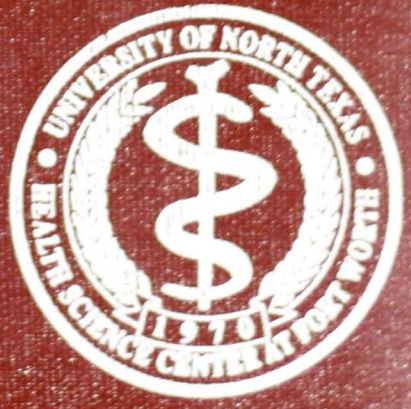
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The effects of chronic morphine treatment on parasympathetic control of the heart and associated cellular mechanisms were examined using a canine model. Vagal bradycardia was significantly blunted in dogs treated for one week with subcutaneous morphine pellets. In a separate group of dogs, heart rate and high frequency fluctuations in heart rate declined during the first three hours of subcutaneous morphine infusion consistent with the vagotonic action of acute morphine. Heart rate remained below baseline on Day 2 of the morphine infusion but had returned to normal by Day 10. Ambient sympathetic tone was increased on Days 2 and 10, and plasma catecholamines were elevated on Day 2. The intrinsic heart rates on Days 2 (160 bpm) and 10 (162 bpm) of morphine treatment were lower than the pre-treatment rate (182 bpm). Suggested mechanisms include a fundamental change in sinoatrial nodal cell function or attenuated tachycardia induced by vasoactive intestinal peptide co-released with acetylcholine from post-ganglionic parasympathetic neurons. The time to 50 % maximal bradycardia during vagal nerve stimulation was increased with chronic and acute morphine suggesting an effect on the rate of acetylcholine synthesis, release or degradation. Muscarinic receptor density in left ventricular and right atrial sarcolemmal membranes from dogs treated

chronically with morphine were 34 % and 17 % higher, respectively, than in control animals. Chronic morphine had no effect on basal or $MnCl_2$-stimulated cyclase activity in either region. Similarly, maximal β-adrenergic and muscarinic receptor/G-protein coupling to adenylate cyclase were not altered by chronic morphine. Atrial norepinephrine content was higher than that in the ventricles and was unaltered by morphine. Ventricular norepinephrine was decreased with chronic but not acute morphine treatment. Epinephrine was evenly distributed throughout the myocardium and was reduced in both the atria and the ventricles by either acute or chronic morphine. This pattern suggests that morphine may reduce extraneuronal uptake of catecholamines. Collectively these studies show that chronic morphine treatment and the accompanying persistent vagal activity may reduce parasympathetic function. This attenuated function, however, is short-lived since sympathetic systems adapt with compensatory responses masking, or perhaps reversing, initial parasympathetic deficits.

CARDIAC PARASYMPATHETIC DYSFUNCTION

IN MORPHINE ADDICTION

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CARDIAC PARASYMPATHETIC DYSFUNCTION IN MORPHINE ADDICTION

DISSERTATION

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CHAPTER 1

INTRODUCTION

History

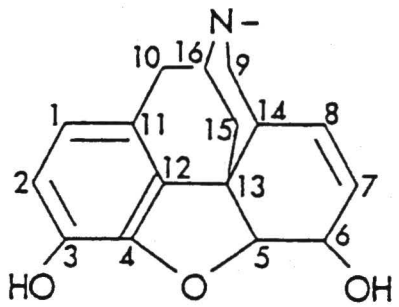
According to historians, opiates were among the first pharmacological substances known to man. Juice extracted from unripe seed capsules of the opium poppy was first used by ancient Babylonians as a sleep-promoting agent as early as 4000 BC. Opium, used as a euphoriant in early religious rituals, was later used by the Greeks and Romans, and became popular in Europe during the Renaissance Period. The addictive properties of opium were first recognized in China where its use sparked the Opium Wars against the British. Morphine, the primary alkaloid component in opium and named after the god of dreams, Morpheus, was isolated in 1806. Morphine was first used clinically as an analgesic in the 1850's after the invention of the hypodermic needle. Thousands of American soldiers became addicted to opiates during the Civil War when the drug was commonly administered to treat pain associated with war injuries. Heroin was synthesized in 1898 and it was around this time that opiate addiction was recognized as a serious problem in the United States. Although morphine could still be used by physicians as an analgesic, the Narcotic Act of 1914 outlawed the general use of opiates (6, 59)

Throughout this century, opiate abuse has occurred in cycles, reaching a peak in the 1970's. Opiate abuse declined following this period and then remained stable until the

recent surge in heroin use. The National Institute on Drug Abuse estimates that up to 1 million people in the United States are addicted to opiates and another 3 million are users with potential to become addicted. In recent years, several cities across the country including Chicago, Boston and Miami have reported increased use of heroin among young people, more admissions to treatment programs, and increased availability and purity of heroin (21). The Drug Abuse Warning Network reported a fourfold increase in heroin-related emergency room visits from 1978 to1992 and an additional twofold increase between 1992 and 1995 (21). Increases in heroin use are also being reported in other countries including Italy, Switzerland, the United Kingdom and Norway (21). Opiate abuse has serious social and economical consequences including increased morbidity and mortality, high crime rates among addicts, the increased spread of blood born pathogens (e.g., AIDS, hepatitis) and the high cost of providing treatment.

Structure, Metabolism and Pharmacological Effects

Morphine, the prototypical opiate alkaloid, has a three-ring motif based on the phenanthrene nucleus. The structure is common to other natural and synthetic opiates with analgesic activity, including codeine and heroin. The compounds differ mainly at the 3 and 6 positions and at the nitrogen, sites known to be involved in binding of the compounds to opiate receptors.



Morphine

Morphine enters the bloodstream rapidly, regardless of the route of administration. Approximately one-third of the drug binds to proteins restricting its transfer through capillary membranes. The unbound morphine, however, is highly lipophilic and accumulates rapidly into various organs. Although the brain is the major site of action for opiates, only about 2% of administered morphine reaches the brain in humans since it crosses the blood-brain barrier slowly. Most opiates are metabolized rapidly by the liver with a duration of action of 4-5 hours. Only traces remain in the body 24 hours after administration. In the liver, morphine is conjugated with glucuronic acid at the 3 and 6 sites giving the major metabolite morphine-3-glucuronide and a minor metabolite morphine-6-glucuronide. Morphine-3-glucuronide is pharmacologically inactive while morphine-6-glucuronide has much greater analgesic potency than morphine and is thought to be responsible for much of the analgesic effect of morphine (5, 59).

Heroin (alternatively named diamorphine or diacetyl morphine) is similar in structure to morphine with acetyl groups attached at the 3 and 6 sites. Heroin is rapidly de-acetylated in the bloodstream by esterases to yield 6-monoacetyl-morphine which is

further hydrolysed by liver enzymes to morphine, the main metabolite of heroin. Most of the analgesic activity of heroin is due to its conversion to morphine. Heroin, however, is more lipophilic than morphine and reaches the brain more rapidly resulting in greater euphoric effects. For this reason, addicts usually prefer heroin over morphine (5, 59).

Opiates are used clinically to alleviate pain and anxiety associated with acute myocardial infarction, traumatic injury and terminal illness (59). The legal use of opiates, as indicated by prescribing practices, increased 26-1423% between 1976 and 1992 in the ten countries that use the most opiates (10). The profound euphoria induced by opiates and their ability to induce tolerance and dependence with chronic use make them prime candidates for abuse. Other pharmacological effects of opiates include sedation, respiratory depression, decreased gut motility and cough suppression (59).

Endogenous Opioids

In 1973, several groups of investigators described specific receptors in the central nervous system that selectively recognize molecules with opiate-like structures (46, 58, 60). This discovery suggested the existence of endogenous substances which normally interact with these receptors. Subsequently, Hughes and Kosterlitz (20) identified two pentapeptides in brain extracts which possessed potent opiate activity. Since then, additional endogenous opioids have been identified and subsequently localized in the central nervous system, pituitary, adrenals, heart, pancreas, kidneys, gastrointestinal tract and autonomic ganglia (2, 6, 18, 59).

Endogenous opioid peptides can be divided into three major classes loosely based on their derivation from three distinct precursor molecules: Pro-opiomelanocortin serves as the precursor for β-endorphin and related peptides. Pro-enkephalin gives rise to the enkephalins and other intermediate-sized derivatives, and pro-dynorphin is the precursor for dynorphin and related peptides. Regardless of their precursor, all endogenous opioids have a common amino-terminus consisting of the amino acids tyrosine-glycine-glycine-phenylalanine. Because of this similarity at the N-terminus, differences in the length and amino acid sequence of the C-terminus determine the pharmacological activity, duration of action and receptor specificity of endogenous opioids (18, 59). The three primary opiate receptors are labeled mu, delta and kappa. Each receptor subtype binds both endogenous and exogenous opiates with varying affinities. Morphine is the prototypical agonist for the mu receptor. Opioids are involved in many physiological systems and affect analgesia, cardiovascular function, respiration, temperature regulation, gastrointestinal activity, endocrine responses, feeding behavior, sexual function, learning and memory and the body's response to exercise.

Opiate Addiction and Cardiovascular Disease

Several lines of evidence indicate a greater incidence of cardiovascular disease following chronic opiate abuse. In one post-mortem study completed at the Heart Lung and Blood Institute, more than 95% of opiate addicts had identifiable cardiac abnormalities and 58% of the deaths were related to cardiovascular disease (14). A much larger retrospective study in Rome also found more cardiovascular disease among opiate

addicts than expected for age matched controls (47). Endocarditis was a common finding among intravenous drug users (1, 39, 47) however, cardiovascular pathologies without obvious connections to infectious processes were also observed more frequently in opiate addicts (9, 14, 34, 44, 47, 62). Most fatalities classified as heroin overdose occur among dependent, regular users, not novices, and often occur several hours following the last use. In the majority of cases, death occurs in the absence of toxic concentrations of the narcotic in question (11). These data collectively suggest that chronic alterations, and not acute effects, may contribute to heroin-related deaths. Lipski *et al.* (38) reported cardiac arrhythmias in more than 50% of otherwise healthy opiate addicts and suggested that the resulting cardiac vulnerability might contribute to narcotic-associated fatalities. The high incidence of bradyarrythmias in this study may indicate a loss of parasympathetic reserve and perhaps the inability to react appropriately to changes in sympathetic influences. Kapoor *et al.* (24) showed attenuated increases in blood pressure in heroin addicts following a standard postural challenge indicating altered baroreflex function.

Vagal Function is Cardioprotective

Several lines of evidence support the notion that increased vagal activity is cardioprotective. In recent years, heart rate variability, defined as the standard deviation of R-R intervals within a given time period, has been used as an index of vagal activity. In short, the sympathetic and parasympathetic nervous systems continuously react to changes in the cardiovascular system, making appropriate alterations to maintain homeostasis. The "response time" of the parasympathetic system is much shorter than that of the

sympathetic system and thus is responsible for the instantaneous adjustments in heart rate to changes in respiration and posture. This beat-to-beat variation in heart rate is, therefore, an indicator of the strength of the vagus nerve in controlling heart rate. Kleiger *et al.* (26) studied 24-hour electrocardiograms from 808 patients who had recently survived acute myocardial infarction. After three years, morality rates were correlated with HR variability and other known post-infarction risk factors. Heart rate variability was more strongly correlated with mortality than any other variable. Patients with high variability (increased vagal control of heart rate) shortly after acute myocardial infarction were 5 times more likely to survive than patients with less variability. The authors suggest that patients with an increased heart rate variability have increased vagal tone which may protect against ventricular fibrillation.

Other investigative approaches have also shown the vagus to be cardioprotective. In dogs with a healed myocardial infarction (one month prior to the study), a coronary artery occlusion was performed during an exercise stress test (63). When the vagus nerve was stimulated during the occlusion, only 10% of the dogs developed ventricular fibrillation compared to 92% of control animals, clearly indicating that vagal activation can prevent fibrillation caused by acute myocardial ischemia. This effect was attributed to the direct reduction in heart rate and indirect antagonism of sympathetic activity. In a related study (8) vagal activity, as measured by direct neural activity of single cardiac vagal efferent fibers, increased 48% during coronary occlusion in cats that survived compared to no change in vagal activity in those that experienced ventricular fibrillation. In addition,

vagal activity in response to a phenylephrine-induced increase in blood pressure prior to the occlusion was significantly greater in cats that survived the subsequent occlusion compared to those that died.

Acute Morphine is Vagotonic

The acute administration of morphine depresses cardiovascular function. Morphine (0.25 mg/kg) injected into the right atria of anesthetized dogs decreased pulse rate, blood pressure, cardiac output and cardiac work (48). Randich *et al.* (51, 52) demonstrated a dose-dependent decrease in HR and BP in rats given intravenous morphine. Bilateral cervical vagotomy virtually abolished the bradycardic response at all doses of morphine (0.1-2.5 mg/kg) and changed the depressor response to a pressor response at higher doses (1.0-2.5 mg/kg). These results suggest that the morphine-induced bradycardia and hypotension resulted from an increase in vagal outflow. Bilateral vagotomy also reversed the bradycardia and prolonged atrioventricular nodal conduction time in dogs given 0.12 mg/kg epidural morphine again indicating that the depressive effects of morphine were due to an increase in vagal activity (19). Acute morphine reduced vulnerability to ventricular fibrillation in psychologically stressed dogs (12, 13). This effect was partially abolished by vagotomy or the administration of atropine indicating that an increase in vagal activity was at least partially responsible for the beneficial effect of morphine. It is important to note that the protective effects of morphine (and of enhanced vagal outflow) are most apparent when the cardiovascular system is at risk due to an enhanced sympathetic neural tone.

Endogenous Opiates and Vagal Pathology

Alterations in the balance between sympathetic and parasympathetic activities are commonly used to explain a variety of cardiovascular disease processes. Endogenous opioids and their receptors may provide the final common pathway by which chronically applied opiates are able to alter this autonomic balance. For example, cardiovascular reflex control (17, 53) and parasympathetic input to the heart (3, 4, 15, 22, 23) are both reduced in congestive heart failure with a presumed increase in the vulnerability to sympathetically mediated events. The disease process was accompanied by a 2-5 fold increase in circulating endorphin, dynorphin and enkephalin (16, 35) which suggests that endogenous opioids are involved in the pathophysiology. The enkephalins have demonstrable vagolytic activity (7, 42, 64) and several are also intrinsic to the myocardium (2). The endogenous opioids are further implicated in this particular example of vagal dysfunction since opiate antagonists are able to return baroreceptor sensitivity toward normal (55).

Effects of Chronic Morphine on Sympathetic Control of Myocardial Function

Most of the studies examining cardiovascular adaptations to chronic opiate administration have evaluated sympathetic nervous function. A morphine-dependent model was developed in rats by adding the drug to drinking water (29, 30) . Increases in blood pressure, pulse rate, and arterial norepinephrine were all significantly reduced during sympathetic stimulation in rats treated chronically with morphine. Acute morphine did not result in similar reduced responses (31-33). Cardiovascular responses to nicotine and

norepinephrine were unimpaired indicating respectively that the release of norepinephrine and the end organ response to it were normal. The addicted animals were, however, more sensitive to ganglionic blockade which indicates that the apparent sympathetic dysfunction involved preganglionic nerve fibers and not their nicotinic target receptors or postganglionic nerve fibers (31, 33). In rats that received chronic morphine from subcutaneous pellets, a bolus dose of morphine caused an increase in atrial catecholamines and a decrease in the rate and force of atrial contraction (49, 50). In isolated guinea pig atria, acute morphine enhanced the inotropic response to sympathetic nerve stimulation (40). The authors attributed this effect to an inhibition of norepinephrine reuptake since the effect of morphine was abolished in the presence of desmethylimipramine and since morphine potentiated the effect of exogenous norepinephrine. Atria obtained from guinea pigs treated chronically with morphine did not exhibit alterations in the response to sympathetic nerve stimulation. The reasons for the disparate results of these studies are unclear but could result from differences in addiction protocols, experimental preparations or species.

Effects of Morphine on Parasympathetic Control of Myocardial Function

Less information is available regarding parasympathetic control of cardiovascular function following chronic morphine treatment. *Acute* morphine reduced the bradycardic response to vagal stimulation in isolated rabbit hearts (25, 64) but had no affect on vagal bradycardia in anesthetized dogs (43). In one of the few studies evaluating the effects of chronic morphine on vagal function, Leung et al (33) found no differences between

morphine-treated rats and controls in vagally-mediated decreases in heart rate and blood pressure. These findings differ from the data obtained in dogs (43, 65). When naloxone was administered to dogs treated with morphine for seven days, heart rate and blood pressure increased sharply (65). The heart rate then remained elevated for four hours despite the return of blood pressure to normal after only one hour. This persistent tachycardia in addicted, but normotensive, animals suggests that chronic morphine may have impaired the ability of the vagus to return heart rate to normal. We also found that vagal bradycardia was impaired in dogs implanted with subcutaneous morphine pellets for seven days (43). The reason for the differences between rats and dogs is unclear but again may reflect the greater sensitivity of dogs to morphine, differences in experimental methods or the addiction protocol. The rats received their morphine in drinking water which produces intermittent morphine exposure and reduces the overall control of administered dosage.

Cellular Adaptations to Chronic Morphine

The intracellular events resulting from agonists binding to β-adrenergic and muscarinic receptors represent the primary mechanisms for autonomic control the heart. Activation of β-receptors stimulates coupling with a stimulatory G-protein, G_s, activation of adenylate cyclase and a subsequent increase in cAMP. As a result, heart rate and contractility are increased. Conversly, activation of muscarinic receptors stimulates coupling with an inhibitory G-protein, G_i, inhibition of adenylate cyclase and a decrease in cAMP. This results in a decrease in heart rate and contractility. Opiate receptor binding

produces a variety of intracellular events including activation of inwardly-rectifying potassium channels, inhibition of voltage-gated calcium channels and, like muscarinic receptors, inhibition of adenylate cyclase. Many of these effectors are mediated through the coupling of opioid receptors to inhibitory G-proteins including G_i and G_o.

The common second messenger systems of opiate and muscarinic receptors and the complex interplay that can occur between opposing intracellular systems have been the focus of extensive research. One line of research has shown that chronic exposure to drugs that inhibit adenylate cyclase, including muscarinic agonists and opioids, can result in enhanced basal adenylate cyclase activity and enhanced cyclase activity in response to stimulatory agents (61). This effect had been demonstrated in cultured nerve cells where it has been described as a model for narcotic tolerance and dependence (56, 57). More recently, this receptor "crosstalk" has been identified in cultured heart cells and heart membrane preparations where it is described as a general type of cellular adaptation to interventions that enhance vagal activity (37, 45). In addition to effects on intracellular events, prolonged exposure to muscarinic agonists can affect extracellular β-adrenergic receptor binding. In isolated rat cardiac myocytes, prolonged incubation with carbachol resulted in decreased β-receptor binding (36, 45). Although both chronic morphine and muscarinic agonist have similar effects on cyclase activity, the effect of carbachol on β-receptor binding had not been duplicated by chronic morphine administration. β-adrenergic receptor binding was unchanged in cardiac preparations from rats treated with subcutaneous morphine for 4 days (28) or from rats that had morphine-treated drinking

water for 1 month (41). Although it has been well-documented that prolonged exposure to muscarinic agonists causes muscarinic receptor down-regulation (27, 54), it is unknown whether other interventions that increase vagal activity, such as morphine administration, induce the same type of response.

Summary

Opiate abuse is a growing public health concern and opiates are increasingly prescribed for the relief of chronic pain. Post-mortem studies of opiate addicts have indicated that ***chronic*** exposure to opiates is associated with a greater incidence of cardiovascular disease. Several studies have identified altered autonomic control of cardiovascular function in heroin addicts and related animal models which may be attributable to vagal dysfunction. ***Acute*** morphine produces hypotension and bradycardia through its ability to stimulate vagal outflow. Morphine is commonly administered during the treatment of acute myocardial infarction when the electrical stability of the heart is threatened by excess sympathetic activity. Much of morphine's clinical utility in this regard derives from its ability to balance autonomic function through the reassertion of "cardioprotective" vagal influences.

Few studies have systematically examined the cardiovascular consequences of chronic opiate administration and most of these have concentrated on sympathetic function. We recently demonstrated impaired vagal bradycardia in dogs treated with morphine for one week. This suggests that chronic morphine produces adaptations which

reduce vagal reserve either directly through the repeated central stimulation of vagal outflow or indirectly through heterologous opiate and muscarinic receptor mechanisms which employ common second messenger systems. The research which follows employed a canine model of morphine addiction to test the hypothesis that **persistent increases in efferent vagal traffic associated with chronic morphine treatment reduce the parasympathetic control of the heart through the sustained down-regulation of muscarinic receptors, their coupling mechanisms and/or their second messenger systems.**

Specific Aims

Specific Aim 1 tested the hypothesis that chronic morphine treatment reduces parasympathetic control of the heart. Parasympathetic contributions to the control of heart rate were determined in conscious animals before, during and after treatment with the aid of power spectral analysis and pharmacologic blockade. The intrinsic heart rate in the absence of neural input was measured to determine whether the heart itself adapts to chronic morphine. Finally, animals were anesthetized and vagal nerve stimulation/heart rate frequency responses were conducted to determine whether the adaptation to chronic morphine includes the efferent vagal/myocardial interface.

Specific Aim 2 tested the hypothesis that chronic morphine treatment reduces either the affinity or the number of available cardiac muscarinic receptors. Muscarinic receptors were evaluated by radioligand binding with [3H]quinuclidinyl benzilate (QNB)

in cardiac sarcolemmal membrane preparations.

Specific Aim 3 tested the hypothesis that chronic morphine treatment impairs the muscarinic receptor coupling system and/or its post-receptor second messenger system. Basal and $MnCl_2$-stimulated adenylate cyclase activity were measured to determine whether the post-receptor effector adapts to chronic morphine. The integrity and responsiveness of the receptor-G-protein-effector coupling was tested with both muscarinic and β-adrenergic agents.

