is inhibited by pregnan. Thus pregnan may suppress the response of receptors with high frequency spontaneous discharge to stimulus, while their basal discharge is unaltered. The results of experiment C indicate chronically-administered pregnan inhibits the response to HF atrial receptors to the stimulus of atrial stretch, while preserving that of LF receptors, i.e. that pregnan mimics the effects of pregnancy on atrial receptor function. As previously described, LF receptors are thought to sense an acute change in volume while HF receptors signal absolute volume (152); thus we propose that pregnan, through inhibition of HF receptor function, is one of the factors which contributes to the gradual increase in fluid volume in pregnancy.

EXPERIMENT D: EFFECT OF ADRENOMEDULLIN ON C-FOS EXPRESSION IN PARAVENTRICULAR NUCLEUS OF HYPOTHALAMUS.

We hypothesized that ADM, which is released in pregnancy in part due to rising levels of pregnan, could alter central processing of the volume receptor reflex. The technique used to quantify central activation in response to balloon inflation, expression of the immediate-early gene *c-fos*, has been validated as an accurate means of localizing central neurons responding to a specific stimulus (36,42,119). As a metabolic marker, it is useful in mapping responses to stimuli throughout the brain. The response of neurons in the PVN was analysed, as opposed to neurons in the brainstem, as the PVN plays a more modulatory role in processing of afferent cardiovascular signals. Our results indicate that

stretching of the venoatrial junction activates neurons in the PVN, as shown by the significant increase in *c-fos* expression. This increase tended to be suppressed, though not significantly, by the low dose of ADM. When a 10-fold higher dose of ADM was given, this effect was significant. In addition, animals that received the bolus dose of ADM without atrial distension showed increased *c-fos* expression in the PVN, indicating ADM plays some role in activating these neurons independent of the volume receptor reflex. As neither balloon inflation nor ADM had any effect on MAP or HR, it is possible to conclude that the observed changes in *c-fos* expression are a reflection of activation of the atrial volume receptor reflex pathway, and are not the results of indirect stimulation of the arterial baroreceptor reflex pathway.

As previously described, ADM is one of the most potent vasodilators in the body (155,179). Thus, it could act locally to dilate uterine arteries supplying the placenta. In addition, ADM plays a role in volume homeostasis, as it has both diuretic and natriuretic properties (181). As an active participant in regulation of both circulatory pressure and volume, ADM could play an essential role in regulation of cardiovascular homeostasis in pregnancy.

In this study, an effort was made to compare *c-fos* expression in the various subdivisions of the PVN. As previously described, the PVN is composed of a heterogenous population of cells which have been grouped into roughly five subdivisions based on cytoarchitecture, afferent and efferent projection patterns, and neuropeptide content (114,196). The increase in *c-fos* expression in the PVN due to balloon inflation was

concentrated in the medial parvocellular subdivision, though significant increases were also observed in the posterior and periventricular parvocellular subdivisions. Both the medial parvocellular and periventricular subdivisions are composed of neurons which mainly project to the external zone of the median eminence (113,184,197). Many of these neurons release corticotrophin-releasing hormone at this site, which serves as the initiating factor in the hypothalamo-pituitary adrenal (HPA) axis (35,83). CRF stimulates the release of ACTH from the anterior pituitary, which in turn enters the systemic circulation and stimulates secretion of glucocorticoids from the adrenal cortex (83). The results of this study have shown that activation of the atrial volume receptor reflex stimulates neurons of the PVN which are involved in the regulation of the HPA axis. Links between the volume regulation pathway and the HPA axis have been previously demonstrated: hemorrhage has been shown to increase corticosteroid levels (32). More directly, it has been shown that distension of the venoatrial junction leads to a decrease in plasma levels of ACTH and a decrease in the level of cortisol, effects which are blocked by vagal cooling (32,71). These results indicate the atrial volume receptor reflex has an inhibitory effect on the function of the HPA axis. The results of this study confirm that the AVR reflex plays a role in modulating the function of central neurons involved in regulation of the HPA axis. Administration of ADM decreased *c-fos* expression in the mp region of the PVN. This indicates ADM plays a role in modulating processing of the AVR reflex by neurons in the mp region. ADM alone also had a stimulatory effect on neurons in the medial parvocellular subdivision. Work by Shan and Krukoff showing that icv ADM stimulates neurons in the medial parvocellular region of the PVN supports these results (188). In addition, it has been shown that ADM suppresses basal secretion

of ACTH, indicating the hormone may be closely linked with regulation of the HPA axis (167). Given that the link between central ADM and volume regulation is well established, it is perhaps not surprising that ADM and the volume receptor reflex should affect excitation of the same group of neurons in the PVN.

