Canine Cardiac Muscarinic Receptors, G Proteins, and Adenylate Cyclase after Long-Term Morphine

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ABSTRACT

Short-term morphine stimulates vagal bradycardia. This led us to propose the hypothesis that chronically administered morphine would down-regulate myocardial muscarinic receptor systems. Dogs received morphine continuously for 2 weeks through an s.c. catheter, and cellular aspects of parasympathetic control of the heart were examined. Contrary to expectations, morphine increased muscarinic receptor density in the right atrium and left ventricle by 17 and 34%, respectively, with no change in the apparent affinity of the receptor (K_D). Morphine also increased the expression of the G protein G_i by 115 and 233%, respectively, in right atrial and left ventricular sarcolemmal membranes. Morphine increased ventricular and atrial G_s to a much lesser degree (49 and 25%). Morphine failed to alter basal or maximally stimulated (forskolin plus MnCl_2) adenylate cyclase activity. The maximum cyclase activation by isoproterenol and the maximum inhibition by carbamol were similarly unaltered by morphine. Morphine reduced the ventricular but not atrial norepinephrine. Both long- and short-term morphine lowered tissue epinephrine content, suggesting that short-term morphine reduces extraneuronal uptake. Potential systemic and cellular models for myocardial adaptation to morphine are proposed, including sequential sympathetic and parasympathetic compensations.

The administration of morphine is accompanied by a reduction in cardiovascular function consistent with increased parasympathetic activity (Pur-Shahriari et al., 1967; Hotvedt and Refsum, 1986; Randich et al., 1991, 1992). The resulting changes include bradycardia, hypotension, and reduced cardiac output. Vagal participation in the responses to short-term morphine is supported by the observation that vagotomy reduces or abolishes these effects (Hotvedt and Refsum, 1986; Randich et al., 1991, 1992). In contrast, parasympathetic control of cardiovascular function may be diminished in opiate addicts and related animal models of long-term opiate administration (Lipsky et al., 1973; Yoshimura et al., 1993; Napier et al., 1995, 1998). Because short-term morphine stimulates vagal activity, long-term morphine may reduce vagal efficacy by down-regulating selective components responsible for neuroeffector coupling.

A variety of myocardial cellular and intracellular mechanisms could contribute to an altered parasympathetic function after long-term 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. Because opiate receptors like muscarinic receptors are also coupled to adenylate cyclase through the inhibitory G protein G_i, a coincident parallel direct effect of morphine could add significantly to that 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 long-term opiate administration.

Prolonged exposure to muscarinic agonists in isolated hearts and cultured cells rapidly desensitizes and down-regulates muscarinic receptors (Klein et al., 1979; Roskoski et al., 1985), presumably reducing the ability of the target organ to respond to subsequent stimulation in vivo. Exposure to muscarinic agonists can also increase vagal efficacy by reducing β-adrenergic receptor responses. For instance, sustained incubation with carbachol decreased β-receptor binding in isolated rat cardiac myocytes (Limas and Limas, 1985; Paraschos and Karliner, 1994). This effect of carbachol on β-receptor binding was not, however, duplicated after long-term morphine administration. β-Adrenergic receptor binding was unchanged in cardiac preparations from rats treated

ABBREVIATIONS: QNB quinuclidinyl benzilate.
with morphine on a long-term basis (Kuriyama et al., 1981; Minneman and Holtzman, 1984).

The activation of one receptor signaling system can alter the effectiveness of opposing systems. For example, both muscarinic agonists and opioids, can result in enhanced adenylyl cyclase activity in response to stimulatory agents such as norepinephrine (Thomas and Hoffman, 1987). This potential model for narcotic tolerance was developed further by Sadee and Wang (1995), who provided evidence that μ receptors assume constitutive activity during continuous exposure to morphine and no longer require agonist for signal transduction. Tolerance develops because fewer agonist-responsive μ receptors are available to oppose an even further up-regulation in the cAMP system. Prolonged exposure to the muscarinic agonist methacholine resulted in an increase in basal adenylyl cyclase activity and catecholamine-stimulated cyclase activity in isolated rat ventricular myocytes (Paraschos and Karliner, 1994). This receptor cross-talk has been described as a general cellular adaptation to interventions that enhance vagal activity (Linden, 1987; Paraschos and Karliner, 1994).