Significance

Opiates are used clinically to alleviate chronic pain associated with injury and terminal illnesses. Outside the medical arena, opiates are abused initially for the profound euphoric properties and habitually due to tolerance and dependence. Thousands of people are treated, often for years, with the synthetic opioid agonist, methadone, as a treatment for opiate addiction. The clinical use of opiates for the relief of chronic pain is becoming more commonplace, and a surge in opiate abuse is evident around the world.

Despite this resurgence of opiate use, relatively little information is available regarding cardiovascular effects of chronic opiate administration. Myocardial abnormalities are common post-mortem findings in opiate addicts, and select studies have indicated altered autonomic control of cardiovascular function in heroin addicts and related animal models. Most studies in this regard have focused on changes in

sympathetic nervous function. Since acute morphine stimulates vagal outflow, the chronic use of opiates may also affect parasympathetic control of myocardial function. This series of studies provides information currently lacking in the literature concerning alterations in parasympathetic function associated with prolonged exposure to opiates. Results have important implications both clinically and with regard to opiate addiction. The results are particularly pertinent for people using opiates who are known to have compromised myocardial function.

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PREFACE TO CHAPTER II

This chapter describes the development of the canine model used to study alterations in cardiovascular function associated with chronic exposure to opiates. Canines were chosen since the majority of work in our laboratory uses this model. A large animal is beneficial when extensive instrumentation is required for *in vivo* cardiovascular analyses, and, in addition, provides for plasma and tissue samples of sufficient size to measure opioid peptides and neurotransmitters in femtomolar concentrations. Results of preliminary cardiovascular studies conducted in these animals are described which become the basis for the hypotheses of this work. Changes in endogenous opioid systems with chronic opiate exposure were also examined since the primary focus of our laboratory has been the role of endogenous opioids in cardiovascular regulation.

CHAPTER II

DEVELOPMENT OF A LARGE ANIMAL MODEL OF OPIATE ADDICTION TO EVALUATE CARDIOVASCULAR FUNCTION

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ABSTRACT

Cardiac abnormalities are common post-mortem findings in opiate addicts. Although endogenous opioids increase in some types of heart disease, few studies have examined cardiovascular adaptations and changes in endogenous opioids as a result of chronic opiate addiction. A chronic morphine addiction model is under development in the dog using subcutaneous implantation of 75 mg morphine pellets. After 7 days, dogs were anesthetized, cardiovascular function evaluated, and samples collected for enkephalin analysis. Plasma morphine increased proportionally in 4- and 8-pellet protocols and was relatively stable on Days 3-7. The bradycardic response to vagal stimulation and the arterial pressor response to bilateral carotid occlusion (BCO) were significantly blunted in addicted dogs when compared to controls. Naloxone did not reverse the reduced responsiveness in addicted animals and acute morphine did not suppress the response in controls. Immunoreactivity eluting with met-enkephalin-arg-phe (MEAP) and Peptide-B was decreased in addicted animals compared to controls. The results indicate that chronic morphine reduces autonomic control of the heart and circulation. Parallel reductions in cardiac enkephalins suggest that endogenous opioids contribute to the adaptation.

Key Words: morphine addiction, animal model, heart, enkephalins, autonomic nervous system

INTRODUCTION

Opiate abuse is a continuing public health concern in the United States. In addition, opiates are routinely prescribed for patients with chronic pain. Cardiac abnormalities are common post-mortem findings in opiate addicts (Dressler et al, 1989). Endogenous opioids are elevated in congestive heart failure (Fontana et al, 1993) and have been associated with the reduced autonomic control in that model (Sakamoto et al, 1989). Products of the proenkephalin (PENK) gene have been identified in canine myocardium (Barron, et al, 1992) and one in particular, met-enkephalin-arg-phe (MEAP), has significant vagolytic activity (Caffrey et al, 1995). Detailed information regarding alterations in cardiovascular function following chronic opiate addiction is difficult to find. A chronic morphine protocol was recently developed in dogs using miniosmotic pumps (Yoshimura et al, 1993). The dog allows for detailed cardiovascular analyses and sample sizes sufficient to measure hormones and neurotransmitters in femtomolar concentrations. In the present study, an addicted dog model was developed to evaluate cardiovascular adaptations to chronic morphine.

METHODS

Morphine-Addiction Protocol

Dogs were anesthetized with 25 mg/kg thiamylal-sodium (Surital) and morphine

pellets (75mg each) were implanted subcutaneously. Dogs were weighed daily and monitored for signs of morphine addiction. Plasma morphine was determined on Days 0, 3, 5 and 7.

Surgical Procedures and Experimental Protocol

After 7 days, dogs were anesthetized with sodium pentobarbital (32.5 mg/kg), intubated and mechanically ventilated. A femoral arterial catheter was inserted to measure arterial blood pressure (MAP). The carotid arteries and vagus nerves were isolated through a midline cervical incision. The heart was exposed through an incision in the left thorax. A Millar catheter-pressure transducer was inserted into the left ventricle via the left atrial appendage to infuse MEAP and to monitor heart rate (HR), left ventricular pressure (LVP) and rate of change in LVP. In morphine-addicted dogs, the right vagus nerve was stimulated (15s, 15V) at frequencies of 0.5, 1, 2, and 4 Hz, and the carotid arteries were occluded for 30 s during infusions of vehicle, 3.00 nmol/min/kg MEAP, and following the opiate antagonist naloxone (100 μg/kg). Following the experiment, the heart was collected for analysis of cardiac enkephalin content. The protocol was repeated in control animals. In these animals, the right vagus nerve was stimulated and BCOs conducted prior to administration of morphine, following acute morphine (1.0-2.5 mg/kg) and following naloxone (100 μg/kg).

Sample Processing

The hearts were diced and boiled for 30 minutes in 2.5 volumes of 1N acetic acid/

0.2 N HCL. The samples were cooled, adjusted to 0.1% 2-mercaptoethanol, homogenized, and centrifuged for 30 minutes at 25,000 x *g*. The pellets were re-extracted in an additional 2.5 volumes of acid. The two supernatants were combined, aliquoted and stored at -20° C. The samples were neutralized with 10 N NaOH and filtered with 2 µm syringe filters. Peptides in two mls of sample were separated by gel-filtration on Bio-Gel P-10 columns and eluted with 0.01M phosphate buffered saline/0.1%gel. Where required, fractions were concentrated on Porapak Q, eluted with ethanol:acetic acid:water (1:1:1) dried under vacuum and stored at -20° C. Samples were assayed using C-terminal-directed antibodies specific for ME (Dandekar & Sabol, 1982) or MEAP (Panula & Lindberg, 1987).

RESULTS

Dogs implanted with morphine pellets initially exhibited transient behavioral signs of morphine treatment (ataxia, salivation, loss of appetite) but subsequently adapted and appeared normal. Baseline cardiovascular parameters did not differ significantly from those of controls.

Plasma morphine levels increased proportionally in 4- and 8-Pellet protocols (Table 1). Acute subcutaneous administration in doses of 1.25-2.50 mg/kg resulted in plasma morphine concentrations of 275-340 ng/ml.

The bradycardic response to vagal stimulation was significantly blunted as a result of chronic morphine treatment, a response which was not changed by the administration of naloxone (Figure 1). In addition, the dynamics of the vagal response were much slower. The time required for the heart rate to reach a new steady state during vagal stimulation and the time to recover afterwards were both extended. MEAP virtually eliminated the remaining vagal bradycardia at all frequencies (not shown). The vagal bradycardia in control animals was unaltered by the administration of acute morphine.

MAP during BCOs was substantially less (although not yet statistically significant) in the chronically treated animals than in controls or animals treated with acute morphine. Again, naloxone administration did not alter the response (Figure 2). The average increase in MAP of 31 mmHg following naloxone in chronically treated animals was not accompanied by a reflex decline in HR.

Total cardiac MEAP-ir was 30 x total ME-ir and the MEAP-ir was concentrated 2:1 in the ventricles. The majority of MEAP-ir eluted with the 3.6 kd intermediate, Peptide-B and the ventricular:atrial ratio for this fraction was much greater (5:1). Chronic morphine treatment resulted in a significant decline in Peptide-B and MEAP without changing PENK (Table 2).

CONCLUSIONS

Implantation of 75 mg morphine pellets for one week resulted in substantial alterations in parasympathetic control of cardiac function and in baroreceptor reflex control of blood pressure. The fact that the rise in blood pressure following naloxone was not accompanied by a reflex decrease in heart rate further indicates altered autonomic control of cardiac function. Naloxone was unable to reverse the diminished responsiveness in addicted animals and non-addicted dogs given acute morphine responded normally. Thus, the observed functional impairments are adaptive responses to the chronic exposure to morphine and not to its acute influence. The reduction in myocardial immunoreactivity associated with products of PENK without a change in PENK itself suggests an alteration in its processing and/or secretion.

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Table 1. *Plasma Morphine Concentrations (ng/ml)*

Protocol	*n*	Day 0	Day 3	Day 5	Day 7
8-Pellet	8	3.0 ± 1.0	109 ± 10	111 ± 10	135 ± 14

Data are means ± SE.

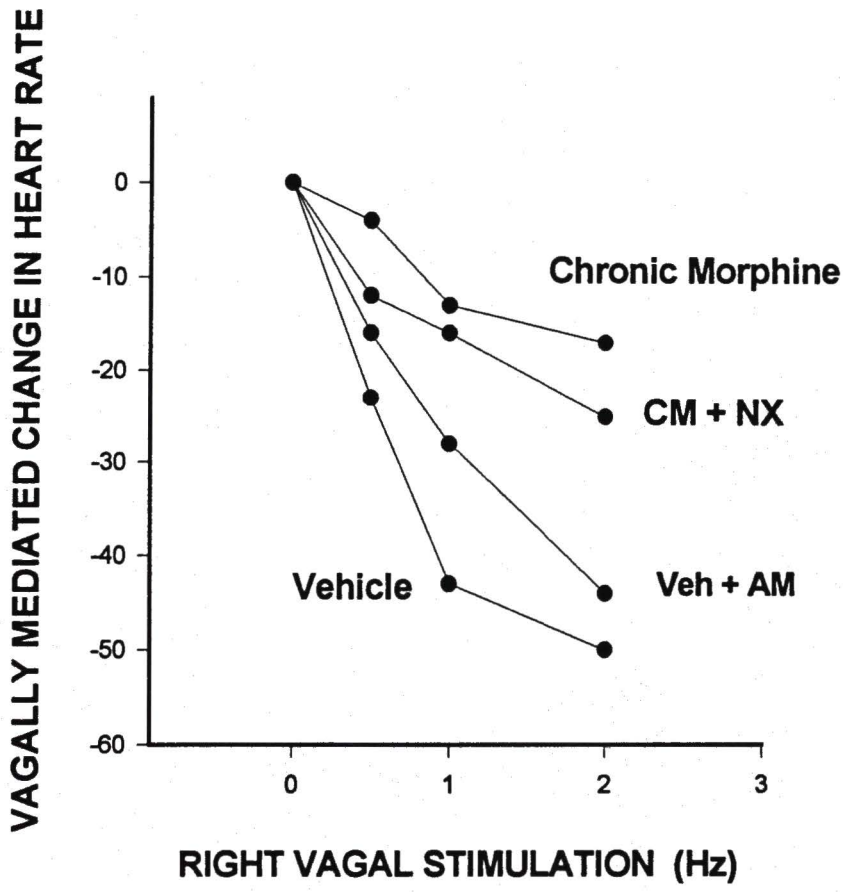

Figure 1. Effect of chronic morphine, naloxone and acute morphine on vagal stimulation. There was a treatment effect of chronic morphine across frequencies ($P < 0.001$). CM=chronic morphine, NX= naloxone, Veh=vehicle, AM=acute morphine.

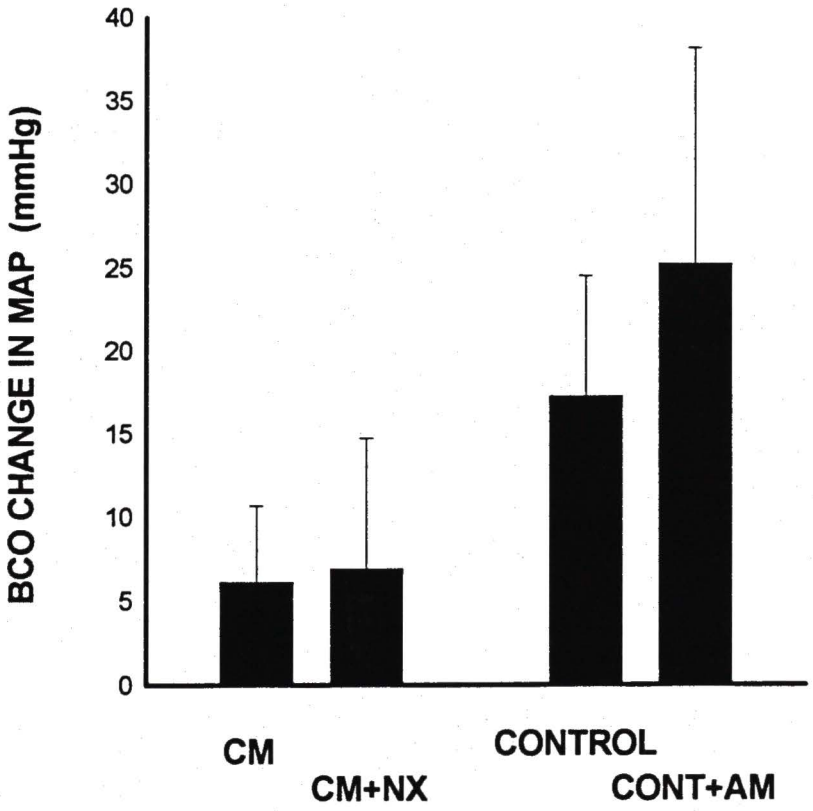

Figure 2. Change in mean arterial pressure (MAP) during bilateral catotid occlusion (BCO). CM=chronic morphine, NX=naloxone, AM=acute morphine.

Table 2. *Cardiac MEAP-immunoreactivity (pmol/gram)*

	Treatment	PENK	Peptide-B	MEAP
Right Atrium	control	2.56 ± 0.81	5.67 ± 0.38	3.19 ± 0.38
	morphine	3.40 ± 1.12	3.03 ± 0.40	1.81 ± 0.46
Left Atrium	control	2.49 ± 0.50	5.97 ± 1.02	1.89 ± 0.23
	morphine	3.61 ± 1.06	3.29 ± 0.57	1.78 ± 0.36
Left Ventricle	control	7.30 ± 1.14	25.15 ± 2.01	6.35 ± 0.88
	morphine	6.68 ± 1.09	14.80 ± 1.05	3.79 ± 0.91
Right Ventricle	control	7.53 ± 0.76	20.99 ± 1.92	3.56 ± 0.75
	morphine	12.25 ± 3.16	13.71 ± 1.55	1.71 ± 0.20
Septum	control	6.19 ± 0.60	21.35 ± 1.59	4.14 ± 0.90
	morphine	5.29 ± 0.71	18.25 ± 2.34	2.44 ± 0.48

Data are means ± SE. $n = 8$. There was a significant treatment effect of chronic morphine for Peptide-B and MEAP across tissues ($P < 0.01$). PENK=proenkephalin, MEAP=met-enkephalin-arg-phe.

PREFACE TO CHAPTER III

The previous chapter described the development of a canine model of opiate addiction and preliminary data indicating altered autonomic function and changes in myocardial enkephalin content in these animals. The most striking consequence of the one week of morphine treatment was the reduced vagal bradycardia which was not reversible with naloxone and was not observed with acute morphine. This suggested a reduction in vagal control of heart rate associated with chronic morphine. Information concerning parasympathetic function with prolonged opiate exposure is virtually nonexistent. It is known that acute morphine stimulates vagal outflow. Given this fact and the aforementioned reduction in vagal bradycardia with chronic morphine, we hypothesized that the persistent increase in efferent vagal traffic associated with chronic morphine would reduce parasympathetic control of the heart and perhaps alter intrinsic myocardial function. This chapter presents studies conducted in conscious dogs to determine parasympathetic contributions to the control of heart rate and intrinsic myocardial function following two and ten days of morphine treatment. Efferent vagal function was further evaluated under anesthesia at the termination of the two week treatment period. Ambulatory infusion pumps were used to deliver subcutaneous morphine since this method is less intrusive and provides for better control of the administered dose than the morphine pellets used in the preliminary studies. The treatment period was extended to two weeks to provide sufficient time for chronic adaptations to occur.

CHAPTER III

AUTONOMIC CONTROL OF HEART RATE IN DOGS TREATED CHRONICALLY WITH MORPHINE

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ABSTRACT

The effects of chronic morphine and the accompanying vagotonic activity on parasympathetic control of the heart were examined in dogs treated with morphine for two weeks. Heart rate and high frequency fluctuations in heart rate declined during morphine initiation. Resting heart rate remained low after two days of treatment (57 bpm) but had returned to the pre-treatment rate (76 bpm) by Day 10 (71 bpm). Ambient sympathetic tone was increased on Days 2 and 10, and plasma catecholamines were elevated on Day 2 suggesting a compensatory sympathetic response to the morphine-induced persistent vagal outflow. The intrinsic heart rate on Days 2 (160 bpm) and 10 (162 bpm) of morphine treatment were lower than the pre-treatment rate (182 bpm). Suggested mechanisms include a fundamental change in sinoatrial nodal cell function or attenuated tachycardia induced by vasoactive intestinal peptide. The time to 50 % maximal bradycardia during vagal nerve stimulation was increased with chronic and acute morphine suggesting an effect of morphine on the rate of acetylcholine synthesis, release or degradation.

Key Words: parasympathetic nervous system, opiates, power spectral analysis, intrinsic heart rate, catecholamines

INTRODUCTION

The acute administration of morphine depresses cardiovascular function in many animal species. Decreases in heart rate, blood pressure, and cardiac output have been observed following epidural, intracardiac and intravenous morphine administration in rats and dogs (15, 38, 39, 40). These depressive effects can often be diminished or completely abolished by bilateral cervical vagotomy (15, 39, 40) indicating that an increase in vagal activity is at least partially responsible for the reduced function. This "vagotonic" action of morphine is beneficial in situations when excess adrenergic activity threatens cardiovascular stability. For example, acute morphine reduced the vulnerability to ventricular fibrillation in psychologically stressed dogs (10, 11), an effect that was significantly diminished by vagotomy or atropine. In addition, morphine is often used to treat acute myocardial infarction when the electrical stability of the heart is threatened by excess sympathetic activity. Much of morphine's clinical utility in this regard derives from its ability to balance autonomic function through the reassertion of cardioprotective vagal influences.

The maintenance of normal vagal control of heart rate is important for long-term survival following acute myocardial infarction. Heart rate variability is often used as an index of the quality of vagal control. Although both the sympathetic and parasympathetic systems participate in the control of heart rate, faster parasympathetic responses are

primarily responsible for the instantaneous adjustments in heart rate which follow changes in respiration and posture. This beat-to-beat variation in heart rate, therefore, indicates the strength of vagal influences in controlling heart rate. Kleiger *et al.* (21) studied heart rate variability in 808 patients soon after myocardial infarctions. Subsequent survival rates were highly correlated with heart rate variability. Patients with high heart rate variability (increased vagal control) shortly after acute myocardial infarction were five times more likely to survive three years than patients with less variability.

Although the vagotonic actions of acute morphine have been well-documented, few studies have examined the effects of chronic morphine administration on the vagal control of cardiovascular function. Leung *et al.* (27) found no differences between morphine-treated rats and controls in vagally-mediated decreases in heart rate and blood pressure. These findings differ from data obtained in dogs (35, 50). When naloxone was administered to dogs treated with morphine for seven days, heart rate and blood pressure increased sharply (50). The heart rate then remained elevated for four hours despite the return of blood pressure to pretreatment control after only one hour. This persistent tachycardia in addicted, but normotensive, animals suggests that chronic morphine may have impaired the ability of the vagus to return heart rate to normal. We recently found that vagal bradycardia was impaired in dogs implanted with subcutaneous morphine pellets for seven days (35). The reason for the differences between rats and dogs is unclear but may reflect the greater sensitivity of dogs to morphine, greater vagal dependence in the dog, or differences in experimental methods.

Disturbances in parasympathetic function may contribute to fatalities in opiate addicts. Lipski *et al.* (29) reported cardiac arrhythmias in more than 50% of otherwise healthy opiate addicts and suggested that the resulting cardiac vulnerability might contribute to narcotic-associated fatalities. The high incidence of bradyarrythmias in this study may indicate a tonic increase in vagal activity, a loss of parasympathetic reserve and, perhaps, the inability to react appropriately to changes in sympathetic influences. In addition, most fatalities classified as heroin overdose occur among dependent, regular users, not novices, and often occur several hours following the last use. In the majority of cases, death occurs in the absence of toxic concentrations of the narcotic in question (9). These data collectively suggest that chronic alterations, and not acute effects, may contribute to heroin-related deaths.

Thus, parasympathetic control of cardiovascular function appears to be altered in opiate addicts and related animal models. This study employed a canine model to test the hypothesis that persistent increases in efferent vagal traffic associated with chronic morphine treatment reduce parasympathetic control of the heart. Parasympathetic and sympathetic contributions to heart rate variability were examined before, during and after chronic morphine treatment with the aid of power spectral analysis. Autonomic blockade was utilized to determine if the heart itself adapts to chronic morphine. Finally, direct vagal nerve stimulations were conducted to determine whether chronic morphine alters the efferent bradycardic response. Results are pertinent to tolerance and dependence models and are particularly important since a resurgence in opiate abuse has occurred in recent

years (16). The study also provides relevant clinical information since opiates are increasingly prescribed for the relief of chronic pain (8).