A significant increase in *c-fos* expression in response to balloon distension was also observed in the posterior division of the parvocellular PVN. This subdivision is composed of autonomic neurons which projects to the spinal cord and to areas of the brainstem (VLM, NTS) (16,185). It has been shown that these descending projections, which arise from the PVN directly or indirectly, via brainstem nuclei, synapse on sympathetic preganglionic neurons in the spinal cord; especially those which project to the kidney (16). Thus the results of this study have shown the AVR reflex activates neurons in the posterior parvocellular subdivision which, both directly and indirectly, control sympathetic outflow to the kidney and consequently excretion of salt and water from the body (114). As was shown in the medial parvocellular subdivision, administration of the high dose of ADM significantly decreased *c-fos* expression in the posterior parvocellular, indicating that ADM plays some role in modulating the response of these neurons to volume receptor reflex stimulation. ADM alone also had a stimulatory effect on these neurons, further confirming that the AVR and ADM both affect autonomic neurons with descending output to the brainstem and spinal cord.

The paraventricular nucleus functionally may be said to represent the hypothalamus on a smaller scale. This mainly underscores the point that the main role of the PVN is to

integrate output in a well-defined pattern of homeostatic responses (14). The results of this experiment indicate that specific areas of the PVN are affected by both the volume receptor reflex and by ADM, and that these areas are involved in both neural and endocrine response patterns.

EXPERIMENT E: EFFECT OF ADRENOMEDULLIN ON PLASMA VOLUME

Acute infusion of ADM at a dose calculated to reproduce plasma levels at end-stage pregnancy, and a dose 10X higher, had no significant effect on plasma volume of conscious rats though there was a trend towards an increase in volume. A great number of studies have shown that ADM exerts various effects in tissues involved in regulation of plasma volume and composition (181). Intrarenal infusion of ADM increased renal blood flow, urine flow and urinary sodium excretion in a dose-dependent manner (51). As well, ADM increases renin secretion (96) and inhibits angiotensin II-stimulated aldosterone release from the adrenal cells (207). Centrally, ADM inhibits water drinking and salt appetite (159,181). This evidence indicates ADM may play a significant role in the regulation of volume and electrolyte balance in different physiological states. While ADM was not seen to have an effect on plasma volume in this study, other studies where ADM was used at higher doses showed a significant effect on fluid balance (183). The dose of ADM used in this study was based on plasma levels of ADM measured at term pregnancy in the rat, and therefore could be said to represent a physiological concentration in the plasma (97). Indeed, it is possible the dose used underestimated actual levels of ADM in the pregnant rat; an accurate measure of level of ADM in the

tissue is difficult to obtain as ADM is an autocrine/paracrine factor released locally by many tissues- thus levels in the plasma may not accurately reflect levels in the tissue (86,156,157).

While alterations in fluid balance in pregnancy may therefore not be attributed to rising levels of ADM alone, it is possible that ADM contributes to the new steady state in pregnancy by contributing to perceived 'underfilling' of the vasculature through vasodilation, and perhaps by supporting or promoting the function of other hormones which more directly affect the volume receptor reflex.

EXPERIMENT F: EFFECT OF ADRENOMEDULLIN ON ATRIAL RECEPTOR DISCHARGE.

As previously described, two subtypes of receptor were identified in the population localized to the right atrium; these receptors exhibited the same properties as were previously shown. The results of this experiment showed that intra-atrial administration of ADM had no effect on either spontaneous or stimulated discharge of HF or LF receptors.