The short-term administration of morphine to patients during acute myocardial events has long been recognized for its beneficial therapeutic profile associated with a reduction in heart rate and afterload. These benefits are thought to arise from reduced pain and anxiety and a shift in the autonomic balance in favor of greater parasympathetic and diminished sympathetic influences. Despite the therapeutic importance of long-term morphine administration for the management of pain and its widespread abuse, surprisingly little is known about the autonomic compensations to its sustained use. This study was conducted to evaluate the hypothesis that long-term opiate exposure to morphine with its accompanying vagotonic activity would produce a compensatory and unfavorable shift in the autonomic balance toward greater sympathetic influence. The proposed mechanisms included a down-regulation of muscarinic receptor systems and a compensatory up-regulation in adenylyl cyclase. Muscarinic receptors, their inhibitory G proteins, adenylyl cyclase activity, and catecholamines were examined in myocardial extracts and sarcolemmal membranes from dogs treated for 14 days with morphine. The companion results presenting detailed autonomic and cardiovascular analyses in conscious and anesthetized animals were reported elsewhere (Napier et al., 1998).

Materials and Methods

Catheter Implantation and Treatment Protocol. Mongrel dogs (15–20 kg) were chosen for the study, and animals were assigned to the control and morphine treatment groups to proceed through the treatment protocol in mixed groups (controls and morphine-treated) of two or three. All dogs were free of heartworms. Three days before initiation of the treatment protocol, dogs were placed under mild sedation (2.5 mg/kg ketamine, 1.5 mg/kg xylazine, 0.15 mg/kg acepromazine), and a sterile field was prepared in the midscapular region. The area was infiltrated with a local anesthetic (5 mg bupivacaine HCl), and a flexible intracatheter (14 gauge × 5.1 cm) was inserted s.c. and sutured in place. A CORMED (Medina, NY) 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, Hawthorne, CA) designed to support the pump. Saline was infused for 3 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 (0.40–0.75 ml/h) and adjusted as required to produce a target concentration of 80 to 120 ng/ml. 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. Sympathetic and parasympathetic evaluations were conducted during the 2-week treatment period and have been reported elsewhere (Napier et al., 1998). These evaluations included construction of heart rate/frequency response relationships in anesthetized animals, pharmacologic determination of autonomic balance and intrinsic heart rate on days 2 and 10 in conscious animals, and collection of multiple blood samples for circulating morphine and catecholamines.

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

Tissue Collection. After 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 with supplemental oxygen, adjustment of minute volume, or administration of 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 for 15 s at 0.5, 1, 2, and 4 Hz. A short-term control group was similarly prepared and tested before and 30 min after the short-term administration of morphine (1 mg/kg). These functional responses were reported elsewhere (Napier et al., 1998). After these in vivo evaluations, hearts were excised and placed immediately into cold isotonic saline/0.1 mM EGTA.

Myocardial Sarcolemmal Membrane Preparation. The right atria and left ventricle were dissected in iced Petri dishes. The superficial blood vessels and the surface epicardium and endocardium were discarded. Four grams of the remaining tissue was 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 MgCl2, 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 three-fourths maximal speed. Homogenates were diluted with an additional 16 ml homogenizing buffer and centrifuged at 12,000g for 15 min at 4°C. The supernatants were collected and centrifuged at 50,000g for 30 min at 4°C. The pellets were resuspended in homogenizing buffer (3 ml/4-g sample) with a motor-driven Potter-Elvejhem homogenizer at full speed. Membrane fractions were aliquoted and stored at −90°C (Quist et al., 1994). Protein concentrations were estimated according to the method of Lowry et al. (1951).