METHODS

Dog Selection and Training

Mongrel dogs (15-20 kg) who tested heart worm-free were accomodated to the laboratory and trained to stand quietly in a restraining sling on at least three occasions prior to beginning the protocol. In addition to size and demeanor, the animals were further selected for their tolerance of the ambulatory vest used to support the infusion pump. Dogs assigned to the control and morphine treatment groups proceeded through the treatment protocol in mixed groups (controls and morphine-treated) of two or three.

Catheter Implantation and Treatment Protocol

A baseline cardiovascular evaluation was performed 4-5 days prior to beginning the treatment protocol (see below). Three days before initiating the protocol, dogs were placed under mild sedation (ketamine, 2.5 mg/kg; xylazine, 1.5 mg/kg; acepromazine, 0.15 mg/kg) and a sterile field was prepared in the mid-scapular region. The area was infiltrated with a local anethesthetic (bupivacaine HCl, 5 mg), and a flexible intracath (14 G x 5.1 cm) was inserted subcutaneously and sutured in place. A CORMED ambulatory infusion pump (model ML-6-6) and accompanying tubing were attached to the catheter, and the dog was fitted with a vest designed to support the pump (Alice Chatham). Saline

was infused for three days in all animals to allow accomodation to the vest and pump. On Day 0, morphine (or saline for controls) was infused at an initial rate of 5.75 mg/kg/day and adjusted as required to achieve a target plasma concentration of 80-120 ng/ml (0.40-0.75 ml/hr). This concentration of morphine is sufficient to induce physical dependence in dogs (50) and, in our experience, is well-tolerated. Morphine was infused for 14 days during which time the dogs were monitored daily. The catheter was flushed daily to ensure patency, and the site was cleaned thoroughly and sprayed with gentamicin sulfate to prevent infection. The treatment protocol is summarized in Table 1.

Serum morphine levels were determined by radioimmunoassay (Coat-A-Count®, Diagnostic Products Corporation, Los Angeles CA).

Power Spectral Analysis Evaluation of Conscious Animals

General Description: The sympathetic and parasympathetic nervous systems are the primary regulators of short-term (seconds to minutes) cardiovascular control. Beat-to-beat fluctuations in heart rate reflect the compensatory responses of these systems to a variety of physiological perturbations. Power spectral analysis (PSA) allows one to assess sympathetic and parasympathetic contributions to heart rate by partitioning the variation into specific frequencies which are characteristics of each (1, 37, 43). Because the parasympathetic nervous systems reacts quickly to changes in the physiological environment, high frequency fluctuations in heart rate (>0.15 Hz) primarily reflect the activity of this system. The sympathetic nervous system responds more slowly and its

activity is associated with variations in heart rate at freqencies below 0.15 Hz. PSA of heart rate in dogs and humans (1, 37, 43) has revealed the presence of three primary frequency peaks: 1) a peak centered at 0.25 Hz corresponding to the respiratory frequency and mediated primarily by the parasympathetic nervous system, 2) a peak near 0.10 Hz important in arterial blood pressure control, and 3) a peak at 0.04 Hz related to peripheral vasomotor control. The two low frequency peaks are mediated by both sympathetic and parasympathetic influences.

Recording and Analysis of Heart Rate Signals: The heart rate signal was captured from a surface ECG and continuously displayed on a monitor (Hewlett Packard 78354A). The signal was relayed to a 12-bit analog-to-digital convertor (ADC, CIO-DAS16 Jr) connected to an IBM-compatible personal computer equipped with a 486 microprocessor.

The system digitized heart rate signals at a rate of 512 Hz. The signals were then analyzed by an algorithm which allows for continuous, on-line PSA in real-time (23). The digitized signals were truncated into 32 s time segments (windows). For each segment, the algorithm used fast Fourier transform to estimate the power density of the spectral components. The components were quantified by integration of the area of power spectral density between specific upper and lower frequency limits. The selected frequency ranges were 1) high frequency (HF)-0.15-0.40 Hz, 2) low frequency (LF)- 0.08-0.15 Hz, and 3) very low frequency (VLF)-0.01-0.08 Hz). The graphical results were continuously and simulaneously displayed on the monitor and printed (NEC Pinwriter P5200). Numerical

output was listed and printed at the end of each recording session. Data were recorded for later retrieval on an external Zip® drive.

Morphine Initiation Protocol: The heart rate power spectrum was observed and recorded during the first three hours of morphine treatment on Day 0 of the protocol. A 10-20 minute baseline recording was obtained during saline infusion. The morphine infusion was then initiated and the power spectrum recorded and printed continuously for three hours. Blood samples for plasma morphine determination were obtained via an intravenous catheter at 15, 30, 60, 90, 120, 150 and 180 minutes. The PSA data were divided into time periods for analysis as follows: 0-15 min, 15-30 min, 30-60 min, 60-90 min, 90-120 min, 120-150 min, and 150-180 min. All data were edited manually for artifacts caused by ectopic or premature beats, ECG signal interference or gross movement. PSA data were also obtained from control animals for the same number of minutes during saline infusion.

Autonomic Blockade Protocol: PSA was conducted before treatment (Day -4), and both early (Day 2) and late (Day 10) in the treatment protocol. A venous catheter was inserted into the forearm to obtain blood samples and to administer drugs. Heart rate was monitored continuously while the dog rested quietly in the sling. Every attempt was made to reduce extraneous noise and other disturbances in the laboratory. The room temperature was controlled at 24-26° C. When a steady state was achieved, the heart rate power spectrum was recorded for 10-20 minutes. The muscarinic antagonist atropine

methyl bromide (AMB, 75 μg/kg i.v.) was then administered to induce parasympathetic blockade. As AMB does not readily cross the blood-brain barrier, the secondary central effects of atropine were avoided (14). The selected dose of AMB results in complete muscarinic blockade without interrupting ganglionic transmission (48). The adequacy of the blockade was verified in each animal by demonstrating no further change in heart rate after doubling the dose. Sympathetic blockade with atenolol (3.0 mg/kg i.v.) was then induced to determine the heart rate in the absence of neural input (intrinsic heart rate). Blockade with atenolol was previously verified in animals similarly pretreated with atropine and challenged with isoproterenol (1 μg). The heart rate power spectrum was recorded continuously during the experiment. Data were edited as described above and five to ten minutes of steady-state data following each injection was used for analysis.

Plasma Catecholamines

Given the complex interplay between parasympathetic and sympathetic control mechanisms and the effects of opiates on autonomic function, plasma catecholamines were measured at pre-treatment and on Days 2 and 10 of the treatment protocol. Venous blood samples were collected into iced tubes containing EGTA and glutathione. The plasma was separated by centrifugation, spiked with metabisulfite, stored at -90° and assayed within 30 days. Catecholamines were adsorbed onto alumina and eluted with 0.1 M perchloric acid. Catecholamines were then separated by HPLC and quantitated amperometrically by integration of the signal from the electrochemical detector (BAS) as previously described (2).

Surgical Procedure

Following the chronic infusion (Day 14), dogs were anesthetized with sodium pentobarbital (32.5 mg/kg), intubated and mechanically ventilated with room air (225 ml/kg/min). Catheters filled with heparinized saline were inserted into the right femoral artery and vein and advanced into the descending aorta and inferior vena cava, respectively. The arterial catheter was attached to a Statham PD 23 XL transducer to monitor arterial pressure. Heart rate was monitored continuously by a tachometer tracking the arterial pulse pressure. The venous catheter was used to obtain blood samples and for the administration of additional anesthetic as required. Arterial blood gases and pH were monitored throughout the experiment using a Corning 178 Blood Gas Analyzer and were adjusted to within normal limits (PO_2, 90-120 mmHg; PCO_2, 30-40 mmHg; pH, 7.35-7.40) by supplementing oxygen, adjusting the minute volume or administering bicarbonate. The right and left vagus nerves were isolated through a midline cervical incision and ligated to eliminate afferently-mediated sympathetic responses during efferent vagal stimulation. Heparin (1500 units/kg) was administered to provide for sustained anticoagulation.

Vagal Stimulation

Following surgical preparation, the animals were allowed to stabilize for 30 minutes during which time blood gases and anesthesia were evaluated and adjusted as required. Test stimulations of the right vagus nerve were conducted in order to determine the supramaximal voltage required for vagal stimulations (usually 15 V). The right vagus

was then stimulated at frequencies between 0.5 and 4.0 Hz according to the protocol presented in Table 2.

Heart rate was sampled during the 15 s of each stimulation. A 105 s interval followed each stimulation to allow heart rate to return to normal. The times to reach the minimum heart rate during vagal stimulation and to return to the pre-stimulation heart rate were recorded for each frequency. Data were sampled at appropriate times, digitized and recorded on-line (MacLab).

In order to separate chronic adaptive responses from effects due to morphine circulating at the time of the experiment, the vagal stimulation protocol was also conducted in animals treated acutely with morphine (1 mg/kg s.c.). This dose produced plasma morphine concentrations of approximately double those observed in chronically-treated animals (230 ± 14 vs 108 ± 5 ng/ml). The dose was selected to produce central nervous system (CNS) morphine concentrations equal to those in animals treated with chronic morphine. Following acute morphine administration, CNS morphine concentrations in dogs are approximately half those observed in plasma (31, 32). Surgical preparation was as described above for chronically-treated animals.

Statistics

Values are expressed as means ± SE. Experiments were analyzed using within-subjects (repeated measures) analysis of variance (ANOVA) and/or between-group

randomized two- or three- factor ANOVA (GB-STAT™, Dynamic Microsystems, Silver Spring, MD). Multiple *post-hoc* comparisons were made using Tukey's protected *t*-test. $P < 0.05$ was accepted as statistically different.

RESULTS

Body Mass

Figure 1 illustrates the weight of saline control and morphine-treated dogs over the 14 day treatment period. The weight of control animals did not change significantly over time. During the initial 24-48 hours, the morphine-treated animals were clearly sedated and ate little. As a result, the weight of morphine-treated dogs declined significantly through day four of the protocol and, although their appetite returned, their weight remained below baseline for the remainder of the treatment period. The maximum weight loss was 1.7 kg observed on Days 4 and 7. By Day 10, dogs receiving morphine were behaviorally indistinguishable from control animals and had begun to regain some of their lost body mass. This is reflected by the return of weight towards baseline by Day 10.

Plasma Morphine

Plasma morphine concentrations during the first three hours of morphine infusion and during the 14 day treatment period are presented in Figure 2. By the end of the initial three hour observation period, plasma morphine concentration had reached an average of 42 ng/ml, or about 50% of the target concentration, 80-120 ng/ml. Throughout the

remainder of the protocol, infusion rates were adjusted as required to maintain plasma concentrations within the target range.

Initial exposure to morphine

Heart Rate: The changes in heart rate during the first three hours of morphine/saline infusion are presented in Figure 3. The average heart rate of dogs treated with saline did not differ significanly from baseline (70 ± 3.95 bpm) at any time during the three hour period. The average heart rate of dogs receiving morphine decreased during the three hours and was significanly different from baseline (78 ± 2.32 bpm) and from control animals after 60 min. This decline corresponded to a plasma morphine concentration of 27 ng/ml and, as indicated in Figure 4, the change in heart rate was significantly correlated with plasma morphine concentration during the three hour initiation period. ($r = -0.74$, $P < 0.01$)

Power Spectral Analysis: PSA data from the three hour saline/morphine initiation period are presented in Table 3. Although the starting heart rates and some spectral data were not statistically different between groups during pre-treatment evaluations, the HF power was different four days later (Day 0) during the baseline determination prior to beginning morphine. This preempted the usefulness of between-group comparisons of these variables over the initial three hour period. Within-group comparisions revealed no significant change in either HF or LF power over time for saline controls. HF power declined with time in the morphine-treated animals and was significantly lower than

baseline by 60-90 min. ($P < 0.05$). When large differences in total power are apparent, normalized units are frequently employed. Normalization of HF and LF power using the formulas HF/(HF+LF) and LF/(HF+LF), respectively, provides an estimate of parasympathetic-sympathetic balance. As illustrated (Figure 5), HF power represented 70-90% of combined power (excluding VLF) while LF power represented the remaining 10-30%. This confirms the predominance of vagal control of heart rate variability in the conscious dog (37). Normalized HF and LF powers were similar in both groups throughout the study, indicating that morphine infusion did not change relative paraympathetic or sympathetic contributions to heart rate variability. The stability of autonomic balance is also demonstrated by LF/HF ratio which does not change significantly during the initial three hours in either group (Table 3). The VLF power represents a small portion of the total power and did not change significantly in either group during the three hours (Table 3).

Early and Late Effects of Chronic Morphine

Heart Rate: Heart rates at rest and during sequential autonomic blockade are presented in Figure 6. In control animals, resting heart rates on Day 2 were higher than those obtained on Day 10. Conversly, resting heart rates declined significantly on Day 2 in morphine-treated animals (76 ± 3.67 to 57 ± 2.60 bpm, $P < 0.05$) but had recovered toward normal by Day 10 (71 ± 4.33 bpm). In control animals, heart rate increased by an average of 170 bpm with the administration of atropine to a resulting heart rate of 239 bpm. This post-atropine heart rate represents a combination of the intrinisic heart rate and

the ambient sympathetic tone and did not differ across days in control animals. However, in morphine-treated animals, the post-atropine heart rate on Day 10 (246 ± 7.80 bpm) was sigificantly higher than the pre-treatment rate (222 ± 11.85 bpm, $P < 0.05$). In addition, the difference between the resting heart rate and the post-atropine heart rate (black bars + hatched bars) was significantly greater on Days 2 and 10 compared to the pre-treatment difference ($P < 0.05$). This difference reflects the amount of vagal activity required to maintain resting heart rate. The subsequent administration of atenolol provides an estimate of the intrinsic heart rate, and the difference between the post-atropine and intrinsic heart rate (area of hatched bars) indicates the ambient sympathetic tone. In control animals, atenolol reduced heart rate by 52 ± 4.04 bpm from the post-atropine rate to reveal an intrinsic heart rate of 187 ± 10.73 bpm. This decrease and the resultant intrinsic heart rate were not different on subsequent days in control animals. The intrinsic heart rates obtained after 2 (160 ± 7.89 bpm) and 10 (164 ± 9.25 bpm) days of morphine were significanly lower than those obtained prior to treatment (182 ± 12.31 bpm) in the same animals ($P < 0.05$) and those obtained in controls on Days 2 and 10 ($P < 0.01$, Figure 7). This difference suggests that chronic morphine alters intrinsic properties of the heart. In an additional set of dogs (n=4), intrinsic heart rates were determined on separate occasions under control conditions and after the acute administration of morphine (1 mg/kg). The intrinsic heart rates were determined 45 min after morphine when plasma morphine concentrations approximated those observed in dogs receiving chronic morphine (135 ± 13 ng/ml). Acute morphine had no consistent effect on intrinisic heart rate in these animals indicating that a direct effect of circulating morphine was not responsible for the

changes in intrinic heart rates observed on Days 2 and 10 of chronic morphine treatment. Chronic morphine also increased ambient sympathetic tone on Days 2 and 10 when compared with measurements made prior to treatment ($P < 0.01$) or in comparable controls on the same days ($P < 0.05$).

Power Spectral Analysis: PSA data obtained prior to treatment and on Days 2 and 10 of the saline or morphine infusion are presented in Table 4. Although the control and morphine-treated dogs differed initially on several variables, no consistent differences were apparent, and within-group analyses indicated no change from baseline in any PSA variable on Day 2 or 10 of saline or morphine infusion. This is best illustrated in the normalized units.

Figure 8 illustrates average PSA results obtained during autonomic blockade studies conducted prior to treatment (morphine group). A representative tracing is presented in Figure 9. The admistration of AMB nearly abolished the HF power verifying that high frequency fluctuations in heart rate primarily reflect vagal influences. The LF power was substantially reduced by atropine indicating that parasympathetic influences also contribute to heart rate variation in this range. As reflected by the normalized units and the LF/HF ratio, AMB shifted the autonomic balance toward greater sympathetic influence. Atenolol further reduced HF power and almost completely abolished the LF power. Relative parasympathetic and sympathetic control of remaining heart rate variability was approximately equal following atenolol as reflected by the HF (52%) and

LF (48%) normalized units and by the LF/HF ratio. The VLF power was reduced by the administration of AMB and almost eradicated with subsequent administration of atenolol. The effects of autonomic blockade on PSA variables were unaffected by morphine on Day 2 or 10.

Plasma Catecholamines

Plasma norepinephrine and epinephrine concentrations remained unchanged in control animals throughout the treatment period (Figure 10). Plasma concentrations of both catecholamines were increased on Day 2 in the morphine-treated dogs when compared to pre-treatment ($P < 0.05$). On Day 10, norepinephrine and epinephrine concentrations were still slightly elevated compared to pre-treatment but were no longer significantly different.

Vagal Stimulation Data

Following the 14-day treatment period animals were anesthetized and prepared for the vagal stimulation protocol. A separate group of non-addicted animals was similarly prepared and given 1mg/kg morphine subcutaneously 30 min prior to the vagal stimulation protocol. The average plasma morphine concentration was 230 ng/ml in these animals. Initial cardiovascular parameters and blood gases are presented in Table 5. Heart rates in dogs treated with morphine acutely were significantly lower than those of controls ($P < 0.05$) or chronically-treated animals ($P < 0.01$) consistent with the bradycardic effect of acute morphine. Mean arterial pressure (MAP) was lowered by both chronic and acute

morphine treatment ($P < 0.01$). Heart rate changes to graded frequency increases in right vagal nerve stimulation are illustrated in Figure 11. The vagus nerves were ligated prior to stimulation to eliminate afferent vagal traffic while obtaining the efferent bradycardic response. In all groups, heart rate declined further with each increase in the frequency of stimulation, reaching a maximum decrease of 55-60 bpm at 4 Hz. The frequency-response relationship was not significantly different between the three groups at any frequency.

The time required for heart rate to reach 50% of the maximum decrease during stimulation and to return to 50% of the pre-stimulation rate are presented in Figures 12 and 13, respectively. In all groups, the heart rate declined more quickly as the frequency of vagal stimulation increased (Figure 12). The down-time intervals were significantly different among the three groups across frequencies ($P < 0.01$) with the control group reaching 50% of maximum decrease more quickly followed by the chronic and then acute morphine groups. In all groups, heart rate returned to the pre-stimulation heart rate more quickly as the frequency of stimulation increased (Figure 13). Although chronic morphine appeared to slow the recovery, these time intervals were not significantly different among treatment groups at any frequency.

DISCUSSION

The experiments presented above were conducted to determine if chronic morphine produced a persistent increase in efferent vagal outflow and a resultant

reduction in parasympathetic control of the heart. Dogs received subcutaneous morphine for two weeks in doses to maintain plasma morphine concentrations between 80 and 120 ng/ml. This concentration of morphine is sufficient to induce physical dependence in dogs. Yoshimura *et al.* (50) implanted miniosmotic pumps subcutaneously in dogs and delivered morphine to produce plasma concentrations of 40-50 ng/ml. After eight days of treatment, the administration of the opiate antagonist naloxone (1 mg/kg) produced both behavioral and physiological signs of withdrawal including hyperactivity, biting, tremors, nausea, tachycardia and changes in the EEG. The rise in blood pressure following naloxone, an index of opiate dependence, was 45-70 mmHg in their dogs. These results are consistent with those we previously obtained in dogs after seven days of subcutaneous morphine which produced plasma concentrations of 100 ng/ml. In those animals, naloxone administration increased blood pressure by 30 mmHg (35).

Morphine reduces heart rate through activation of vagal mechanisms. Pur-Shahriari *et al.* (38) demonstrated a decline in heart rate in dogs following the right atrial injection of morphine. Hotvedt and Refsum (15) provided evidence that morphine-induced bradycardia arises from an increase in vagal activity. They demonstrated that the reduction in heart rate following thoracic epidural morphine administration in dogs was reversed by bilateral cervical vagotomy. Similar results have been demonstrated in rats following intravenous morphine administration (39, 40). The decline in heart rate observed during the first three hours of morphine infusion in the present study is consistent with this bradycardic effect of acute morphine.

The amplitude and frequency of heart rate power spectral peaks can vary considerably in conscious dogs both between animals and within the same animal over minutes or days (4). Many factors, including breathing patterns, posture and personality can affect the power spectrum. Efforts were made to control for factors that could potentially influence autonomic tone and balance, and heart rates were not significantly different between groups prior to treatment. Nevertheless, between-group differences in HF and LF power developed prior to beginning morphine practically limiting some experiments to within-subject comparisons.

Since morphine stimulates vagal outflow, we predicted that HF fluctuations in heart rate would increase with morphine treatment. However, the power of the HF component represents *fluctuations* in heart rate mediated by efferent vagal activity and not vagal tone per se (30). If morphine produces an invariant or *persistent* vagal outflow, fluctuations in heart rate could decrease. In morphine-treated animals, the HF power decreased during the first three hours consistent with this theory. Lee *et al.* (25) measured the effects of the opioid anesthetic fentanyl on the arterial pressure power spectra in anesthetized rats. The power spectral components associated with fluctuations in arterial pressure reflect activity of the same control mechanisms as the heart rate power spectral peaks (37). Fentanyl substantially reduced HF power in these animals which may also reflect an invariant increase in vagal activity. In the present study, HF power following two days of morphine treatment was slightly lower than that obtained pre-treatment indicating that an enhanced vagal effect might still be present. By Day 10, however, the

HF power was slightly higher than that observed pretreatment suggesting a compensatory adaptation to the initial response on Day 0. Given the large variability in these measurements, the changes on Days 2 and 10 were not statistically different from baseline or from each other preventing definitive conclusions in this regard. The gradual development of an opposing adaptation by Day 10 is supported by the observation that the decline in resting heart rate seen on Day 2 had returned to normal by Day 10.

Sequential autonomic blockade provided several indices of parasympathetic and sympathetic activity. The heart rate following parasympathetic blockade with AMB represents a combination of the intrinisic heart rate and ambient sympathetic tone. The *difference* between resting heart rate and this post-AMB heart rate has been described as the vagal effect (36) since it reflects the amount of vagal activity which was required to maintain resting heart rate prior to atropine. Subsequent administration of atenolol produced complete autonomic blockade revealing the intrinsic heart rate. The difference between the post-AMB heart rate and intrinsic heart rate is a measure of ambient sympathetic tone.