Levels of ADM, a vasoactive peptide involved in fluid and electrolyte homeostasis, increase progressively in the plasma in pregnancy (97). It has been shown that this increase is linked to rising levels of pregnan as pregnancy progresses (97). As pregnan has been shown to suppress the volume receptor reflex at the central level, and at the

peripheral level (Experiment C), it was our hypothesis that ADM may contribute to altered volume homeostasis in pregnancy in a similar fashion. In Experiment D it was found that ADM and atrial receptors affected the same groups of neurons in the PVN, yet it was unclear what effect this might have on reflex function. In this experiment, the effect of ADM on the volume receptor reflex was investigated at the peripheral level. Little research has been conducted to determine what effect ADM has on neuronal function outside the central nervous system. The sister peptide of ADM, CGRP, with which it shares 20% homology has, in contrast, been well-studied in terms of its effects in nerve tissue, and has been described as a transmitter in neurons of both the central and peripheral nervous systems (157). Functions of CGRP and ADM are closely linked, as several of the effects of ADM are mediated through the CGRP receptor (24,52,100,157). There is evidence that ADM and CGRP may also mediate their effects through a single receptor- the CRLR (calcitonin receptor-like receptor)- which becomes specific for each hormone by the attachment of certain receptor activity modifying proteins (RAMPs) (148,157). RAMPs, which chaperone the receptor to the surface of the cell and remain part of the functional receptor structure, thus alter the pharmacology of the CRLR: CRLR with RAMP-1 preferentially binds CGRP, while CRLR with RAMP-2 or –3 preferentially binds ADM (80). Studies examining distribution of CGRP receptivity in different regions of the cardiovascular system have shown that the greatest densities of CGRP binding sites in the heart are localized in the right atrium (139,158,206). Thus it would appear that CRLR/RAMP expression is concentrated in this region. It has been speculated that tissue-specific RAMP expression could be regulated by physiological conditions, and that this expression has the potential to change a tissue from CGRP-

responsive to ADM-responsive (19,80,156). In support of this theory, it has been shown that expression of specific RAMPs may be altered in certain pathological conditions; for example, CRLR and RAMP-2 mRNA are up regulated along with ADM in the atria and ventricles of rats with congestive heart failure (156), and RAMP-3 is up regulated in the lymphoid organs in sepsis (163). Thus it is possible that expression of RAMPs may be differentially up or down-regulated in specific physiological conditions, such as pregnancy. This receptor system, therefore, allows for the possibility that peripheral sensory receptors in cardiac tissue, which are normally responsive to CGRP, could, in certain conditions, become responsive to ADM.

This experiment was therefore carried out to determine whether the response of sensory receptors in the atrium of the rat heart to the stimulus of stretch could be modified by intra-atrial infusion of ADM. The nonmyelinated fibers comprising these sensory receptors have not been investigated in terms of their receptivity to various hormones, yet one may assume that, like other sensory nerve fibers, these fibers would possess receptors for a multitude of peptides (140). Ligand-gated receptors in these nerve terminals are most likely linked to ion channels which modify nerve function by depolarizing or hyperpolarizing the neuronal membrane (194). Thus a peptide hormone such as ADM, possibly acting through a CRLR-RAMP structure at the cell surface, could potentially modify sensory neuronal function. The results of this experiment have shown, however, that the response of both HF and LF receptors were unaffected by the presence of ADM. Administration of ADM through the jugular vein at a dose 10X greater than that seen in the plasma in pregnancy did not alter the response to balloon inflation seen in either

baseline or recordings following administration of vehicle. In addition, neither ADM nor vehicle had any effect on spontaneous discharge of HF or LF receptors. It may be that the acute effects of ADM on the atrial volume receptor reflex are confined to modulation of processing in central nuclei of the brainstem and/or the hypothalamus.

EXPERIMENT G: EFFECT OF SNAP ON ATRIAL RECEPTOR DISCHARGE.

In this study nearly all of the receptors localized had a low spontaneous discharge of <5Hz, and responded to atrial stretch with an increased, irregular discharge that began to fade a few seconds following administration of the stimulus. As shown previously, discharge of these receptors was unrelated to the degree of atrial stretch. Two receptors were found which discharged with a higher frequency that was related to the degree of atrial stretch. It has been shown in other studies, and in the experiments described above, that receptors discharging with a higher frequency generally make up a significantly smaller component of the total atrial receptors population (84,199).