Muscarinic Receptor Binding. Membrane preparations from control, chronically treated, and acutely treated animals were subjected to saturation binding studies using the high-affinity muscarinic antagonists quinuclidinyl benzilate to determine the equilibrium dissociation constant (Kd) for the antagonist and maximal muscarinic receptor binding (Bmax). Because there are few, if any, m1 muscarinic receptors in the heart, this nonsselective muscarinic antagonist was used because of its superior efficiency. Saturation studies were performed in a total volume of 1 ml in 50 mM HEPES, pH 7.4, 0.5 mM MgCl2, 1.0 mM NaCl, 0.1 mM EGTA, and 100 mM NaCl. Assays included 100 μl of [3H]quinuclidinyl benzilate (0.005–1.0 nM) and 25 μg of membrane protein ± 100 μl atropine (1 μM) to determine nonspecific binding (Quist et al., 1994). All assays were incubated at 37°C for 30 min in duplicate.
bated 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 of 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 (Prism;
GraphPAD, San Diego, CA).

Expression of G Protein α Subunits. A modified membrane
preparation was used for G protein analysis (Zavecz et al., 1985). The
outermost epicardium and endocardium were trimmed from right
atrial and left ventricular samples. Samples were frozen at −90°C
until analysis. Tissues were later thawed, minced, and homogenized
in Tris buffer (50 mM, pH 7.2) containing 5 mM MgCl₂, 2 mM EGTA,
and 1 mM dithiothreitol (35 mg/g tissue). The homogenate was fil-
tered through 225-μm polyethylene mesh to remove connective tis-
se and centrifuged for 10 min at 360g. The supernatant was cen-
trifuged at 45,000g for 10 min, and the pellet was washed twice by
resuspension and centrifugation from fresh buffer (original volume).
The final pellet was suspended in 1 ml of Tris buffer without EGTA.
The membranes (6–8 mg/ml protein) were stored at
−70°C until further analysis.

Proteins (25–50 µg) were electrophoretically separated by SDS-
polyacrylamide gel electrophoresis in 10% acrylamide. Separated
proteins were transferred to Immobilon P membranes and incubated
overnight in primary antisera (AS/7 for G₁₁, or G₁₂, and R/1 for G₃₃;
New England Nuclear, Boston, MA), washed, and then incubated
with secondary antisera coupled to horseradish peroxidase. Proteins
were visualized by enhanced chemiluminescence after exposure of
the membrane to X-ray film. Density of the bands was quantified
with NIH Image software. Three membranes from each of the three
treatment groups were included in these experiments for control and
comparison purposes. Real development and exposure time of films
varied in this study due to the irregular passage of film through the
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darkroom (Zavecz et al., 1995).

Adenylate Cyclase Studies. Sarcolemmal membranes were pre-
pared as described above for muscarinic receptor binding determi-
nations. Membranes from control, chronically treated, and acutely
treated animals were assayed in a final volume of 200 µl in medium
containing 0.8 mM MgCl₂, 10 µM GTP, 50 mM HEPES, pH 7.4, 0.3
mM dithiothreitol, 100 mM NaCl, 0.3 mM KCl, 5.0 mM creatinine
phosphate, 5 U creatine phosphokinase, 0.5 mM 3-isobutyl-1-
methyl-xanthine, 2 to 4 µg membrane protein, alamethicin (1 µg/µg
protein), and 1 mM ATP (Jones et al., 1980; Quist et al., 1994).
Adenylate cyclase activity was measured under basal conditions and
after the addition of isoproterenol (10.0 µM), carbachol (10.0 µM),
and a combination of both. MnCl₂ (10 mM) was used to stimulate
receptor-independent cyclase activity. In a separate set of incuba-
tions, a combination of MnCl₂ and forskolin was used to evaluate
receptor-independent cyclase activity. In a separate set of incuba-
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receptor-independent cyclase activity.