The vagal influence on resting heart rate and the ambient sympathetic tone were increased on Days 2 and 10 in morphine-treated animals. The increase in sympathetic tone again suggests a compensatory response to the increase in vagal activity induced by morphine treatment. This increase in sympathetic tone may have been responsible for returning the resting heart rate to normal on Day 10. The intrinsic heart rate on Day 2

was depressed to the same degree as the resting heart rate suggesting that the decline in intrinsic heart rate was responsible for the change in resting heart rate. However, since sympathetic tone was also greater, an increase in parasympathetic activity must have accompanied this enhanced ambient sympathetic activity. The intrinsic heart rate remained depressed on Day 10. The lower intrinsic heart rate on Days 2 and 10 are not due to the acute influence of morphine since intrinsic rate was not lower in animals following acute morphine administration.

A decline in intrinsic heart rate may reflect a fundamental change in the function of sinoatrial nodal cells. Several possibilities have been suggested in this regard including alterations in mechanical factors (28), cellular metabolism (19) or potassium kinetics (17). The intrinsic heart rate following double blockade may also involve contibutions from other chronotropic agents. The neuropeptide, vasoactive intestinal peptide (VIP), is co-released with acetylcholine from postganglionic parasympathetic neurons and produces an opposing increase in heart rate (42). This "excess tachycardia" is demonstrated by an additional reduction in heart rate after ganglionic blockade or vagotomy subsequent to the usual double receptor blockade (5, 41, 45). Thus, the reduction in the intrinsic heart rate suggests chronic morphine may reduce ganglionic transmission, the synthesis of VIP or the release of VIP from the parasympathetic nerve terminals thereby reducing the excess tachycardia and lowering the apparent intrinsic heart rate. Alternatively, the chronic parasympathetic activation associated with morphine treatment may deplete the neurons of their stores of VIP.

The intrinsic heart rates obtained in control animals (185 bpm) in the present study were somewhat higher that those reported for dogs elsewhere in the literature (12). Lower intrinsic heart rates in other studies may reflect a reduction in the excess tachycardia described above due to gangliolytic properties of higher doses of atropine. The dose of AMB selected for our study was specifically titrated to provide for complete muscarinic blockade without blocking the ganglion.

An increase in plasma catecholamines paralleled the augmentation of ambient sympathetic activity after two days of morphine treatment. The sympathetic activity remained high through Day 10 despite the return of plasma catecholamines toward the baseline concentrations. This trend would be consistent with observations by Leung *et al.* (26) who found no change in arterial catecholamines in rats following three weeks of morphine. Plasma concentrations were not determined during their treatment protocol which precludes comparisons with our early observations on catecholamines.

Contrary to expectations, efferent vagal control of the heart was not altered by chronic morphine treatment as indicated by the bradycardic responses to vagal nerve stimulations. This contrasts with our earlier observation when we found a significant attenuation of efferent vagal bradycardia in dogs treated with subcutaneous morphine pellets for seven days (35). These conflicting results were not due to different plasma morphine concentrations since the concentrations were similar throughout the two protocols. The animals may have become tolerant to the vagal effects of morphine

between days 7 and 14. Leung *et al.* (27) similarly found no effect of chronic morphine on vagal bradycardia in rats that had received morphine in drinking water for three weeks. The administered dose of morphine had been increased in this study but only during the first eight days. Thereafter, the dose remained constant. Increasing the dose of morphine during week two may reduce the likelihood that animals accomodate to the effects of the drug.

Kosterlitz and Taylor (22) first reported the ability of morphine to inhibit vagal bradycardia in anethetized rats and rabbits but were unable to show this effect in guinea pigs. Acute morphine has been shown to reduce the bradycardic response to vagal nerve stimulation (49) and to stimulation of cholinergic fibers in the sinoatrial node (20) in isolated rat heart preparations. The reduced vagal function was attributed to morphine-mediated inhibition of acetylcholine release from post-ganglionic nerve fibers (20). Musha *et al.* (34) found that acute morphine in doses similar to those used presently did not alter vagal bradycardia in anethetized dogs. Similarly, acute morphine in doses sufficient to produce central concentrations equal to those in chronically-treated animals also had no effect on efferent vagal bradycardia in the present study. These conflicting results may be due to the different experimental preparations (isolated versus intact) or to species differences in the distribution of μ receptors.

Although neither acute nor chronic morphine treatment had any effect on the maximal bradycardic response to vagal nerve stimulations, the time to reach 50% of the

maximum decrease in heart rate was longer in dogs that had received chronic morphine. This time was extended even further in animals treated with morphine acutely. These results suggest that acute morphine altered the kinetics of vagal bradycardia by decreasing the rate of acetylcholine release or by increasing its rate of degradation. Acute morphine inhibits acetylcholine release in a number of systems (3, 13, 44, 46), but the effects of chronic morphine treatment are less consistent. Some studies have found no change in release (6, 33) while others have reported a decrease in release with chronic morphine (18, 24). Although applicable to the present experiments, these studies did not measure the *rate* of acetylcholine release. Brain acetylcholine turnover rate, used as an index of acetylcholine synthesis, decreased in rats following acute and chronic morphine treatment (7) and increased in mice dependent on morphine but not after acute exposure. In contrast, neither acute nor chronic morphine influenced choline uptake in rat brain (6). Acute morphine inhibits serum and tissue cholinesterase *in vitro* albeit at very high morphine concentrations (47). A chronic effect of morphine on myocardial cholinesterase has not been determined.

In summary, we predicted that chronic morphine with the accompanying increase in persistent efferent vagal traffic would attenuate parasympathetic control of myocardial function. The initial decrease in heart rate and in the high frequency power spectrum are consistent with the increase in vagal activity with morphine. Heart rate was still decreased on Day 2 but had returned to normal by Day 10. Autonomic blockade revealed a progressive increase in ambient sympathetic activity with two and ten days of morphine,

and plasma catecholamines were increased on Day 2. Collectively, these results suggest an adaptive, compensatory response in sympathetic nervous activity to chronic morphine or the resultant vagal outflow. The lower intrinsic heart rates with chronic morphine could reflect a fundamental change in sinoatrial nodal cell function or alternatively, attenuated tachycardia induced by VIP. The altered kinetics of vagal bradycardia suggests an effect of morphine on acetylcholine synthesis, release or degradation. Thus, if morphine decreased parasympathetic function, the resultant compensation by the sympathetic system masked, or perhaps partially reversed, these deficits.

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Table 1. *Treatment protocol for dogs treated for 14 days with saline or morphine.*

DAY	-4	-3	0	2	4	7	10	14
Procedures	CVA	Catheter Insertion & Saline Infusion	CVA PM PC	CVA PM PC	PM	PM	CVA PM PC	PM Terminal CVA Heart Collection

↑

Begin Morphine/Saline Infusion

CVA= cardiovascular analyses; PM= plasma morphine; PC= plasma catecholamines.

Table 2. *Vagal nerve stimulation protocol.*

Frequency (Hz)	0		0.5		1		2		4	
Stimulator	OFF	OFF	ON	OFF	ON	OFF	ON	OFF	ON	OFF
Time (sec)	15	105	15	105	15	105	15	105	15	105

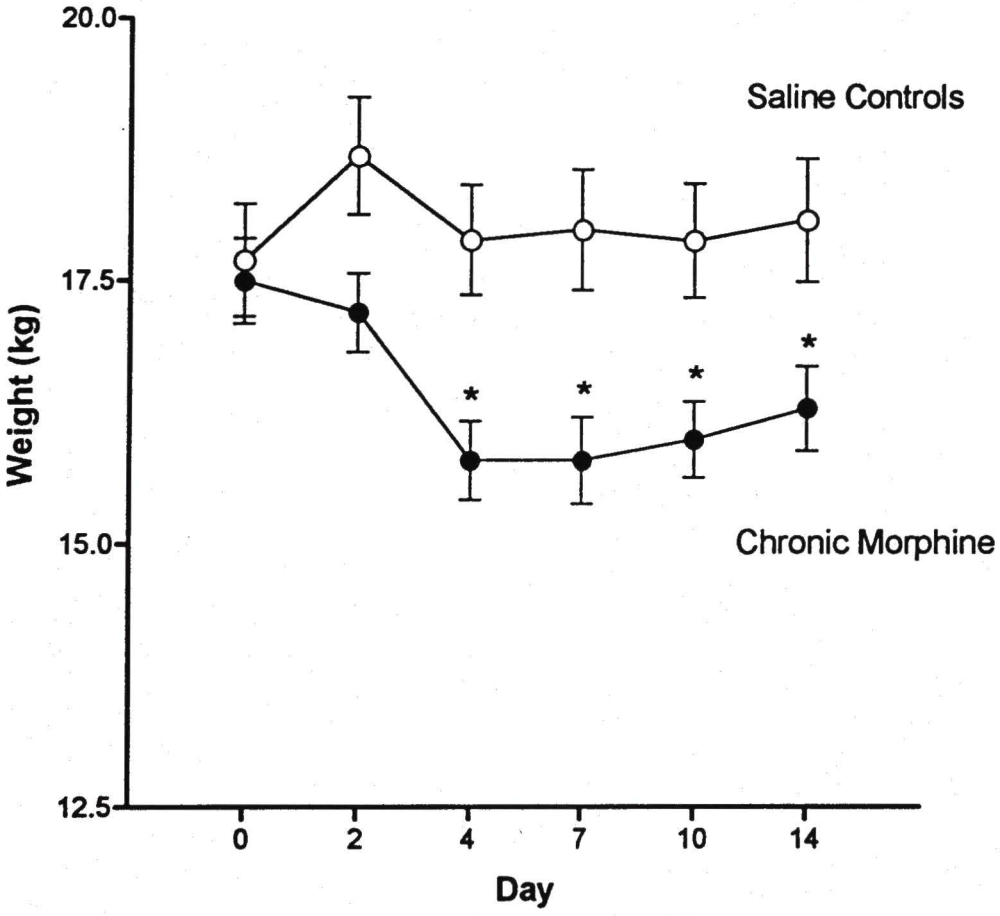

Figure 1. Weight (kg) of saline control and chronic morphine dogs during the 14-day treatment period. Values are means ± SE. *n* = 12 for saline controls; *n* = 11 for chronic morphine. *Signficantly different (*P* < 0.01) from Day 0 and from controls on the same day.

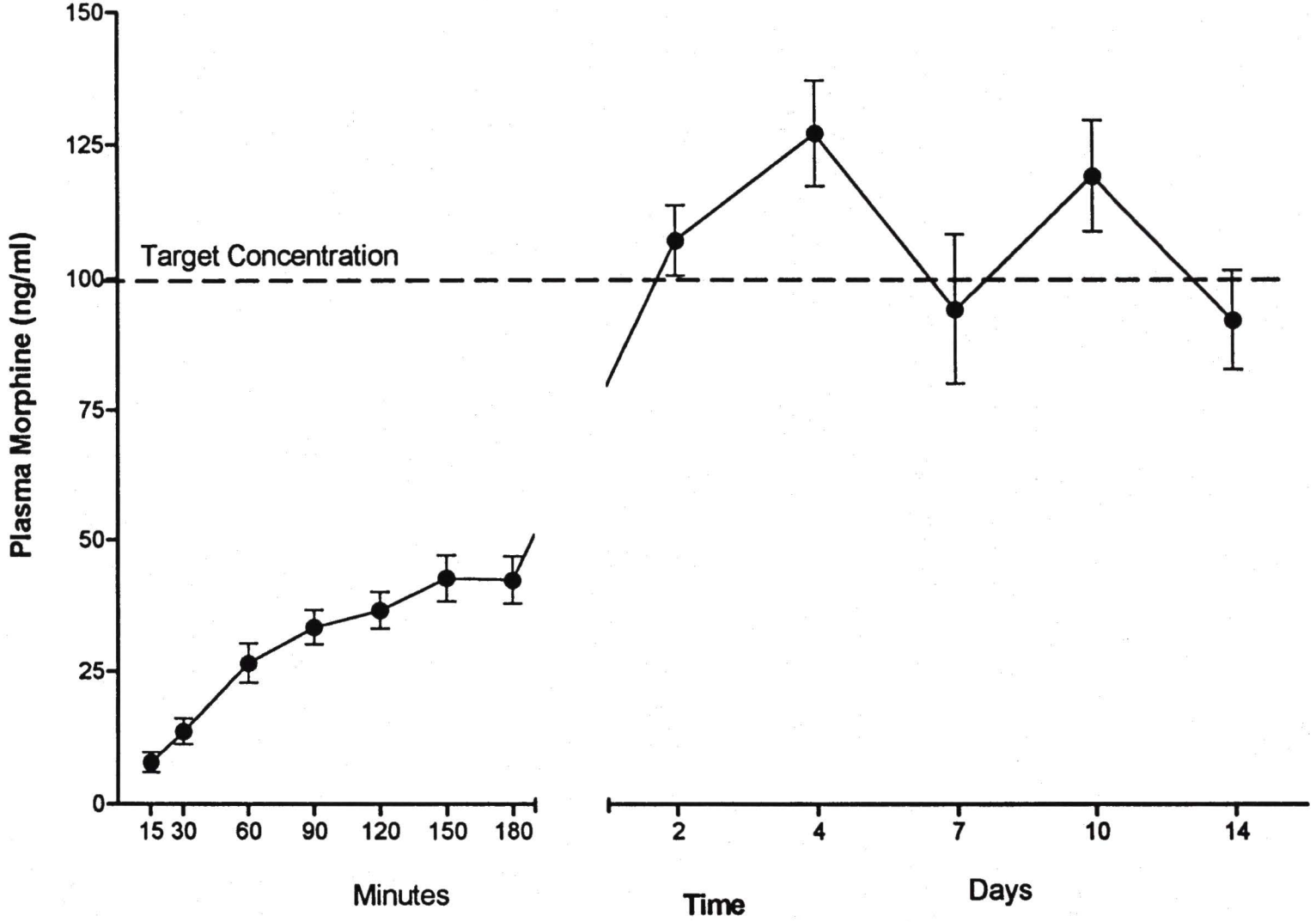

Figure 2. Plasma morphine concentrations (ng/ml) during the first three hours of morphine infusion and during the 14-day treatment period. Values are means ± SE. *n* = 10-11.

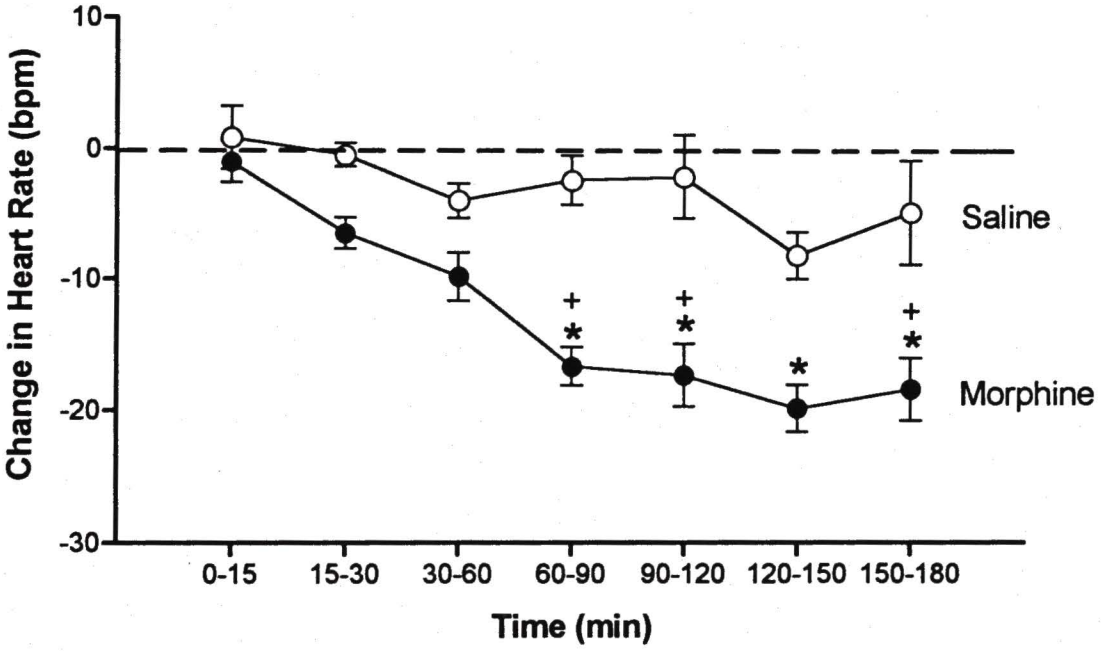

Figure 3. Change in heart rate (bpm) during the first three hours of saline or morphine infusion. Values are means ± SE. *n*=7 for saline controls; *n*=10 for morphine-treated. *Significantly different ($P < 0.01$) from baseline; $^{+}$Significantly different ($P < 0.05$) from saline controls at the same time.

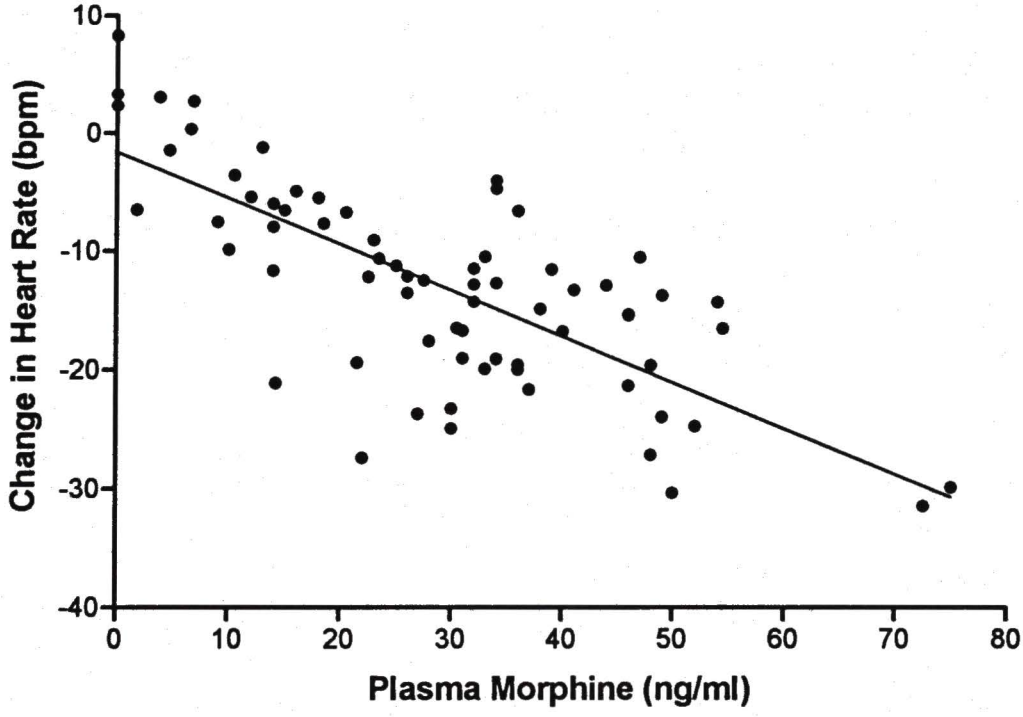

Figure 4. Linear regression analysis of the change in heart rate (bpm) as a function of plasma morphine concentration (ng/ml) during the first three hours of morphine infusion. $r = -0.74$ ($P < 0.01$).

Table 3. *Heart rate power spectral analysis data for the first three hours of morphine/saline infusion.*

	HF (bpm^2)	LF (bpm^2)	VLF (bpm^2)	LF/HF (bpm^2)
Baseline				
Saline	14.23 ± 2.79	4.12 ± 0.63	4.17 ± 0.82	0.43 ± 0.08
Morphine	126.37 ± 26.37	22.85 ± 7.35	6.15 ± 0.86	0.19 ± 0.04
0-15 min				
Saline	17.94 ± 3.95	3.22 ± 0.36	3.43 ± 0.43	0.35 ± 0.05
Morphine	112.25 ± 23.64	34.92 ± 11.10	6.45 ± 0.86	0.27 ± 0.06
15-30 min				
Saline	34.74 ± 11.63	6.16 ± 0.59	6.19 ± 0.92	0.27 ± 0.06
Morphine	100.42 ± 0.10	29.4 ± 9.71	6.48 ± 0.84	0.37 ± 0.09
30-60 min				
Saline	31.08 ± 4.82	4.03 ± 0.42	5.07 ± 1.26	0.28 ± 0.07
Morphine	90.49 ± 17.22	12.59 ± 2.46	8.45 ± 1.62	0.29 ± 0.07
60-90 min				
Saline	28.59 ± 2.90	5.00 ± 0.31	7.13 ± 1.07	0.28 ± 0.05
Morphine	*74.52 ± 11.03	13.08 ± 3.70	5.28 ± 0.73	0.18 ± 0.03
90-120 min				
Saline	22.68 ± 1.86	5.09 ± 0.31	6.52 ± 0.87	0.33 ± 0.04
Morphine	89.83 ± 14.72	11.59 ± 2.30	6.48 ± 0.89	0.21 ± 0.05
120-150 min				
Saline	44.41 ± 6.44	8.51 ± 1.73	4.35 ± 0.56	0.31 ± 0.08
Morphine	*79.44 ± 13.86	10.96 ± 2.05	5.89 ± 0.86	0.20 ± 0.04
150-180 min				
Saline	32.44 ± 4.93	7.34 ± 1.71	7.06 ± 1.22	0.36 ± 0.11
Morphine	88.27 ± 12.58	7.56 ± 0.49	4.81 ± 0.41	0.16 ± 0.03

Values are means ± SE. n=7 for saline controls and n=10 for morphine-treated. *Significantly different from baseline ($P < 0.05$). HF=high frequency, LF=low frequency, VLF=very low frequency, bpm=beats per minute.