There is evidence supporting the idea that NO plays a role in modulation of cardiovascular reflexes. Baroreflex control of renal sympathetic nerve activity and heart rate is attenuated by intracerebroventricular infusion of an NO donor (146). L-NAME suppresses excitability of neurons in the NTS, while NO donor, microinjected into the RVLM, attenuate renal sympathetic nerve activity and lowers blood pressure (137,190). A recent study has shown that NO also suppresses the baroreceptor reflex at the level of the sensory receptors by inhibiting activation of Na channels in the afferent fibers (146).

It was speculated that calcium influx into the nerve terminals during activation of the sensory receptor leads to the activation of nNOS, and the subsequent increase in NO inhibits Na channels and subsequent nerve discharge (146). As the physiology and function of baroreceptor and volume receptor reflexes are highly similar, the purpose of this experiment was to determine whether infusion of an NO donor (SNAP) into the systemic circulation would attenuate atrial receptor discharge in response to the stimulus of atrial stretch. It had no such effect. It may be that, whereas the activation properties of atrial volume receptors are not altered by NO, other cardiovascular receptors, such as the arterial baroreceptors, are responsive. In a related experiment, female rats were implanted with cardiac balloons, mated, and subsequently treated with either the NOS inhibitor L-NAME or its inactive enantiomer D-NAME. Brain tissue was then removed and prepared for visualization of hypothalamic *c-fos* expression. The results of this experiment showed that NOS inhibition (via L-NAME infusion) restored the response to atrial distension in the pregnant animals (D-NAME had no effect), indicating that the suppression of central processing of the AVR reflex in pregnancy is, at least in part, mediated by NO (198). These two experiments have thus shown that, while NO does not appear to affect atrial receptor reflex function at the peripheral level, it is involved in the suppression of the central component of the reflex.

CHAPTER 5.

CONCLUSION

Based on the results presented in this thesis and their interpretation in the discussion the following conclusions are made:

1. Atrial receptors in anesthetized virgin female rats may be subdivided into high and low frequency subgroups which, based on differences in stimulated discharge patterns, appear to signal an increase in absolute circulating volume and an acute change in volume load, respectively.
2. In pregnancy, discharge of HF atrial receptors in response to distension of the venoatrial junction is suppressed, while that of LF receptors is preserved. This indicates the ability to sense acute changes in volume is retained in pregnant animals, while the sensation of absolute volume is suppressed.
3. Acutely administered pregnan had no effect on high or low frequency receptor discharge in response to atrial stretch, whereas chronically administered pregnan suppressed HF response and preserved LF response. Thus chronic pregnan mimicked the effects of pregnancy on atrial receptor function.
4. ADM activates the same areas of the PVN which are involved in the atrial volume receptor reflex, but has no effect on atrial receptor discharge.
5. NO has no effect on atrial receptor afferent discharge; its effect on the atrial volume receptor reflex may be limited to modulation of central processing of the sensory stimulus.
6. Volume expansion in pregnancy is due, at least in part, to hormonally-mediated suppression of both peripheral and central function of the AVR reflex.

Figure 23 provides a general overview of the proposed mechanism of hormonal regulation of the atrial volume receptor reflex in pregnancy