Tissue Catecholamines. Atrial and ventricular tissues were
dissected on ice and dried 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 homogenizer; Brinkmann Instruments,
Westbury, NY), and the supernatant was separated by centrifuga-
tion at 25,000g for 30 min. The pellet was rehomogenized with an
additional 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 perchlo-
eric acid. Catecholamines were separated by HPLC and quantified
amperometrically through integration of the signals from the elect-
rochemical detector (BAS) as previously described (Barron et al.,
1985).

Results

Plasma Morphine. Plasma morphine concentrations measured
on days 2, 4, 7, 10, and 14 of the protocol are presented in Table 1. Statistical analysis revealed no significant
differences in plasma morphine concentrations across
days. Infusion rates were adjusted as required to maintain
plasma concentrations within the 80- to 120-ng/ml target
range.

Myocardial Mass and Protein Recovery. Average
weights (g tissue/kg b.wt.), total protein recovery (mg pro-
tein/g tissue), and sarcolemmal membrane protein recovery
(mg/ml) for myocardial tissue regions are presented in Table
2. Neither long- nor short-term morphine administration had
any statistically significant effect on these variables.

Muscarinic Receptors. The density and equilibrium dis-
sociation constant (Kᵦ) of muscarinic receptors in left ventricle
and right atrial sarcoplemmal membranes were ex-
amed to determine whether they were altered by morphine or the resulting persistent vagal outflow. Receptor density in
left ventricular and right atrial membranes from dogs
treated with morphine on a long-term basis was 34 (p < .01)
and 17% (p < .05) higher, respectively, than that in control
animals (Fig. 1). Representative saturation curves are pre-
sented in Fig. 2. Similar but smaller increases after short-
term morphine failed to reach statistical significance.

Within each treatment group, the Kᵦ value of muscarinic
receptor binding was slightly but not significantly higher in
the right atria than in the left ventricle. The Kᵦ (±S.E.)
values for left ventricular muscarinic receptors were 58 ± 3,
63 ± 4, and 67 ± 3 pM for saline controls, chronically treated
animals, and acutely treated animals, respectively. Right
atrial Kᵦ values were 66 ± 5, 74 ± 5, and 77 ± 5 pM for
controls, chronically treated animals, and acutely treated
animals, respectively. There were no significant differences
between treatment groups in either the left ventricle or right
atria.

Expression of G Protein α Subunits. The expression of
G₃₃ was measured to determine whether long-term morphine

TABLE 1
Plasma morphine concentrations during the 14-day treatment period

<table>
<thead>
<tr>
<th>Day</th>
<th>Plasma morphine (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>107 ± 6.58</td>
</tr>
<tr>
<td>4</td>
<td>127 ± 9.89</td>
</tr>
<tr>
<td>7</td>
<td>94 ± 14.1</td>
</tr>
<tr>
<td>10</td>
<td>119 ± 10.4</td>
</tr>
<tr>
<td>14</td>
<td>92.3 ± 9.41</td>
</tr>
</tbody>
</table>

Values are mean ± S.E. (n = 11).
affected the coupling mechanism associated with muscarinic receptors. Long-term morphine treatment significantly increased the expression of $G_i\alpha$ in both right atrial (115%) and left ventricular (233%) membranes ($p$, .05, Fig. 3). Short-term morphine had no affect on $G_i\alpha$ in either the right atria or left ventricle. Long-term morphine also provoked significant, although smaller, increases in $G_s\alpha$ of 49 and 25% in ventricle and atria, respectively.

**Adenylate Cyclase Activity.** Basal, MnCl$_2$-stimulated, and maximal (MnCl$_2$ plus forskolin) adenylate cyclase activity were examined to determine if long-term morphine treatment altered the postreceptor effector. Maximal muscarinic and $\beta$-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 activities expressed as cAMP production in left ventricular and right atrial sarcolemmal membranes from dogs treated with saline, long-term morphine, or short-term morphine are presented in Figs. 4 (left ventricle) and 5 (right atria). The results of separate assays in which cyclase was stimulated by isoproterenol, forskolin, MnCl$_2$, and a combination of forskolin and MnCl$_2$ are presented in Fig. 6.