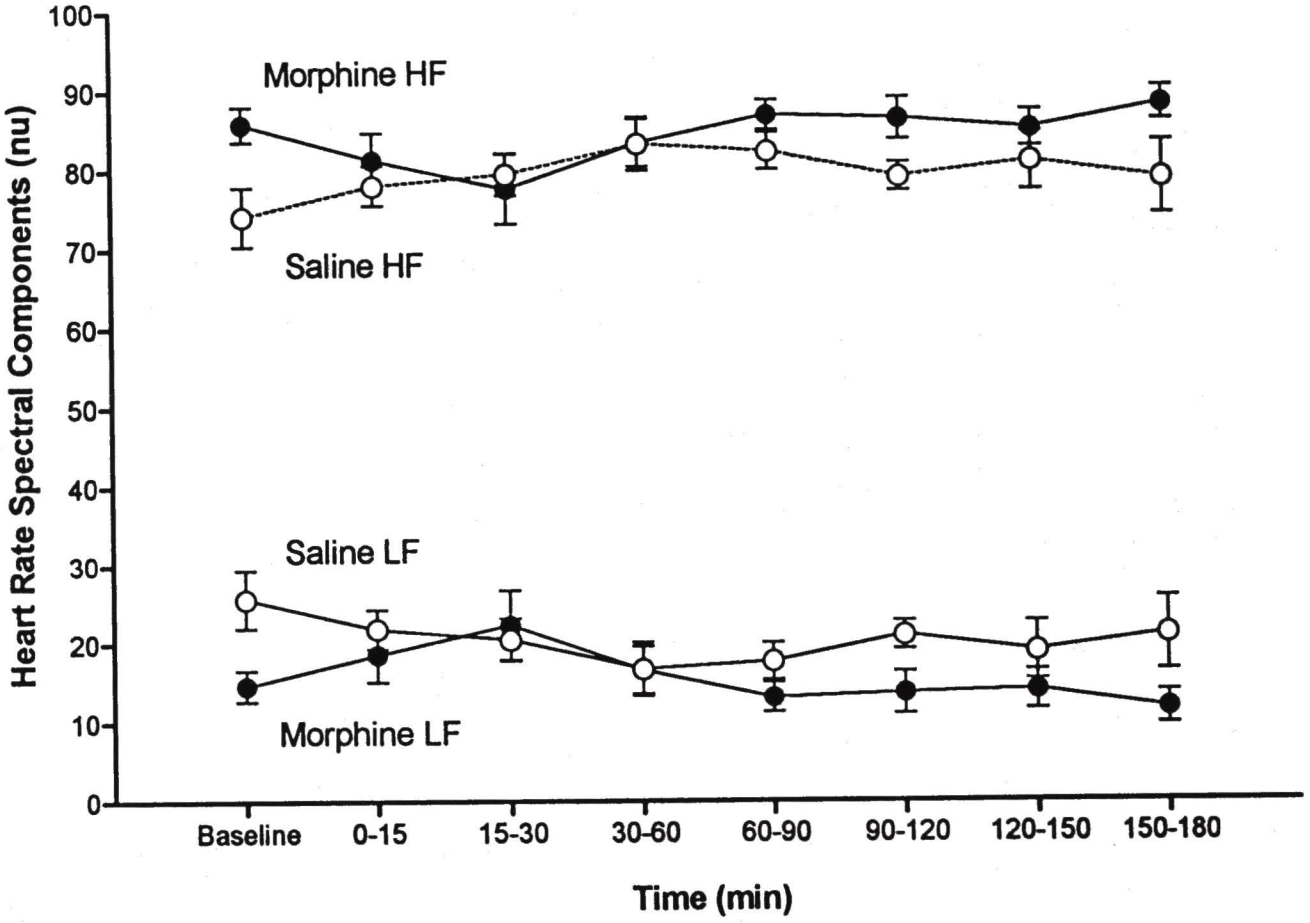

Figure 5. High frequency (HF) and low frequency (LF) power spectral components of heart rate expressed in normalized units (nu) for the first three hours of saline or morphine infusion. Values are means ± SE. *n* = 7 for saline controls, *n* = 10 for morphine-treated.

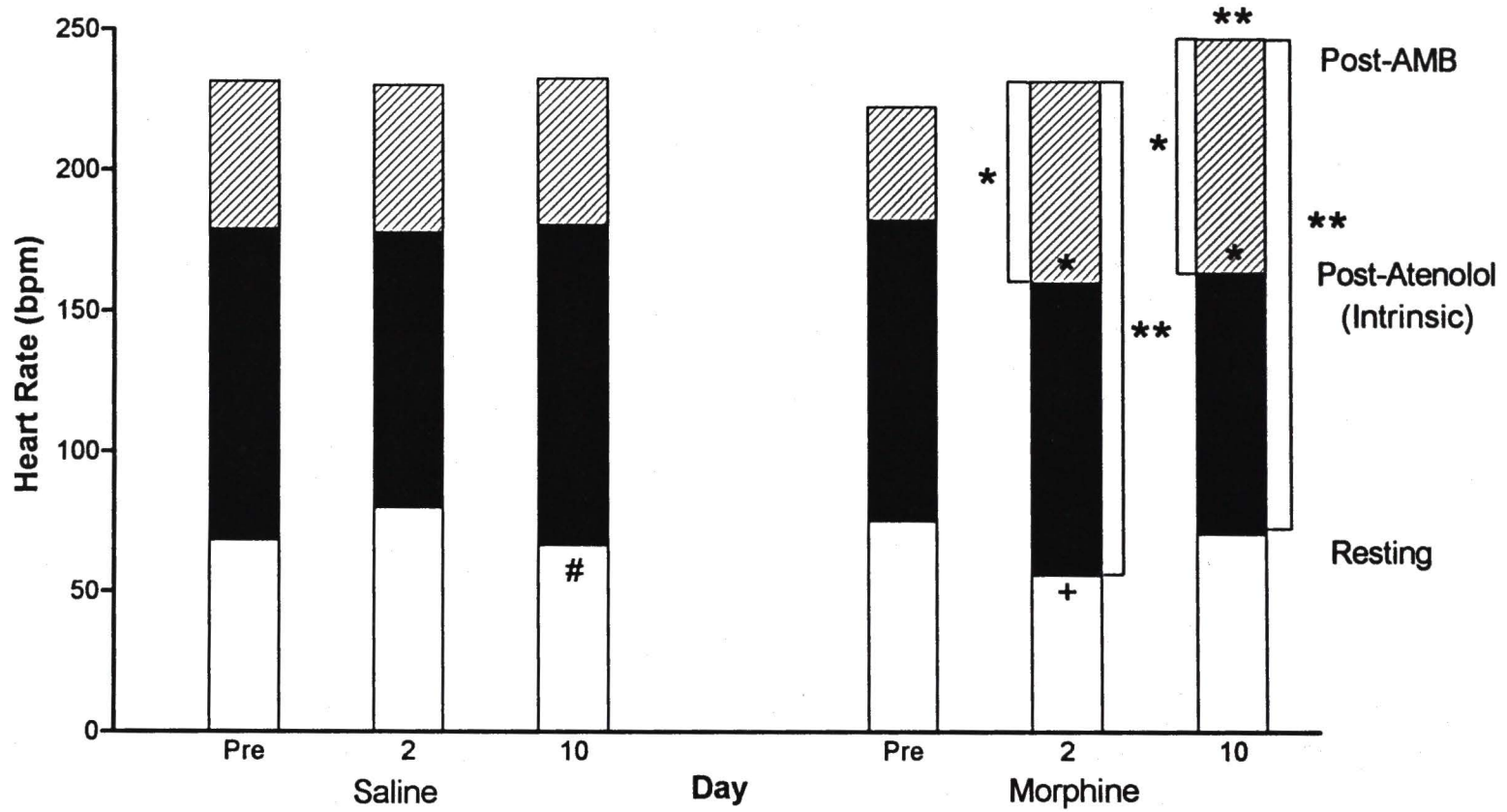

Figure 6. Heart rates at rest, post-atropine methyl bromide (AMB) and post-atenolol in dogs prior to treatment and on Days 2 and 10 of saline or morphine infusion. Values are means. n = 8 and 10 for saline and morphine dogs, respectively. #Different from saline Day 2; +Different from saline Day 2 and from morphine Pre and Day 10; *Different from saline on same day and from morphine pre-treatment; **Different from morphine pre-treatment. All degrees of significance are at $P < 0.05$.

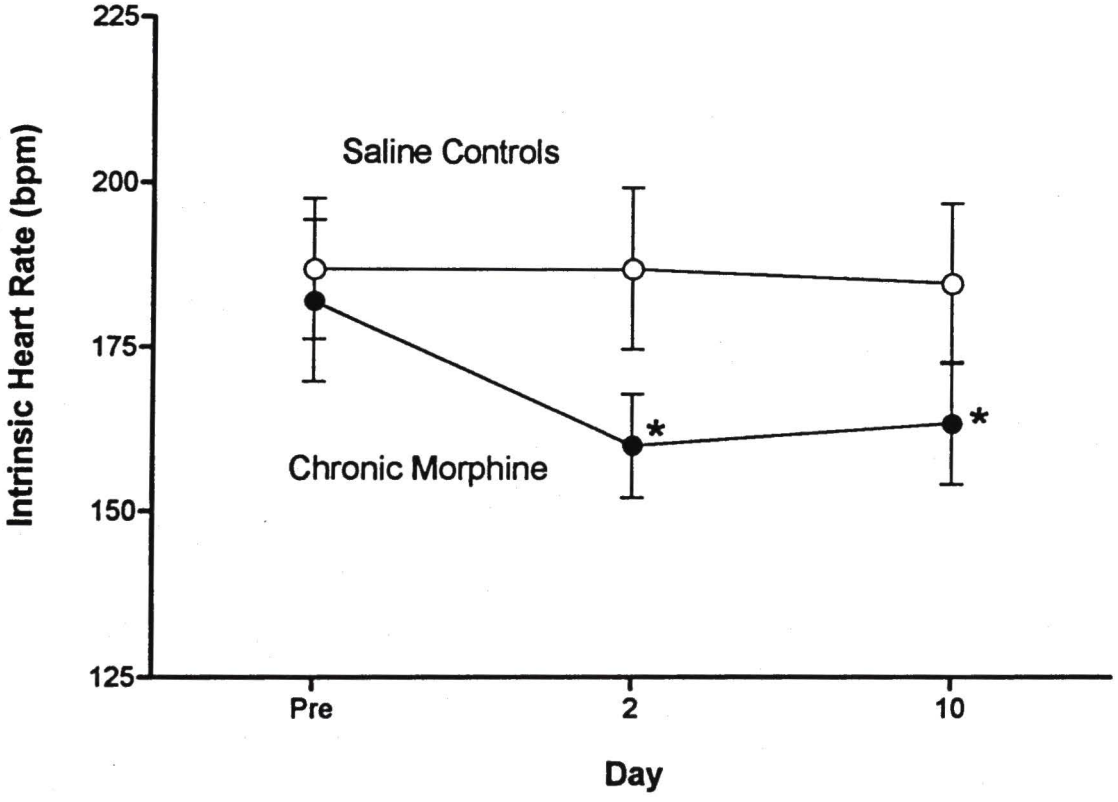

Figure 7. Intrinsic heart rates for morphine-treated and saline control dogs pre-treatment and on Days 2 and 10 of the treatment period. Values are means ± SE. n = 8 for saline controls, n = 9 for morphine-treated. *Significantly different from pre-treatment and from saline controls on the same day ($P < 0.05$).

Table 4. *Heart rate power spectral analysis data prior to treatment and on Days 2 and 10 of morphine or saline infusion.*

	Normalized Units		Spectral Components (bpm^2)			
	HF	LF	HF	LF	VLF	LF/HF
Pre						
Saline	*76.23 ± 3.33	*23.77 ± 3.33	19.92 ± 3.08	5.86 ± 1.17	5.16 ± 1.14	*0.43 ± 0.09
Morphine	90.03 ± 1.76	8.30 ± 0.94	87.03 ± 9.78	7.77 ± 1.17	4.35 ± 0.57	0.10 ± 0.01
Day 2						
Saline	83.32 ± 2.60	16.68 ± 2.60	36.65 ± 2.60	5.47 ± 1.49	3.05 ± 0.34	0.24 ± 0.04
Morphine	87.78 ± 1.60	12.22 ± 1.60	66.43 ± 11.51	8.46 ± 1.16	3.23 ± 0.51	0.16 ± 0.02
Day 10						
Saline	78.43 ± 3.21	21.58 ± 3.21	22.61 ± 3.90	5.85 ± 1.03	3.30 ± 0.35	0.34 ± 0.06
Morphine	81.32 ± 3.59	18.68 ± 3.59	102.41 ± 26.75	18.26 ± 5.06	5.42 ± 1.05	0.21 ± 0.04

Values are means ± SE. $n = 8$ for saline controls, $n = 9$ for morphine-treated. *$P < 0.05$ marked for pre-treatment between-group differences only. HF=high frequency, LF=low frequency, VLF=very low frequency, bpm=beats per minute.

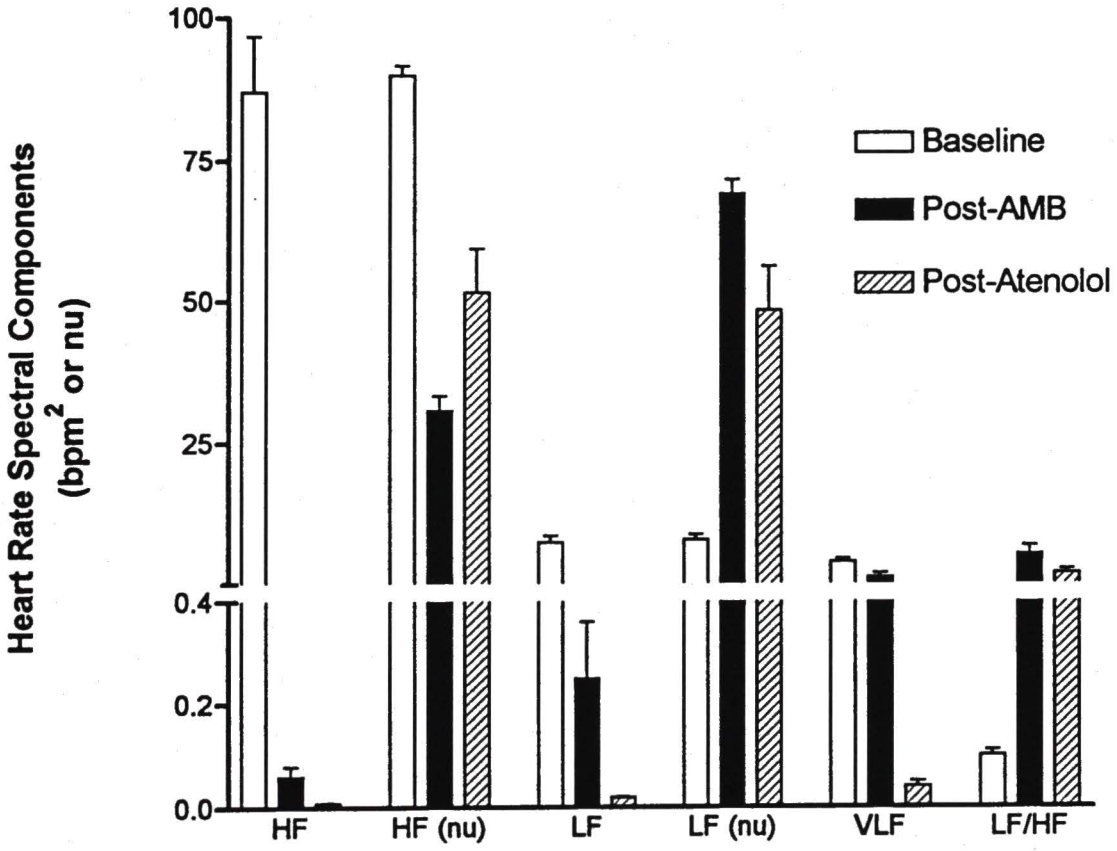

Figure 8. Effects of autonomic blockade on heart rate power spectral components. Values are means ± SE for morphine group pre-treatment. *n* = 9. HF=high frequency, LF=low fequency, VLF=very low frequency, nu=normalized units, AMB=atropine methyl bromide, bpm=beats per minute.

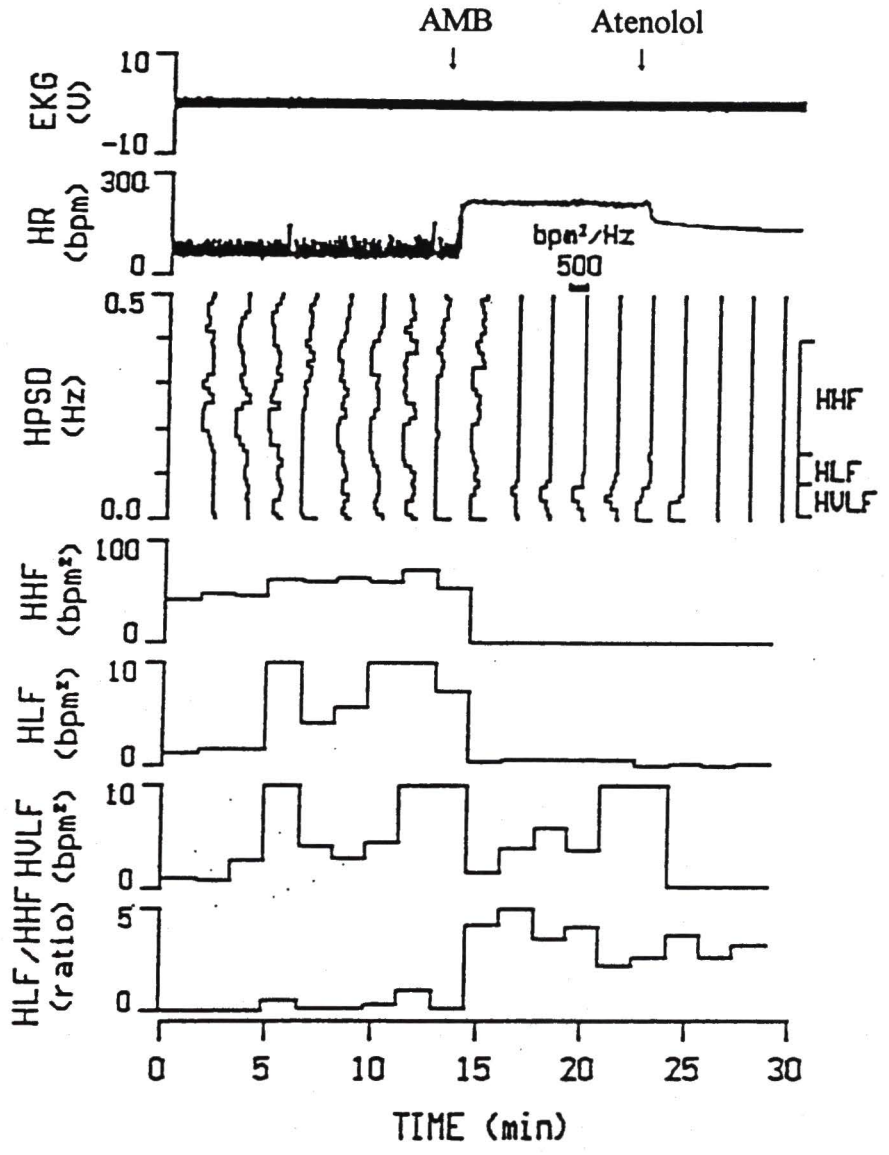

Figure 9: Representative tracing of continuous, on-line, real-time power spectral analysis of heart rate variability during sequential autonomic blockade. HR=heart rate, HPSD=heart rate power spectral density, HHF=heart rate high frequency, HLF=heart rate low frequency, HVLF=heart rate very low frequency, AMB=atropine methyl bromide.

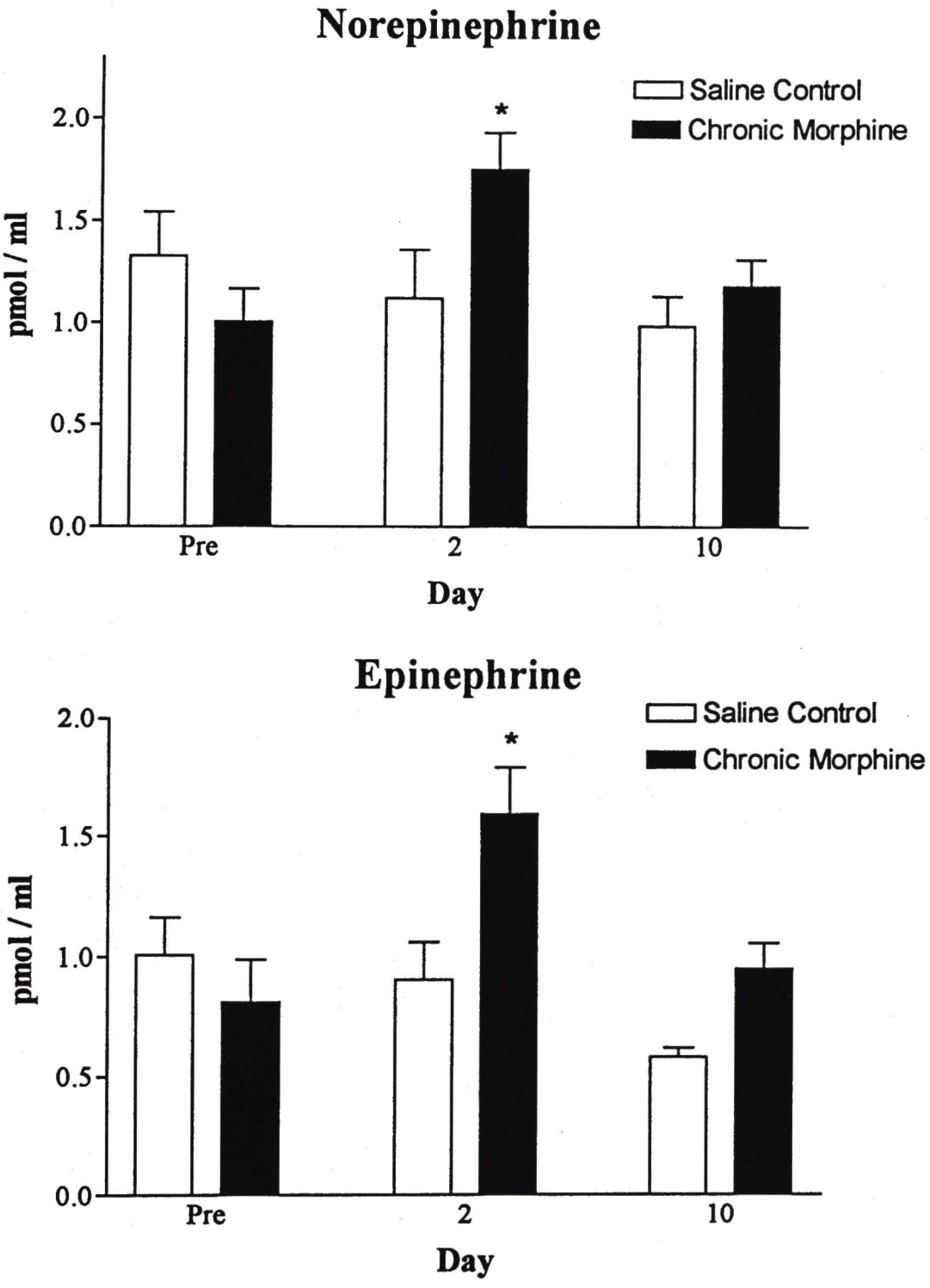

Figure 10. Plasma catecholamines in saline control and morphine-treated dogs pre-treatment and on Days 2 and 10. Values are means ± SE. n = 12 for both groups. *Significantly different from pre-treatment ($P < 0.05$).