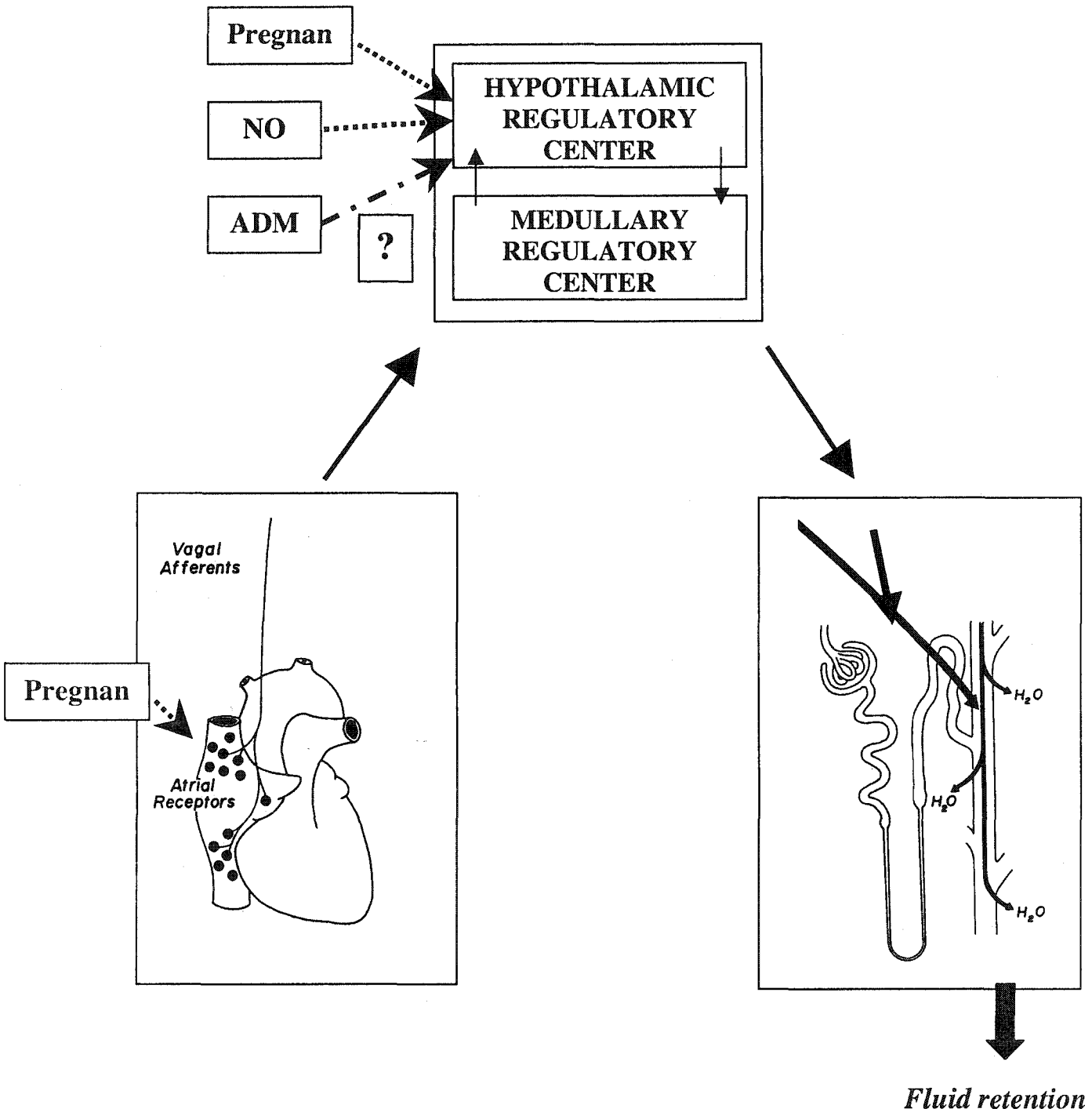

Figure 24. Representation of mechanism of hormonal regulation of atrial volume receptor reflex in pregnancy.

Methodological considerations and limitations

There are several considerations supporting the use of rats as an experimental model in the study of volume regulation in pregnancy: 1) the timelines of human and rat pregnancy are similar: human gestation is 9 months, divided into three trimesters of 3 months each, and rat gestation is 3 weeks, divided into three trimesters of 1 week each, 2) this short gestation period facilitates experimental design and 3) many of the physiological alterations observed in human pregnancy (i.e. renal function, Na+ metabolism) also occur in the rat at a corresponding time in gestation (2,130).

As each of the experiments described in this discussion have involved activation of the atrial volume receptors, it is important to discuss the implications of the type of stimulus used. A great deal has been written on the subject of what constitutes an appropriate, physiological stimulus to the atrial receptors. As described by Hainsworth, an ideal stimulus must fulfill at least two requirements: 1) it must be localized to the area of interest, and 2) the response the stimulus elicits must actually be a reflex (71). The first point addresses the concern that a stimulus which takes the form of an inflatable device may obstruct venous flow through the right atrium, consequently altering cardiac output and systemic arterial pressure. This would in turn alter activity of the arterial baroreceptors, thus eliciting a host of additional reflex responses which would effectively mask the atrial receptor reflex (71). Though it would be imagined that inflation of a balloon in the venoatrial junction of the rat would cause these changes, the anatomy of the rat is unique to other mammals in that blood from the left jugular vein enters the inferior vena cava, and drains from the head into the left superior venal cava via cross circulation in the head and neck. Thus when the balloon in the right superior vena cava is