Left ventricular adenylate cyclase activity was approximately twice that observed in the right atria for all treatment groups (Figs. 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 cyclase 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, whereas carbachol attenuated cyclase activity by an average of 69% in the left ventricle and 81% in the right atria. Neither long- nor short-term treatment with morphine altered maximal $\beta$-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 (isoproterenol plus carbachol) to produce a response in which the inhibition by carbachol was not maximal. No difference in this

### TABLE 2

<table>
<thead>
<tr>
<th></th>
<th>Left Ventricle</th>
<th>Right Ventricle</th>
<th>Whole Atria</th>
<th>Left Atria</th>
<th>Right Atria</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Myocardial mass, g tissue/kg b.wt.</strong></td>
<td>4.19 ± 0.16</td>
<td>1.38 ± 0.05</td>
<td>0.67 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Recovered tissue protein, mg/g</strong></td>
<td>204 ± 4.93</td>
<td>198 ± 4.94</td>
<td>174 ± 3.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Recovered sarcolemmal protein, mg/ml</strong></td>
<td>2.05 ± 0.17</td>
<td>2.15 ± 0.17</td>
<td>3.13 ± 0.26</td>
<td>2.12 ± 0.19</td>
<td>2.97 ± 0.22</td>
</tr>
</tbody>
</table>

Values are mean ± S.E. ($n = 11$ or 12 for saline controls, 11 for chronic morphine, 7–10 for acute morphine).
integrated response was noted with long- or short-term treatment with morphine. To determine whether morphine treatment altered the adenylate cyclase available for stimulation, cyclase activity was measured in the presence of MnCl₂, a direct stimulator of adenylate cyclase that 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 MnCl₂. Neither long- nor short-term treatment with morphine altered MnCl₂-stimulated adenylate cyclase in myocardial membranes. Similar results were obtained when maximal cyclase activity was determined by combining forskolin and MnCl₂. Left ventricular adenylate cyclase increased by 2677% and was unaffected by morphine (Fig. 6).

### Tissue Catecholamine Content

Norepinephrine and epinephrine contents in myocardial tissue extracts from dogs treated with saline, long-term morphine, or short-term morphine were examined to determine whether morphine treatment resulted in an adaptive response in tissue catecholamines. As we reported previously (Barron et al., 1992, 1995), left atrial norepinephrine content was consistently about twice that in the ventricles (Fig. 7). Morphine had no apparent effect on atrial norepinephrine. Ventricular norepinephrine was distributed evenly between the ventricles and was significantly lower ($p < .01$) in dogs treated chronically with morphine compared with animals treated with saline or short-term morphine (Fig. 7). Epinephrine content was evenly distributed throughout the atria and ventricles as previously reported (Barron et al., 1995) and was significantly lower in every region after long-term morphine (Fig. 8). Epinephrine was also lower in tissue extracts from dogs treated with morphine on a short-term basis. This effect was significantly different from saline controls in the left atria and right ventricle (Fig. 8, $p < .01$).

### Discussion

Previously, parasympathetic function appeared to be impaired after 1 week of morphine treatment in dogs (Napier et al., 1995). In the present study, the morphine was extended to 2 weeks to allow adaptations to develop more completely. Systemic adaptations were first evaluated in vivo (Napier et al., 1998), and then tissues were extracted to identify subcellular adaptations in vitro. Muscarinic receptors, G proteins, adenylate cyclase, and catecholamines were examined.

Gross heart size was unaltered by morphine despite prior reports indicating increased myocardial mass in opiate addicts at autopsy (Dressler and Roberts, 1989; Turnicky et al., 1992). Although the post-mortem studies did not indicate the duration of addiction, death among opiate addicts usually occurs among long-term users (Darke and Zador, 1996). In this study, 2 weeks may have been insufficient to produce an appreciable change in myocardial mass.