Table 5. *Post-anesthesia cardiovascular parameters and blood gases.*

	HR (bpm)	MAP (mmHg)	PO_2 (mmHg)	PCO_2 (mmHg)	pH
Saline Control	149 ± 5.21	128 ± 3.94	105 ± 3.08	39 ± 1.57	7.36 ± 0.01
Chronic Morphine	155 ± 5.25	$^{+}$106 ± 4.65	113 ± 2.89	39 ± 1.17	7.37 ± 0.01
Acute Morphine	*123 ± 7.37	$^{+}$97 ± 5.36	116 ± 2.96	40 ± 1.65	7.37 ± 0.01

Values are means ± SE. n = 12 for controls, n = 11 for chronic morphine, n = 8 for acute morphine. *Significantly different from control ($P < 0.05$) and from chronic morphine ($P < 0.01$). $^{+}$Significantly different from control ($P < 0.01$). HR = heart rate, bpm = beats per minute, MAP = mean arterial pressure.

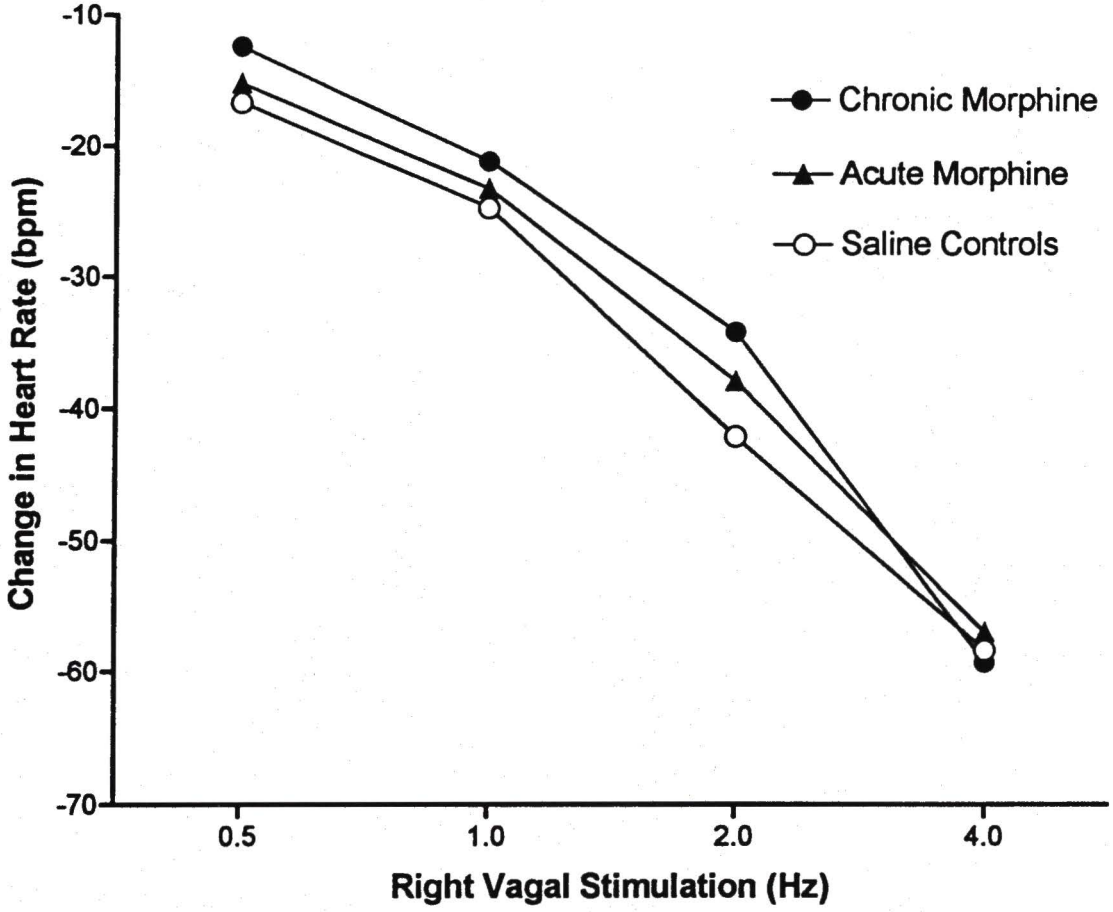

Figure 11. Decreases in heart rate mediated by right vagal stimulation in saline controls and in dogs treated with chronic or acute morphine. Values are means. Error bars have mean ommited for clarity. n = 12 for controls, n = 11 for chronic morphine, n = 8 for acute morphine.

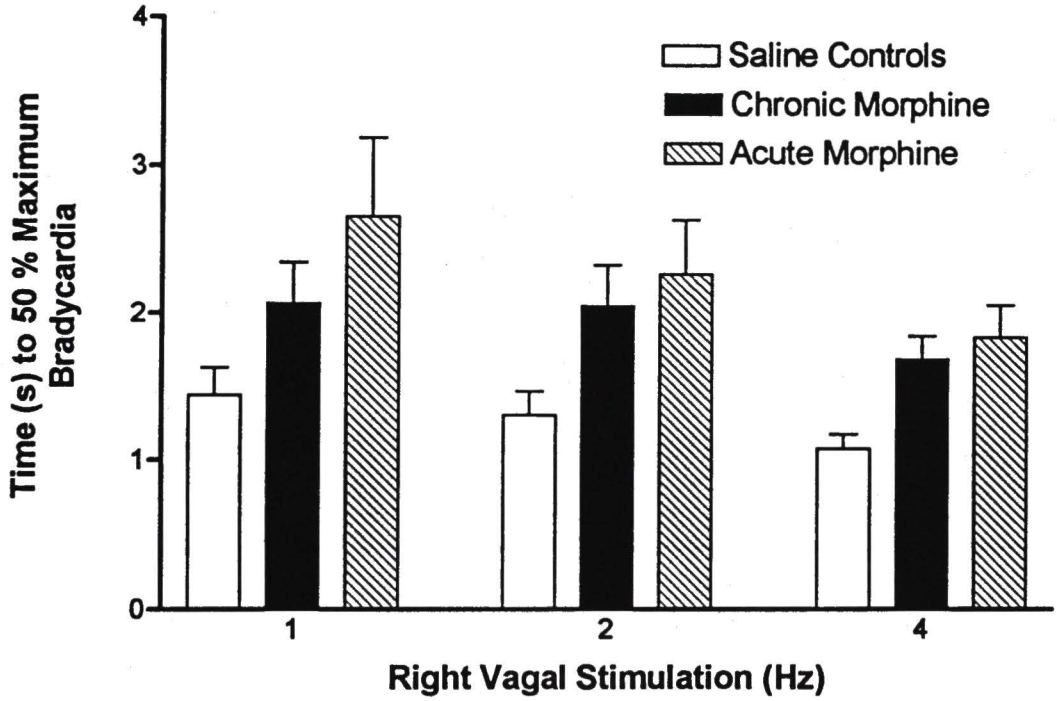

Figure 12. Time (seconds) for heart rate to reach 50% maximum bradycardia during right vagal nerve stimulation for controls and for dogs treated with acute or chronic morphine. Values are means ± SE. n = 10 for controls, n = 11 for chronic morphine, n = 7 for acute morphine. Times were significantly different between the three groups across frequencies ($P < 0.01$).

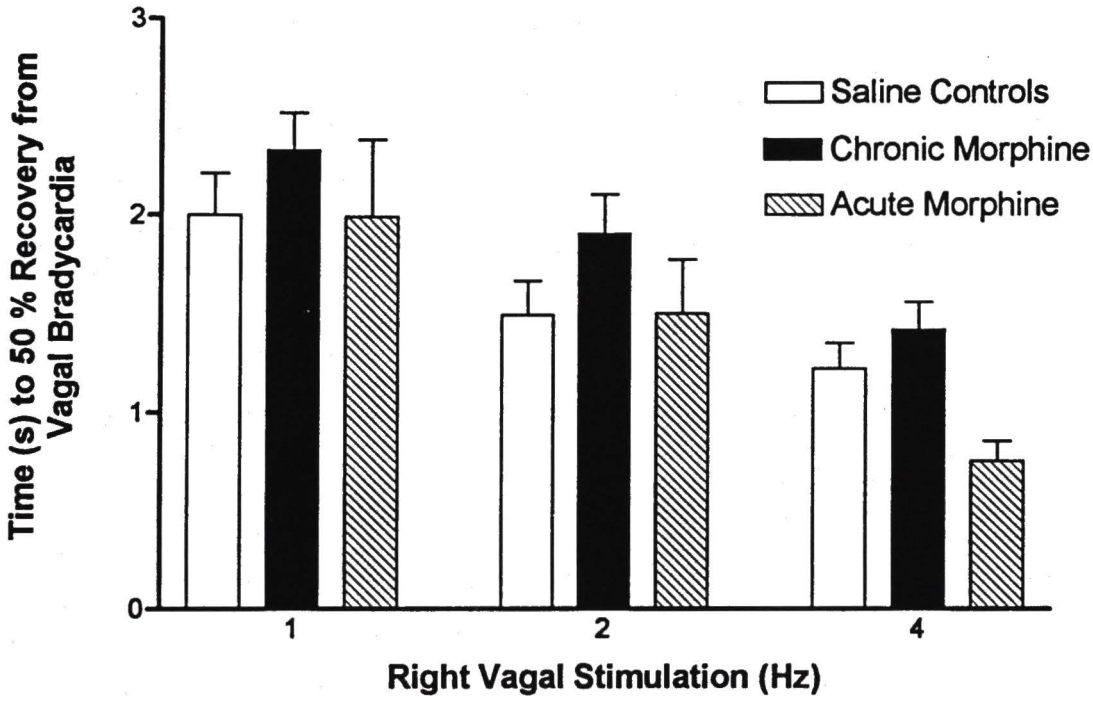

Figure 13. Time (seconds) for heart rate to return to 50 % pre-stimulation heart rate upon termination of right vagal nerve stimulation for controls and for dogs treated with acute or chronic morphine. Values are means ± SE. *n* = 10 for controls, *n* = 11 for chronic morphine, *n* = 7 for acute morphine.

PREFACE TO CHAPTER IV

This chapter describes a series of *in vitro* experiments designed to compliment those on whole animals presented in Chapter III. These studies were conducted concurrently to identify possible cellular mechanism contributing to parasympathetic dysfunction associated with chronic morphine treatment. It was hypothesized that continuous vagal outflow would result in down-regulation of cardiac muscarinic receptors and alter post-receptor second messenger systems activated by muscarinic or opposing adrenergic receptors.

CHAPTER IV

CANINE CARDIAC MUSCARINIC RECEPTORS AND ADENYLATE CYCLASE WITH CHRONIC MORPHINE

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ABSTRACT

Acute morphine stimulates vagal outflow while chronic morphine attenuates parasympathetic control of myocardial function. Dogs were treated with morphine for two weeks to identify cellular and intracellular mechanisms contributing to the reduced vagal function. Muscarinic receptor density in left ventricular and right atrial sarcolemmal membranes from dogs treated chronically with morphine were 34 % and 17 % higher, respectively, than in control animals. The increased receptor densities were not accompanied by changes in affinity (K_D). Chronic morphine had no effect on basal or $MnCl_2$-stimulated cyclase activity in either region. Similarly, maximal β-adrenergic and muscarinic receptor/G-protein coupling to adenylate cyclase were not altered by chronic morphine. An integrated, biphasic response to chronic morphine is proposed. Atrial norepinephrine content was higher than that in the ventricles and was unaltered by morphine. Ventricular norepinephrine was decreased with chronic but not acute morphine treatment. Epinephrine was evenly distributed throughout the myocardium and was reduced in the atria and ventricles by both acute and chronic morphine. This pattern suggests that morphine may reduce extraneuronal uptake of catecholamines.

Key Words: parasympathetic nervous system, heart, opiates, catecholamines, canine

INTRODUCTION

The administration of morphine is accompanied by a reduction in cardiovascular function consistent with increased parasympathetic activity (7, 25, 27, 28). The resulting changes include bradycardia, hypotension, and reduced cardiac output. Vagal participation in the responses to acute morphine is supported by the observation that vagotomy reduces or abolished these effects (7, 27, 28). In contrast, parasympathetic control of cardiovascular function may be diminished in opiate addicts and related animal models of chronic opiate administration (15, 20, 21, 40). Since acute morphine stimulates vagal outflow, chronic morphine may reduce vagal efficacy by downregulating selective components responsible for neuroeffector coupling.

A variety of myocardial cellular and intracellular mechanisms may contribute to altered parasympathetic function following chronic opiate administration. Cholinergic activation of cardiac muscarinic receptors reduces heart rate and contractile activity due in part to the activation of the inhibitory G-protein, G_i, which inhibits adenylate cyclase and the subsequent production of cAMP. This opposes the positive chronotropic and inotropic actions of β-adrenergic agonists which increase cAMP via activation of the stimulatory G-protein, G_s. Since opiate receptors, like muscarinic receptors, are also coupled to adenylate cyclase through the inhibitory G-protein, G_i, direct effects of morphine may add significantly to those mediated indirectly through its activation of the

vagus nerve. Thus, the complex interplay that can occur among opposing muscarinic, opioid and adrenergic systems and their common second messengers provides multiple opportunities for altered autonomic function during chronic opiate administration.

Prolonged exposure to muscarinic agonists rapidly desensitizes and down regulates muscarinic receptors (10, 31) reducing the ability of the target organ to respond to subsequent stimulation. Other interventions which increase vagal activity, such as morphine administration, may induce similar responses. Exposure to muscarinic agonists can also increase vagal efficacy by reducing β-adrenergic receptor responses. Sustained incubation with carbachol, for instance, decreased β-receptor binding in isolated rat cardiac myocytes, (13, 23). This effect of carbachol on β-receptor binding was not, however, duplicated after chronic morphine administration. β-adrenergic receptor binding was unchanged in cardiac preparations from rats treated chronically with morphine (12, 19).

The activation of one receptor signaling system can alter the effectiveness of opposing systems. For example, both methacholine and the opioid peptide leucine-enkephalin reduce the adrenergic activation of adenylate cyclase in the myocardium (24, 38). However, chronic exposure to drugs that inhibit adenylate cyclase, including muscarinic agonists and opioids, can result in enhanced basal adenylate cyclase activity and enhanced cyclase activity in response to stimulatory agents such as norepinephrine (35). Chronic exposure to morphine *in vitro* produced an increase in basal cyclase

activity in cultured nerve cells (33, 34). This potential model for narcotic tolerance was developed further by Wang et al (32) who have provided evidence that μ receptors assume constitutive activity during continuous exposure to morphine and no longer require agonist for signal transduction. Tolerance develops since fewer agonist-responsive μ receptors are available to oppose an even further upregulation in the cAMP system. Prolonged exposure to the muscarinic agonist, methacholine, resulted in an increase in basal adenylate cyclase activity and catecholamine-stimulated cyclase activity in embryonic chick hearts, cultured chick heart cells (14) and isolated rat ventricular myocytes (23). This receptor "crosstalk" has been described as a general cellular adaptation to interventions that enhance vagal activity (14, 23). Pertussis toxin-sensitive G-proteins may be involved in this adaptation since pre-incubation with the toxin prevented the increase in adenylate cyclase activity following prolonged incubation with carbachol in neuroblastoma/glial cells (35).

This study was conducted to determine if chronic opiate exposure to morphine with its accompanying vagotonic activity would in fact lower vagal responses by reducing muscarinic receptors and produce a compensatory upregulation in adenylate cyclase. Muscarinic receptors, adenylate cyclase activity and catecholamine content were examined in myocardial extracts and sarcolemmal membranes from dogs treated for 14 days with saline or morphine.

METHODS

Catheter Implantation and Treatment Protocol

Mongrel dogs (15-20 kg) were chosen for the study, and animals assigned to the control and morphine treatment groups proceeded through the treatment protocol in mixed groups (controls and morphine-treated) of two or three. All dogs were heart worm-free. Three days before initiating the treatment protocol, dogs were placed under mild sedation (ketamine, 2.5 mg/kg; xylazine, 1.5 mg/kg; acepromazine 0.15 mg/kg), and a sterile field was prepared in the mid-scapular region. The area was infiltrated with a local anesthetic (bupivacaine HCl, 5 mg), and a flexible intracath (14 G x 5.1 cm) was inserted subcutaneously and sutured in place. A CORMED ambulatory infusion pump (model ML-6-6) and accompanying tubing were attached to the catheter and the dog was fitted with a vest (Alice Chatham) designed to support the pump. Saline was infused for three days in all animals to allow for accommodation to the vest and pump. Morphine (or saline for vehicle controls) was then infused at an initial rate of 5.75 mg/kg/day and adjusted as required to produce a target concentration of 80-120 ng/ml (0.40-0.75 ml/hr). Morphine was infused for 14 days during which time the dogs were monitored daily. The catheter was flushed daily to maintain patency, and the site was cleaned thoroughly and sprayed with gentamicin sulfate to prevent infection. Autonomic evaluations were conducted during the two-week treatment period and have been reported elsewhere (21). These evaluations included the pharmacologic determination of intrinsic heart rate on Days 2 and 10 and collection of multiple blood samples for the determination of

circulating morphine and catecholamines.

Plasma Morphine Determinations

Plasma morphine concentrations were determined throughout the protocol by radioimmunoassay with Coat-A-Count® Serum Morphine Kit obtained from Diagnostics Products Corporation, Los Angeles CA.

Tissue Collection

Following the 14 day infusion, morphine-treated and control animals were anesthetized with sodium pentobarbital (32.5 mg/kg), intubated and ventilated with room air (225 ml/kg/min). Arterial blood gases and pH were monitored using a Corning 178 Blood Gas Analyzer and maintained within normal limits by supplementing oxygen, adjusting minute volume or administering bicarbonate. The vagus nerves were isolated and ligated, and the heart rate responses to vagal stimulation were briefly determined by stimulating the right vagus nerve at 0.5, 1, 2 and 4 Hz. A second control group was similarly prepared and tested before and 30 min after the acute administration of morphine (1mg/kg). These functional responses were reported elsewhere (21). Following these *in vivo* evaluations, hearts were excised and placed immediately into cold isotonic saline/0.1mM EGTA.

Myocardial Sarcolemmal Membrane Preparation

The right atria and left ventricle were dissected in iced petri dishes. The outermost

epicardium and endocardium were discarded, and 4 g of the remaining tissue were placed into 50 ml centrifuge tubes. The tissue was minced with scissors in 10 ml of iced saline\EGTA and washed twice with the same solution. The samples were suspended in 18 ml cold homogenizing buffer containing 10 mM Hepes, pH 7.4, 2 mM $MgCl_2$, 0.5 mM dithiothreitol and 0.1 mM EGTA. The tissue was homogenized for 10 s four times at 30 s intervals with a Polytron set at 3/4 maximal speed. Homogenates were diluted with an additional 16 ml homogenizing buffer and centrifuged at 12,000 *g* for 15 min at 4° C. The supernatants were collected and centrifuged at 50,000 *g* for 30 min at 4° C. The pellets were resuspended in homogenizing buffer (3 ml per 4 g sample) with a motor driven Potter-Elvehjem homogenizer at full speed. Membrane fractions were aliquoted and stored at -90° C (26). Protein concentrations were estimated by the method of Lowry (17).

Muscarinic Receptor Binding

Membrane preparations from control, chronically-treated and acutely-treated animals were subjected to saturation binding studies using the high affinity muscarinic antagonist quinuclidinyl benzilate (QNB) to determine differences in the muscarinic receptor equilibrium dissociation constant (K_D) for the antagonist and maximal muscarinic receptor binding (B_{max}). Saturation studies were performed in a total volume of 1 ml in 50 mM Hepes, pH 7.4, 0.5 mM $MgCl_2$, 1.0 mM NaN_3, 0.1mM EGTA and 100 mM NaCl. Assays included 100 µl [^{3}H]QNB (.005-1.0 nM) and 25 µg membrane protein ± 100 µl atropine (1µM) to determine nonspecific binding (26). All assays were incubated for 60

min at 25° C. Assays were stopped by applying the reaction mixture to glass-fiber filters under suction and washing three times with 3 ml 25 mM Tris HCl, pH 7.6. Filters were placed in 7 ml Cytoscint™ and counted in a scintillation counter. All determinations were performed in triplicate and analyzed with the aid of standard computer based curve fitting algorithms (GraphPad, Prism®).