inflated, there are no accompanying changes in central venous pressure, arterial pressure or mean arterial pressure (42,110). Other studies examining atrial receptor afferent discharge in the rat have attempted to activate the receptors via injection of a warmed bolus of saline into the atrium. Although this technique may be said to be more physiological than balloon inflation, when unaccompanied by receptor localization, it is impossible to state with any certainty which receptors in the cardiovascular system have been activated: a large (0.3 ml) bolus of saline activates stretch receptors not only in the atrium, but also elsewhere in the heart (84). The balloon, in contrast, provides a localized, discrete and consistent stimulus, which, when accompanied by receptor localization, can provide a great deal of information concerning receptor behavior. One limitation must be noted, however- due to the absence of a direct measure of right atrial pressure, neither the threshold of receptor activation nor the pressure at which maximal receptor response occurs may be recorded, as is possible when other means of stimulation, such as intra-atrial bolus administration or occlusion of the ascending aorta, are used (84,199). However, it must be noted that progressive inflation of the balloon to a volume of 50μl results in a receptor response pattern similar to what is seen when atrial pressure is progressively increased from 2 to 20 mmHg (84,199). Another possible criticism of the technique of balloon inflation is that it does not account for potential changes in dimensions of the heart and its chambers in pregnancy, which could alter the response of groups of intracardiac receptors to a stretch stimulus. Previous work by Jacobs et al. has shown that there is no significant change in unstressed right atrial size or atrial compliance during pregnancy, in the rat (108). Therefore it may be safely concluded that a stretch stimulus administered to pregnant animals is not quantitatively

different than that administered to nonpregnant animals, and would subsequently activate the same populations of receptors.

Despite the localized nature of the stimulus of balloon inflation, additional precautions were taken in the experiments to ensure recorded receptors were in fact atrial receptors. Balloon placement in the venoatrial junction was visually confirmed prior to recording, and receptors were localized at the termination of the experiment. Hainsworth's second requirement for an adequate stimulus of the atrial receptors- that the effects elicited truly belong to the reflex being studied- has been consistently answered by the intracardiac balloon: it has been previously shown that a volume of inflation of 50μl elicits an increase in urine output and plasma ANF in virgin female rats (107), and an increase in neuronal activation in the PVN or male and female rats (42,107).

In investigating the effect of ADM on the central response to atrial distension, localization of c-fos expression in neurons of the PVN was used as a semi-quantitative measure of activation. Immediate-early gene expression has been widely used as a means of mapping pathways of neuronal activation in response to a specific pharmacological or physiological stimulus (135). Immunocytochemistry for the protein product Fos thus clearly marks neurons which have responded to a specific stimulus by an increased spike frequency- only neurons which have been strongly activated express the c-fos gene (48). Despite the apparent clarity of results based on the use of c-fos, extreme caution must be used when interpreting these results; particular attention must be paid to Hoffman's use of the Walle Nauta quote: 'absence of proof is not the proof of absence' (88). Hoffman's

warning was in reference to the interpretation of negative results when c-fos is used. Should a neuron be over-stimulated, for example, the result could be activation of intracellular mechanisms that inhibit chronic c-fos expression (88). It has also been shown that, depending on the nature of the stimulating input, some neurons express different combinations of immediate-early genes. Thus when c-fos expression is absent, expression of other factors must be measured before an absence of activation may be concluded (88). When interpreting the results of experiment D, it is therefore impossible to rule out the suggestion that an absence of c-fos expression in a neuron stimulated by both ADM and atrial receptor activation may be the result of 'over-activation' as opposed to an inhibition of activation. All that may be safely concluded is that ADM and the volume receptor reflex both stimulate the same population of neurons in the PVN.

As the experiments described in previous sections have been designed to connect hormonal modulation of the volume receptor reflex with alterations in reflex function during pregnancy, some justification for the doses used in these experiments must be given. Very few studies have been conducted investigating ADM in pregnancy: both in humans (44) and in rats (97), plasma levels of the peptide hormone have been shown to increase progressively during pregnancy. In the rat, levels were shown to increase 3-fold from 100 pg/ml in virgin animals to 400 pg/ml at 21 days of pregnancy (97). Plasma levels of ADM in rats in the experiments here described (Experiments D and E) were calculated to roughly reproduce levels seen in the plasma at end-stage pregnancy. Indeed, though a 10-fold higher dose was also used in both chronic and acute preparations, it is important to note that plasma levels of ADM in pregnancy are most

likely to be an under-representation of that seen by the tissues. As ADM is mainly believed to act as an autocrine/paracrine factor, it is more than likely that our dose falls far below what is actually present in cardiovascular tissues during pregnancy (181).