The hypothesis predicted that the persistent vagal stimulation associated with long-term morphine treatment would down-regulate myocardial muscarinic receptors. The density of muscarinic receptors was greater in the atria than in the left ventricle in rats (Wei and Sulakhe, 1978; Roskoski, 1983), rabbit (Fields et al., 1978; Wei and Sulakhe, 1978), and cat (Ransnas et al., 1986). Muscarinic receptors were more evenly distributed throughout the myocardium in guinea pigs (Wei and Sulakhe, 1978; Roskoski, 1983) and dogs (Wei and Sulakhe, 1978). Because parasympathetic rate control primarily innervates the sinoatrial node, a prominent down-regulation was expected in the right atria. Ventricular muscarinic receptors were examined primarily for comparison because they receive only 20% of the cardiac parasympathetic innervation (Loffelholz and Pappano, 1985). The rhythm generation and contractile activity of the atria are exquisitely sensitive to vagal input, whereas ventricular effects are less prominent. Contrary to the hypothesis, down-regulation was not observed and muscarinic receptors increased in both regions. The similarities in ventricular and atrial muscarinic receptor densities and their similar adaptive responses to morphine are difficult to reconcile with known vagal influences in the ventricle and suggest an unknown or unappreciated parasympathetic role in ventricular function.

Little information is available concerning muscarinic receptors in the heart after long-term morphine, and the increase in receptor number was unexpected. Long-term morphine had no effect on muscarinic receptor binding in rat brain (Das et al., 1986), but psychopharmacological evidence suggested that muscarinic receptors were more sensitive to agonist (Vasquez et al., 1974). They postulated that morphine had suppressed acetylcholine release and induced a compensatory increase in muscarinic receptors. A change in sensitivity could result from changes in receptor number or affinity state (Roskoski et al., 1985). The increase in receptor number observed in the present study was not accompanied by an increase in the $K_D$ value or in responsiveness during direct vagal stimulation. Roskoski et al. (1985) reported that the affinity state of muscarinic receptors could be rapidly and reversibly shifted into lower-affinity states after exposure to agonist in vitro. The proportion of high- and low-affinity receptors was not determined in this study, but a persistent shift of receptors into lower-affinity states could represent an intracellular feedback loop responsible for the up-regulation in both receptor numbers and G proteins. The added $G_{ins}$ would presumably increase the probability of finding receptors in the high-affinity state and might represent a cellular strategy to moderate desensitization and reduce the down-regulation in critical systems such as vagal control of the heart.
The hypothesis predicted a decrease in inhibitory G proteins to accompany the expected down-regulation of muscarinic receptors. However, after morphine, myocardial G
contents were significantly elevated to a greater extent than the increased receptor numbers. Because muscarinic receptors and their associated G proteins are believed to be tightly coupled, the absence of an increase in response to carbachol is understandable if the new G proteins are actually surplus and are constitutively active and/or not well coupled. Functionally, these increases may be compensatory responses to the rising sympathetic activity demonstrated in these same animals (Napier et al., 1998).

Basal and MnCl₂-stimulated adenylate cyclase activities were consistently 50% higher in ventricular tissue than in corresponding atria. This had not been previously reported for dog. The finding contrasts with higher adenylate cyclase activity in rabbit, cat, and rat atria; all attributed to a greater atrial parasym pathetic innervation (Löffelholz and Pappano, 1985). An extraction artifact seems unlikely because the recovery of membrane protein was equivalent in atria and ventricles.

The hypothesis predicted that continuous vagal activation with morphine would induce a compensatory increase cyclase activity. Again, contrary to expectations, long-term morphine did not increase basal or MnCl₂-stimulated adenylate cyclase activity. Niroomand et al. (1996) reported a similar finding for canine cardiac adenylate cyclase after the short-term addition of the µ receptor agonist morphiceptin. The maximal inhibition by carbachol was also unimpaired even when the maximal inhibition was prevented by raising the cyclase activity with isoproterenol. Compensatory increases in adenylate cyclase activity has been reported in other models after prolonged exposure to morphine or other drugs that inhibit adenylate cyclase activity (Thomas and Hoffman, 1987; Paraschos and Karliner, 1994). Our new data do not support an increase in adenylate cyclase activity and suggest that the vagal impairments we observed after 1 week of morphine were transient and not likely explained by increased adenylate cyclase activity (Napier et al., 1995).