Adenylate Cyclase Studies

Adenylate cyclase activities in sarcolemmal membranes from control, chronically-treated and acutely-treated animals were assayed in a final volume of 200 µl in medium containing 0.8 mM $MgCl_2$, 10 µM GTP, 50 mM HEPES, pH 7.4, 0.3 mM dithiothrietol, 100 mM NaCl, 0.3 mM KCl , 5.0 mM creatinine phosphate, 5 U creatinine phosphokinase, 0.5 mM 3-isobutyl-1-methyl-xanthine, 2-4 µg membrane protein, alamethicin (1ug/ug protein) and 1mM ATP (8, 26). Adenylate cyclase activity was measured under basal conditions and following the addition of isoproterenol (10.0 µM), carbachol (10.0 µM) and a combination of both. $MnCl_2$ (10 mM) was used to stimulate receptor-independent cyclase activity. Tubes were kept on ice until reactions were initiated by incubation at 30° C for 15 min. Reactions were stopped by the addition of 1.8 mls 80-90° C H_2O. CAMP was measured directly by radioimmunoassay with [^{125}I]-cyclic-AMP-tyrosine methyl-ester as the radioligand and counted in a gamma spectrometer (9). All enzyme assays and subsequent cAMP determinations were performed in duplicate.

Tissue Catecholamines

Atrial and ventricular tissues were dissected on ice and diced as described. Tissue sections were boiled in 2.5 volumes of 1 N acetic acid with 0.2 N HCl for 30 min. After cooling, 0.1% β-mercaptoethanol was added. The tissue was then homogenized (Polytron, Brinkman), and the supernatant was separated by centrifugation at 25,000 *g* for 30 min. The pellet was re-homogenized with another 2.5 volumes of fresh acid and β-mercaptoethanol. The homogenate was centrifuged again, and the two supernatants were combined and stored at -90° C for no longer than 30 days. Thawed samples were adsorbed onto alumina and eluted with 0.1 M perchloric acid. Catecholamines were separated by HPLC and quantitated amperometrically by integration of the signals from the electrochemical detector (BAS) as previously described (2).

RESULTS

Plasma Morphine

Plasma morphine concentrations measured on Days 2, 4, 7, 10 and 14 of the protocol are presented in Table 1. Infusion rates were adjusted as required to maintain plasma concentrations within the 80-120 ng/ml target range.

Myocardial Mass and Protein Recovery

Average weights (g tissue/kg body weight), total protein recovery (mg protein/g tissue) and sarcolemmal membrane protein recovery (mg/ml) for myocardial tissue regions

are presented in Table 2. Neither chronic or acute morphine had any significant effect on these variables.

Muscarinic Receptors

The density and equilibrium dissociation constant (K_D) of muscarinic receptors in left ventricular and right atrial sarcolemmal membranes were examined to ascertain whether they were altered by morphine or the resulting persistant vagal outflow. Receptor density in left ventricular and right atrial membranes from dogs treated chronically with morphine were 34% ($P < 0.01$) and 17% ($P < 0.05$) higher, respectively, than in control animals (Figure 1). Representative saturation curves are presented in Figure 2. Similar but smaller increases following acute morphine failed to reach statistical significance.

Within each treatment group, the K_D of muscarinic receptor binding was slightly but not significantly higher in the right atria than in the left ventricle (Figure 3). There were no treatment effects among groups in either the left ventricle or right atria.

Adenylate Cyclase Activity

Basal and $MnCl_2$-stimulated adenylate cyclase activity were examined to determine if chronic morphine treatment altered the post-receptor effector. Maximal muscarinic and β-adrenergic receptor/G-protein coupling to adenylate cyclase were examined by determining the cyclase activity in the presence of carbachol and isoproterenol. These two

agents were also tested together. Adenylate cyclase activity expressed as cAMP production in left ventricular and right atrial sarcolemmal membranes from dogs treated with saline, chronic morphine or acute morphine is presented in Figures 4 (left ventricle) and 5 (right atria).

For all assay conditions and treatment groups, right atrial adenylate cyclase activity was at least 50% lower than left ventricular activity (Figures 4 and 5). Basal adenylate cyclase activities were 480 and 175 pmol cAMP/mg protein/min, respectively, for left ventricular and right atrial membranes across treatment groups. No difference in basal adenylate activity was noted as a result of morphine treatment. Isoproterenol resulted in an average increase from baseline of 52% in the left ventricle and 67% in the right atria while carbachol attenuated cyclase activity by an average of 69% in the left ventricle and 81% in the right atria. Neither chronic nor acute treatment with morphine altered maximal β-adrenergic (isoproterenol) or muscarinic (carbachol) receptor/G-protein coupling to adenylate cyclase. Adenylate cyclase activity was decreased by an average of 36% in the left ventricle and 39% in the right atria when opposing adrenergic and muscarinic coupling systems were activated simultaneously by adding isoproterenol and carbachol together. No difference in this integrated response was noted with chronic or acute treatment with morphine. To determine if morphine treatment altered the quantity of adenylate cyclase available for stimulation, cyclase activity was measured in the presence of $MnCl_2$, a direct stimulator of adenylate cyclase which bypasses the receptor/G-protein coupling mechanism. Cyclase activity was increased above baseline by 246 % in the left ventricle

and 366% in the right atria across treatment groups in the presence of $MnCl_2$. Neither chronic nor acute treatment with morphine altered $MnCl_2$-stimulated adenylate cyclase in myocardial membranes.

Tissue Catecholamine Content

Norepinephrine and epinephrine content in myocardial tissue extracts from dogs treated with saline, chronic morphine or acute morphine were examined to determine whether morphine treatment resulted in an adaptive response in tissue catecholamines. As we reported previously (1,2), left atrial norepinephrine content was consistently higher than ventricular content (Figure 6). Morphine had no apparent effect on atrial norepinephrine. Ventricular norepinephrine was distributed evenly throughout the ventricular tissues and was significantly lower ($P < 0.01$) in dogs treated chronically with morphine when compared with animals treated with saline or acute morphine (Figure 6). Epinephrine content was evenly distributed throughout both the atria and ventricles as previously reported (2) and was significantly lower in every region after chronic morphine (Figure 7). Epinephrine was also lower in tissue extracts from dogs treated acutely with morphine. This effect was significantly different from saline controls in the left atria and right ventricle (Figure 7, $P < 0.01$).

DISCUSSION

The present experiments were conducted to identify cellular and intracellular mechanisms contributing to attenuated parasympathetic control of myocardial function associated with chronic opiate administration. Muscarinic receptors, adenylate cyclase activity and catecholamine content were examined in myocardial extracts and sarcolemmal membranes from dogs treated with saline or morphine for 14 days or given morphine acutely for 30 min. Morphine doses were adjusted as required throughout the treatment period to maintain plasma morphine at concentrations known to induce physical dependence (20, 40).

Following the treatment protocol hearts were excised and weighed. Two weeks of morphine treatment had no effect on myocardial mass. Dressler and Roberts (5) reported cardiomegaly in 68% of hearts from opiate addicts examined at autopsy. Another post-mortem study reported enlarged hearts in approximately 50% of addicts when compared to the mean heart weight of non-addicted patients (36). The two weeks of morphine treatment in the present study may have been insufficient to produce appreciable changes in myocardial mass. Although the aforementioned studies of drug addicts did not provide information as to the duration of addiction, death among opiate addicts usually occurs among chronic users (3) suggesting that the autopsy cases most likely represented addicts who had abused drugs for extended periods of time.

We hypothesized that the persistent vagal outflow accompanying chronic morphine treatment would result in down-regulation of myocardial muscarinic receptors. Since the parasympathetic innervation associated with rate control projects primarily to the sinoatrial node, it was expected that down-regulation would be prominent in the right atria. Left ventricular muscarinic receptors were examined for comparison purposes. Contrary to the hypothesis, the number of muscarinic receptors was increased as a result of chronic exposure to morphine, an effect that was slightly more evident in the left ventricle than in the right atria. The literature is contradictory with respect to muscarinic receptor distribution and parasympathetic innervation in the myocardium. The density of muscarinic receptors is greater in the atria compared to the left ventricle in rat (30, 39), rabbit (6, 39) and cat (29). Muscarinic receptors are more evenly distributed throughout the myocardium in guinea pigs (30, 39) and, consistent with the present study, in dogs (39). The ventricles are generally recognized to receive parasympathetic innervation (16). In man, it is estimated that the ventricles receive about one-fifth the cholinergic innervation of the atria. The existence of parasympathetic innervation in the ventricles is supported by the ability of cholinergic agents or vagal stimulation to depress ventricular function (16). Thus, changes in parasympathetic activity with morphine treatment could provoke cholinergic adaptations in the ventricles.

Little information is available concerning muscarinic receptors in the heart following chronic morphine treatment. Chronic morphine had no effect on muscarinic receptor binding in rat brain (4). However, psychopharmacological evidence in rats

treated with chronic morphine suggested that muscarinic receptors had become supersensitive to agonist stimulation (37). Consistent with the present study, the authors suggested that supersensitivity could reflect an increase in the number of central and peripheral cholinergic receptors. Further, they postulated that the inhibition of acetylcholine release sometimes observed following morphine treatment may have been responsible for a compensatory upregulation of muscarinic receptors. An increase in muscarinic receptor affinity could also explain the reported supersensitivity. However, we found no change in the K_D in either the right atria or left ventricle following chronic morphine treatment. Plasma catecholamine concentrations and ambient sympathetic activity were both increased in our dogs during morphine treatment (21). The upregulation of muscarinic receptors may reflect compensatory cholinergic adaptations to this increase in sympathetic activity.

Basal and $MnCl_2$-stimulated adenylate cyclase activities were consistently 50% higher in left ventricular tissue than in corresponding atria. This result contrasts with earlier reports of higher adenylate cyclase activity in the atria of other species including rat, chicken, rabbit and cat (16). The higher atrial cyclase activity has been attributed to the greater degree of parasympathetic innervation in this region. This comparison has not been made for similarly prepared canine tissues. An extraction artifact can not be ruled out but seems unlikely since the recovery of membrane protein was equivalent in both atria and ventricles. The small animals referenced above have characteristically high resting heart rates compared to dogs. Discrepancies in the atrial/ventricular distribution of

cyclase activity between large and small animals may reflect differences in the relative importance of heart rate and stroke volume in determining the cardiovascular response to autonomic control.

Our hypothesis predicted that the continuous application of agents which inhibit adenylate cyclase would provoke a compensatory increase in cyclase activity. Contrary to expectations, chronic morphine did not result in upregulation of adenylate cyclase. As expected, however, the acute administration of morphine *in vivo* had no effect on the basal or $MnCl_2$-stimulated cyclase activity measured in right atrial and left ventricular membranes *in vitro*. Niroomand *et al.* (22) also found no acute effect of the μ receptor agonist morphiceptin on adenylate cyclase activity when added to canine cardiac sarcolemma *in vitro*. Several studies have, however, shown a compensatory increase in adenylate cyclase activity in other models following prolonged exposure to morphine or to other drugs known to inhibit adenylate cyclase activity (14, 23, 33, 34, 35). The data presented above obtained in dogs do not corroborate these findings since chronic morphine had no effect on basal or $MnCl_2$-stimulated cyclase activity, suggesting that vagal impairments cannot be explained by an increase in cyclase activity.

Given that the cyclase activity remained constant and the number of inhibitory muscarinic receptors increased, a compensatory decrease in muscarinic agonist-receptor coupling could explain a decrease in vagal efficiency. However, the maximal inhibition of cyclase activity by carbachol was unaltered by either acute or chronic morphine.

In the absence of a compensatory increase in the enzyme effector, adenylate cyclase, reduced vagal function also could result from an increase in opposing adrenergic receptor coupling. Isoproterenol-stimulated cyclase activity can provide an estimate of β-adrenergic receptor-effector coupling. However, maximal β-adrenergic stimulation (isoproterenol) of adenylate cyclase was unaffected by acute or chronic morphine treatment. This observation is consistent with findings in rat heart where β-receptor number was unchanged following chronic treatment with morphine (12, 19). In contrast, our findings differ from data obtained in other models where adrenergic stimulation of adenylate cyclase activity was enhanced by chronic morphine treatment (33, 34, 35).

Tissue catecholamine content was examined to further characterize adaptations in sympathetic function during chronic morphine treatment. Changes in catecholamine content could reflect differences in their synthesis, release or uptake. Atrial norepinephrine content was consistently higher than that in the ventricles reflecting the greater density of sympathetic nerves in this region (1). Atrial norepinephrine was unaltered by morphine, but chronic morphine decreased ventricular norepinephrine. Ko *et al.* (11) found no change in ventricular norepinephrine content in rats treated chronically with morphine. These conflicting results may reflect differences in the species sensitivity to morphine, route of drug delivery or experimental design. Acute morphine did not alter ventricular norepinephrine in the present study in agreement with the results found by Ko *et al.* (11) in rats. Mantelli *et al.* (18), however, found that acute morphine potentiated the inotropic response to sympathetic nerve stimulation in isolated guinea pig atria. This

effect was blocked by desmethylimipramine, a drug which inhibits neuronal uptake of norepinephrine, indicating an effect of acute morphine on reuptake of norepinephrine by sympathetic nerve terminals. Epinephrine was evenly distributed throughout the myocardium (1, 2) reflecting perhaps its primary route of accumulation, uptake from the circulation. Morphine delivered either chronically or acutely decreased atrial and ventricular epinephrine content. This pattern suggests that morphine may reduce extraneuronal uptake which prefers epinephrine over norepinephrine and presumably is proportionally more important in the less densly innervated ventricles. Acute morphine produced a somewhat less consistent reduction in myocardial catecholamines suggesting that this effect may have both acute and chronic components.

In summary, we predicted that chronic exposure to morphine with its attendant vagotonic activity would attenuate paraympathetic control of myocardial function by reducing myocardial muscarinic receptors and increasing adenylate cyclase activity. We found, however, that chronic morphine treatment *increased* left ventricular and right atrial muscarinic receptor density without any coincident change in adenylate cyclase activity or its response to muscarinic and β-adrenergic stimulation. The doses of carbachol and isoproterenol were chosen to elicit a maximal response so these results do not preclude subtle changes in *responsiveness* of adenylate cyclase to lower concentrations of carbachol and isoproterenol had full dose-response curves been practical. Both plasma catecholamines and the ambient adrenergic activity were elevated earlier in the treatment period in these same animals (21). The augmented sympathetic activity may represent a

secondary adaptation to sustained parasympathetic activation associated with chronic morphine. This suggests a biphasic response to morphine treatment whereby sympathetic systems compensate for the drug-mediated sustained parasympathetic outflow by increasing ambient adrenergic activity. This is followed by a subsequent upregulation of parasympathetic systems as evident in this case by an increase in muscarinic receptor density.

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Table 1. *Plasma morphine concentrations during the 14-day treatment period.*

Day	2	4	7	10	14
Plasma Morphine	107 ± 6.58	127 ± 9.89	94 ± 14.1	119 ± 10.4	92.3 ± 9.41

Values are means ± SE. $n = 11$.

Table 2. *Mass (g tissue/kg body weight), recovered protein (mg/g) and recovered sarcolemmal protein (mg/ml) of myocardial regions from dogs treated with saline, chronic morphine or acute morphine.*

		Left Ventricle	Right Ventricle	Whole Atria	Left Atria	Right Atria
Myocardial Mass	Saline Controls	4.19 ± 0.16	1.38 ± 0.05	0.67 ± 0.02	--------	--------
	Chronic Morphine	4.11 ± 0.16	1.32 ± 0.07	0.74 ± 0.04	--------	--------
	Acute Morphine	4.37 ± 0.24	1.40 ± 0.07	0.73 ± 0.04	--------	--------
Recovered Tissue Protein	Saline Controls	204 ± 4.93	198 ± 4.94	--------	174 ± 3.68	--------
	Chronic Morphine	208 ± 5.08	211 ± 6.14	--------	173 ± 5.64	--------
	Acute Morphine	211 ± 7.13	212 ± 8.31	--------	182 ± 10.9	--------
Recovered Sarcolemmal Protein	Saline Controls	2.05 ± 0.17	--------	--------	--------	3.13 ± 0.26
	Chronic Morphine	2.15 ± 0.17	--------	--------	--------	3.21 ± 0.35
	Acute Morphine	2.12 ± 0.19	--------	--------	--------	2.97 ± 0.22

Values are means ± SE. n = 11-12 for saline controls, n = 11 for chronic morphine, n = 7-10 for acute morphine.

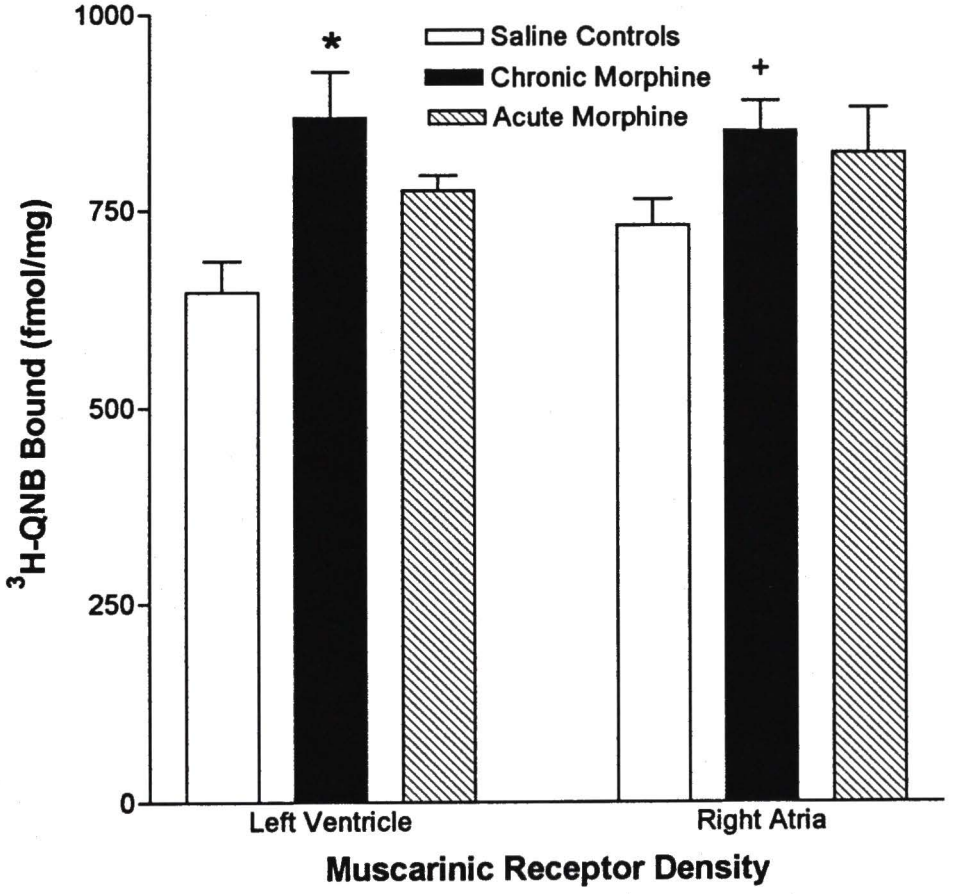

Figure 1. Muscarinic receptor densities (B_{max}) in left ventricular and right atrial sarcolemma from saline control, chronic morphine and acute morphine dogs. Values are means ± SE. n = 12 for saline controls, n = 11 for chronic morphine, n = 10 for acute morphine. *Significantly different from control ($P < 0.01$). +Significantly different from control ($P < 0.05$).

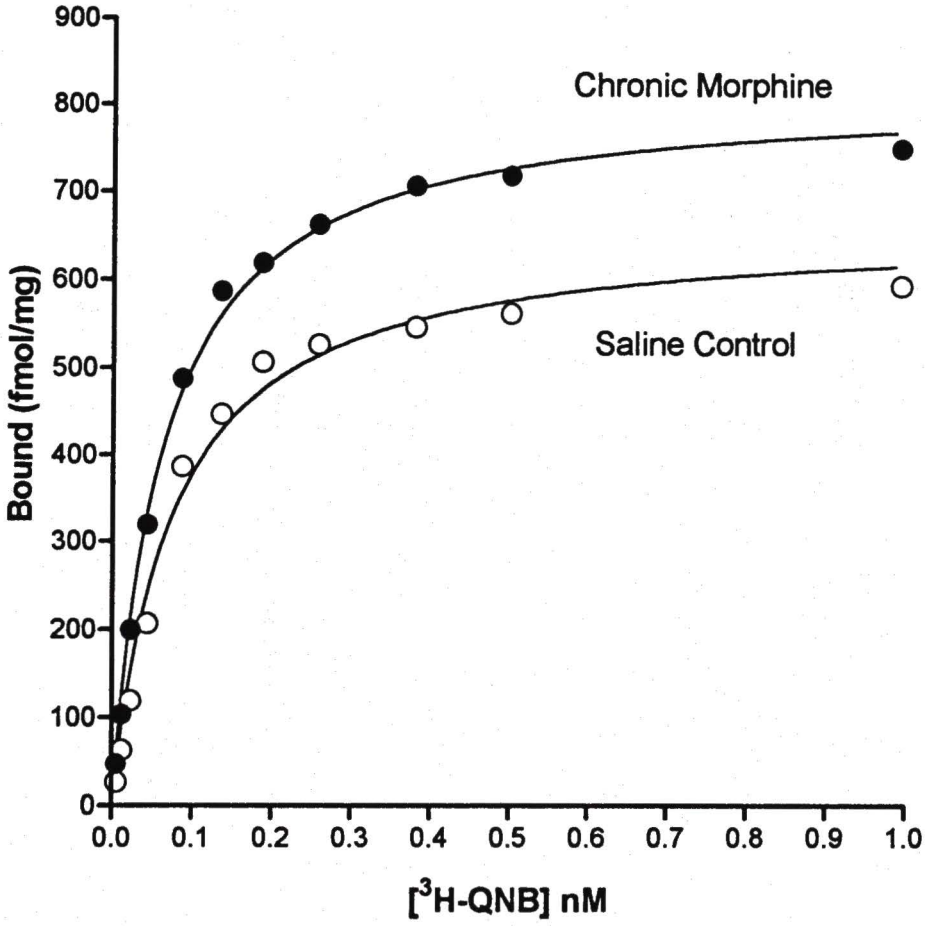

Figure 2. Representative saturation plots of muscarinic receptor binding to left ventricular sarcolemmal membranes from one saline control and one chronic morphine dog.