As with ADM, plasma levels of pregnan increase progressively in pregnancy. Indeed, they are highly correlated with progesterone levels in women during the menstrual cycle and in pregnancy (170). In the rat, levels of pregnan increase from 10 ng/ml to roughly 30-40 ng/ml by the last stage of gestation (28,133). It has been shown that exogenous pregnan at a dose of 5 mg/kg/day causes an increase in plasma levels to roughly 40 ng/ml (17). Thus the dose of 2.5 mg/kg/day administered to rats over a two day period in experiment B would lead to a plasma level of pregnan roughly approximating that seen at midpregnancy (20 ng/ml). The acute pregnan dose administered in experiment C was similarly calculated to achieve mid- to late-pregnancy plasma levels of the hormone (30 ng/ml). It has been shown that this dose of pregnan mimics the effects of pregnancy in potentiating arterial baroreflex sympathoinhibition (122).

Possible future experiments

The experiments here described have provided evidence for the proposal that specific hormones may modulate the sensory component of the volume receptor reflex. A future course of investigation would most appropriately focus on the exact mechanism of the alteration in function of high frequency atrial receptors in the presence of pregnan. By isolating atrial receptors in culture (such as was done by Li et al. with baroreceptor neurons) following chronic treatment of the animal with pregnan, a great deal could be

learned of the exact ionic mechanism of altered function following prolonged exposure to the hormone (i.e. a down-regulation of stretch-induced current) (127).

While it did not appear that ADM had any effect on the peripheral component of the atrial volume receptor reflex when administered acutely, the effect of chronically-administered hormone (made technically difficult by the short half-life of ADM) has yet to be explored. In addition, the effect of ADM on central processing must be investigated in greater detail. A series of studies conducted by Shan and Krukoff have demonstrated that ADM, which accesses the brain through the area postrema, has significant effects on the activity of neurons in the PVN, and many of these neurons also produce NO (188,189). Whole cell recording of neurons in the PVN following atrial receptor stimulation, and in the presence of ADM, would help to shed light on the precise role of the peptide hormone in mediation of central processing. Further study of the role of NO in modulation of atrial receptor signaling should also be considered. For example, an investigation of the effect of chronic NOS blockade on atrial receptor afferent activity would help to shed light on the extent of NO involvement in alteration of sensory processing.

Future studies investigating the importance of hormonal regulation of the volume receptor reflex in pregnancy must also address how these regulatory systems are altered in the disease state preeclampsia. The first manifestation of this condition, which leads to fetal growth restriction, is a 'contraction' of the plasma volume, and the severity of the disease state as it develops is proportional to the degree of volume contraction (58). We

may speculate, based on the findings presented in this thesis, that the absence of expansion of the plasma volume in preeclamptic pregnancy may be due, in part, to the body's inability to 'reset' the volume receptor reflex to a level where volume is permitted to accumulate. Future studies investigating atrial receptor reflex function in preeclamptic-like pregnancies, in conjunction with measurements of circulating 'modulatory' hormone levels, would help to shed light on the physiological importance of hormone-induced modulation of the reflex.

In conclusion, the results of experiments A-F provide support for the role of hormones in the establishment of a state of expanded plasma volume in pregnancy. It has been established that many of the physiological alterations in pregnancy, which occur to allow the mother to fully support and protect the fetus for the duration of gestation, are the result of varying levels of circulating hormones in the maternal plasma. Given the results of the experiments that comprise this thesis, regulation of plasma volume in pregnancy is, at least in part, the result of combined peripheral and central modulation of the atrial volume receptor reflex. The clinical significance of these findings arises from the observation that an absence of appropriate hormonally-mediated modification of the volume receptor reflex in pregnancy could lead to an attenuation of the progressive volume expansion which has been shown to be essential for healthy fetal development.

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