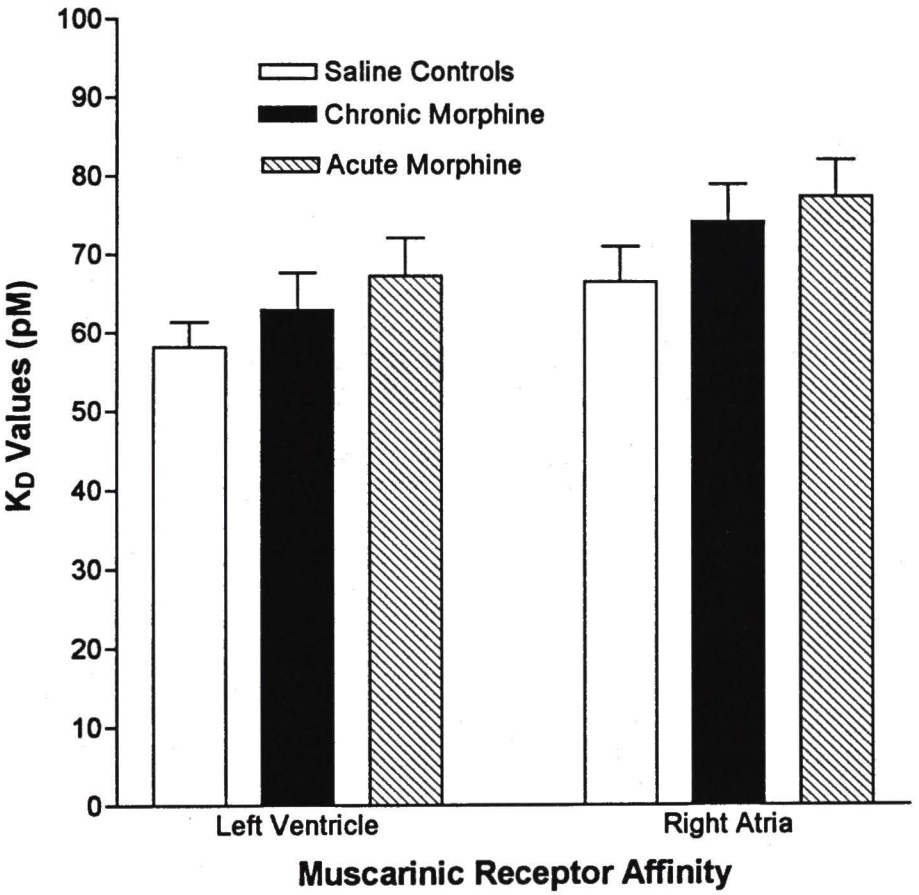

Figure 3. Muscarinic receptor affinities (K_D) in left ventricular and right atrial sarcolemma from saline control, chronic morphine and acute morphine dogs. Values are means ± SE. n = 12 for saline controls, n = 11 for chronic morphine, n = 10 for acute morphine.

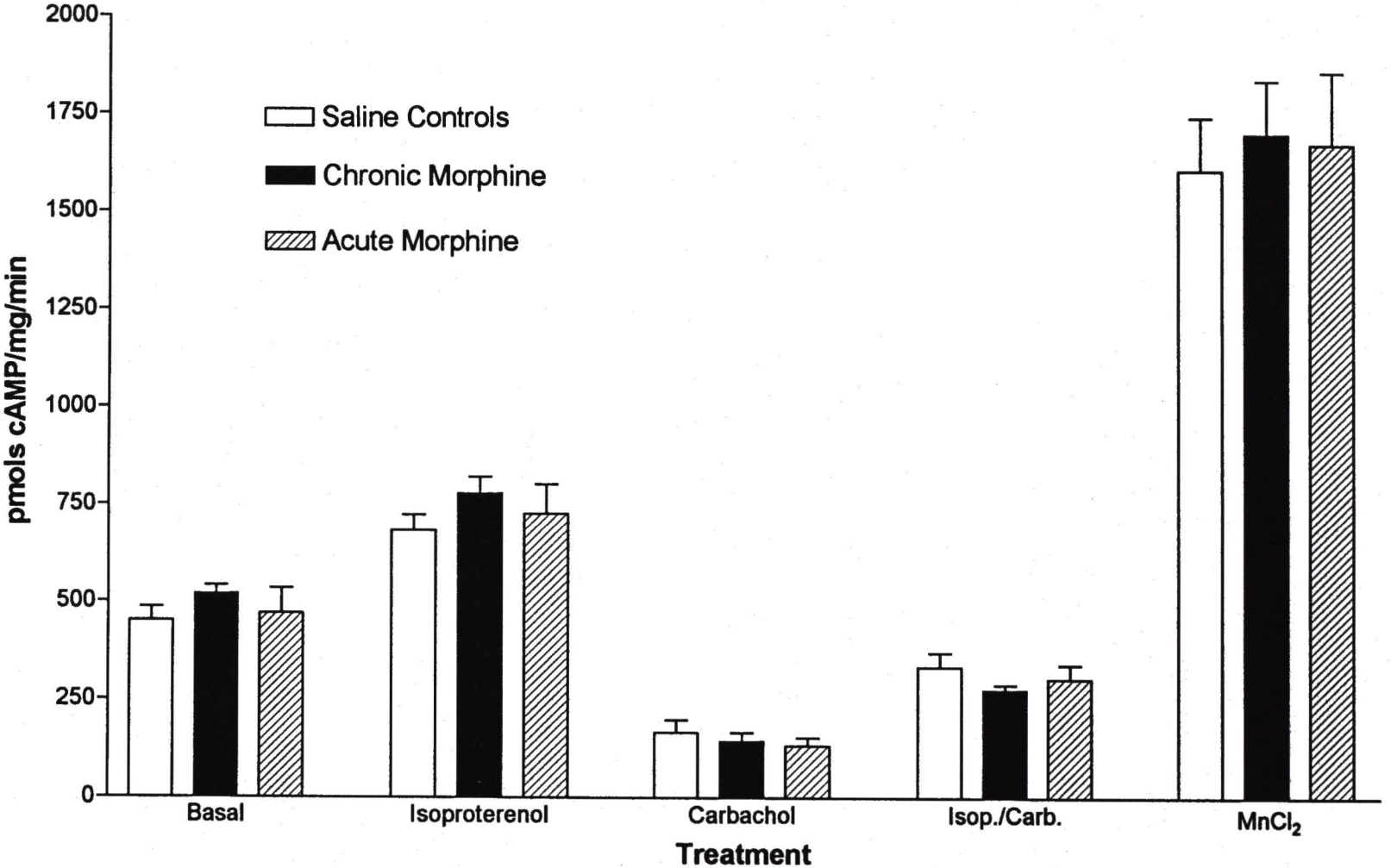

Figure 4. Adenylate cyclase activity in left ventricular sarcolemma from dogs treated with saline, chronic morphine or acute morphine. Values are means ± SE. *n* = 12 for controls, *n* = 11 for chronic morphine, *n* = 10 for acute morphine.

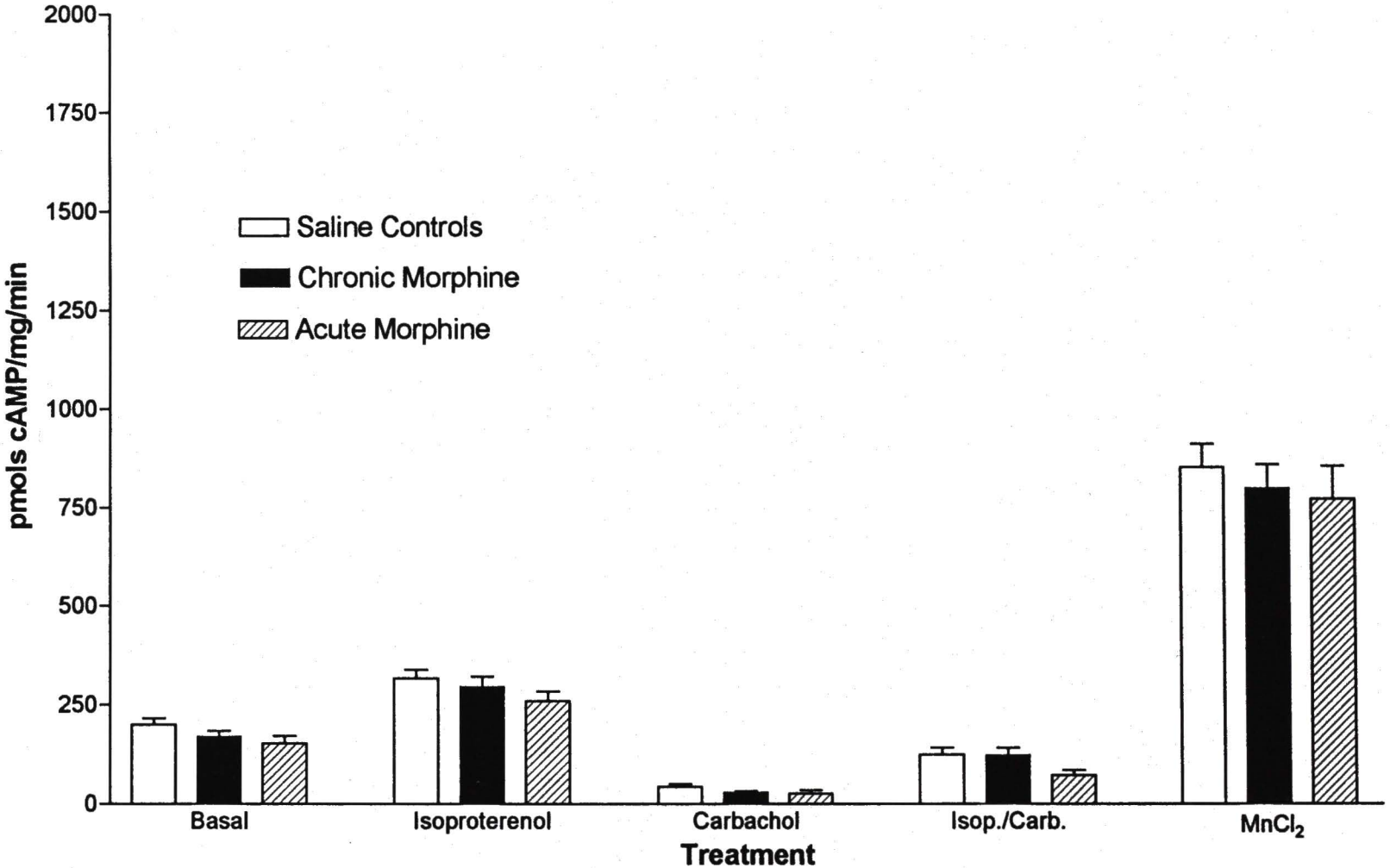

Figure 5. Adenylate cyclase activity in right atrial sarcolemma from dogs treated with saline, chronic morphine, or acute morphine. Values are means ± SE. *n* = 12 for controls, *n* = 11 for chronic morphine, *n* = 10 for acute morphine.

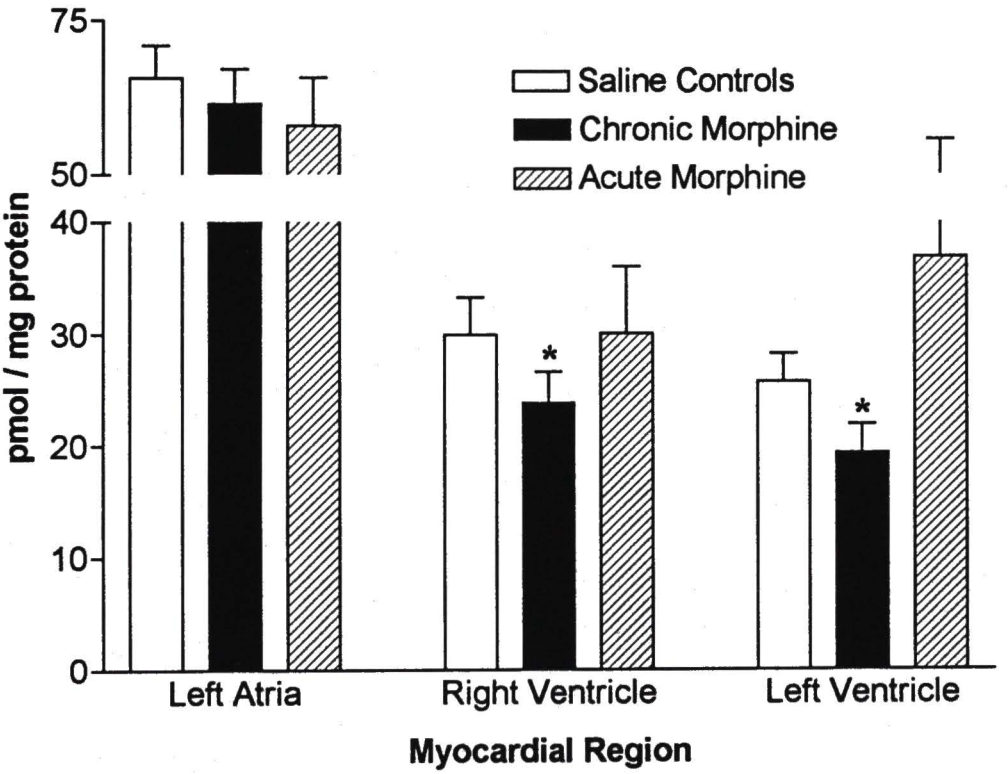

Figure 6. Tissue norepinephrine content in dogs treated with saline, chronic morphine and acute morphine. Values are means ± SE. n = 12 for controls, n = 10 for chronic morphine, n = 7 for acute morphine. *Significantly different from control ($P < 0.01$).

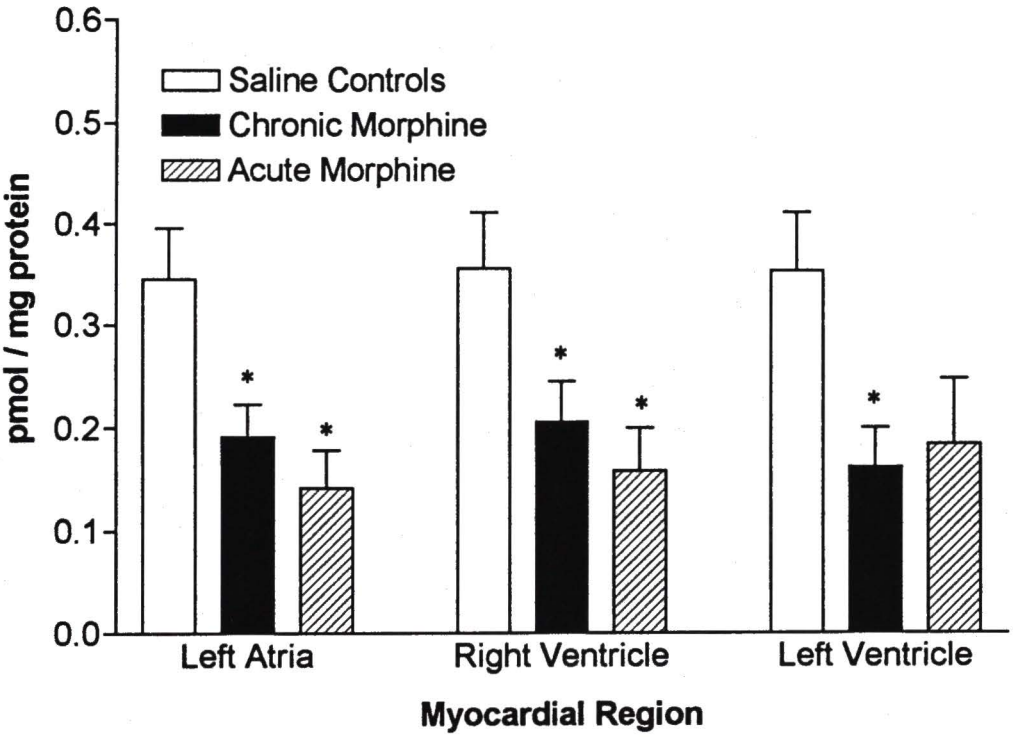

Figure 7. Tissue epinephrine content in dogs treated with saline, chronic morphine and acute morphine. Values are means ± SE. n = 11 for controls, n = 10 for chronic morphine, n = 7 for acute morphine. *Significantly different from control ($P < 0.01$).

CHAPTER V

CONCLUSIONS

Preliminary data showed reduced vagal bradycardia following one week of morphine treatment. Based on this observation, we hypothesized that chronic (two weeks) morphine exposure and the accompanying vagal activity would reduce parasympathetic control of the heart as indicated by a decrease in the bradycardic response to vagal nerve stimulation. Further, we predicted that a down-regulation of muscarinic receptors and a compensatory increase in adenylate cyclase activity would contribute to the attenuated function. In this regard, we expected a decrease in cyclase activity in response to carbachol and an increase in the cyclase response to isoproterenol. Contrary to expectations, we were unable to reproduce the decrease in vagal bradycardia observed in initial experiments. In addition, the cellular adaptations to chronic morphine were not as predicted. These and other major findings of this work were as follows:

1) Heart rate and high frequency fluctuations in heart rate declined during the first three hours of morphine infusion. Heart rate remained low after two days of morphine. These results are consistent with the vagotonic activity of acute morphine.

2) Ambient sympathetic tone and plasma catecholamines were increased during the two week treatment period, and resting heart rate had returned to baseline by Day 10,

suggesting a sympathetic compensation to the sustained parasympathetic activation.

3) Intrinsic heart rate was significantly lower on Days 2 and 10 of morphine treatment compared to that obtained pre-treatment. A lower intrinsic heart rate could reflect a fundamental change in sinoatrial nodal cells or a decrease in tachycardia induced by vasoactive intestinal peptide which is co-released with acetylcholine from post-ganglionic parasympathetic neurons.

4) While neither acute nor chronic morphine reduced the maximal bradycardic response to vagal nerve stimulation, the time to reach 50% of the maximum decrease in heart rate was longer in dogs treated with chronic morphine and extended even further in animals given morphine acutely. These results suggest that acute morphine altered the kinetics of the bradycardic response by decreasing the rate of acetylcholine release or by increasing its rate of degradation.

5) Muscarinic receptor densities were increased in left ventricular and right atrial sarcolemmal membranes from dogs treated with chronic morphine. This increase in receptor number was not accompanied by a change in receptor affinity (K_D).

6) Morphine treatment had no effect on basal or $MnCl_2$-stimulated adenylate cyclase activity or in the maximal β-adrenergic or muscarinic receptor coupling to cyclase as indicated with isoproterenol or carbachol, respectively.

7) Chronic morphine decreased ventricular norepinephrine content, while both chronic and acute morphine decreased atrial and ventricular epinephrine. This pattern suggests that morphine may reduce extraneuronal uptake of catecholamines in the myocardium.

A biphasic response to chronic morphine treatment may explain some of the unexpected findings. Chronic morphine and the ensuing persistent vagal outflow may have reduced parasympathetic function. This attenuated function, however, was short-lived since sympathetic systems adapted with compensatory responses which masked, and perhaps reversed, any parasympathetic deficits. Parasympathetic components may subsequently respond to the increase in adrenergic activity by upregulating, as evident in this case by the increase in muscarinic receptor density. Alternatively, the sympathetic upregulation in response to parasympathetic activation accompanying morphine treatment may have prevented any resultant parasympathetic deficits. Parasympathetic components would, therefore, upregulate instead of downregulate as expected.

The reduction in the intrinsic heart rate with chronic morphine treatment is a novel finding, and additional research will be required to determine the precise mechanisms involved.

CHAPTER VI

PROPOSAL OF FURTHER RESEARCH

The following studies are proposed to further clarify the autonomic and myocardial adaptations to chronic morphine treatment observed in the present study:

1) Infuse morphine for one week and re-evaluate vagal bradycardia. Evaluate muscarinic receptors and adenylate cyclase activity to determine if the predicted cellular alterations are present following one week of morphine treatment.

2) Examine adenylate cyclase activity in response to a range of isoproterenol and carbachol concentrations to determine if chronic morphine produces subtle changes in the responsiveness of cyclase to lower concentrations of these agents.

3) Determine if vasoactive intestinal polypeptide (VIP) is involved in the lower apparent intrinsic heart rate following double receptor blockade in morphine-treated animals. In this regard, one could determine the myocardial and plasma concentrations of VIP, examine myocardial VIP receptors and examine the tachycardic response to exogenous VIP. Since VIP stimulates adenylate cyclase activity, one could also measure cyclase activity in response to VIP *in vitro* to determine if the VIP receptor-coupling mechanism is altered by chronic morphine.

4) Determine whether a change in the rate of acetylcholine synthesis, release or degradation is responsible for the slower vagal bradycardia observed in the presence of morphine by examining acetylcholine release, choline uptake and acetylcholine esterase activity.

5) Determine whether neuronal (Type 1) or non-neuronal (Type 2) uptake is responsible for the reduction in myocardial epinephrine and norepinephrine content with morphine treatment by blocking each type of uptake separately and examining the uptake of radiolabelled catecholamines.