In the absence of an increase in adenylate cyclase, reduced vagal function could accomplished by increased opposition

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**Fig. 4.** Adenylate cyclase activity in left ventricular sarcolemma from dogs treated with saline, long-term morphine, or short-term morphine. Values are mean ± S.E. (n = 12 for controls, n = 11 for long-term morphine, n = 10 for short-term morphine).

**Fig. 5.** Adenylate cyclase activity in right atrial sarcolemma from dogs treated with saline, long-term morphine, or short-term morphine. Values are mean ± S.E. (n = 12 for controls, n = 11 for long-term morphine, n = 10 for short-term morphine).
from adrenergic receptors. However, maximal adrenergic stimulation of adenylate cyclase with isoproterenol was unaffected by morphine, and the observed increase in $G_s$ was very modest. This observation is consistent with findings in the rat where receptor numbers were unchanged after treatment with morphine (Kuriyama et al., 1981; Minneman and Holtzman, 1984) but differs from findings in other models in which adrenergic stimulation of adenylate cyclase activity was enhanced by long-term morphine treatment (Thomas and Hoffman, 1987).

Tissue catecholamine content was examined to further characterize sympathetic adaptations after morphine. Changes in catecholamine content could reflect differences in synthesis, release, or uptake. Atrial norepinephrine was consistently higher than that in the ventricles, reflecting the greater density of sympathetic nerves in the atrium (Barron et al., 1992). Atrial norepinephrine was unaltered by morphine, but ventricular norepinephrine was decreased. This differs from Ko et al. (1988), who reported ventricular norepinephrine unchanged. These conflicting results may reflect differences in the species sensitivity to morphine, route of drug delivery, or experimental design. Mantelli et al. (1987) found that short-term morphine potentiated the inotropic response to sympathetic nerve stimulation in isolated guinea pig atria. This effect was blocked by desmethylimipramine, indicating an effect of short-term morphine on reuptake of norepinephrine by sympathetic nerve terminals. Epinephrine was evenly distributed throughout the myocardium (Barron et al., 1992, 1995), 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 that prefers norepinephrine over epinephrine and presumably is proportionally more important in the less densely innervated ventricles. Short-term morphine produced a somewhat less uniform reduction in myocardial catecholamines, suggesting the effect may have short- and long-term components.
In summary, the hypothesis predicted that long-term exposure to morphine with its attendant vagotonic activity would attenuate parasympathetic control of myocardial function by reducing myocardial muscarinic receptors and increasing adenylate cyclase activity. However, long-term morphine treatment increased muscarinic receptor densities without any change in basal, maximal, or agonist-stimulated adenylate cyclase activity. The doses of carbamol and isoproterenol were chosen to elicit maximal responses, so these results do not preclude subtle changes in responsiveness of adenylate cyclase had full dose-response curves been practical. The proportion of receptors in the high- and low-affinity states was not assessed, although this information could provide additional insight into the complex compensations at work. We propose two potential models. First, if the continued exposure to agonist shifted the muscarinic receptors into a lower-affinity state, the observed increase in receptor numbers and G proteins could represent cellular feedback intended to return the critical vagal control back to normal. "Spare receptors" would provide a modest leftward shift in the dose response, and the disproportionate increase in G\sub{i} might serve to rectify the desensitization process further by increasing the probability of receptors resuming the high-affinity state and thereby limiting their subsequent rate of down-regulation. As an alternative, the "surplus" inhibitory G proteins could act without agonist to constitutively moderate the cyclase activity. Beneficially, this would enable the heart to resist the costly effects of the increasing sympathetic activity without giving up critical moment-to-moment vagal control of cardiac rhythm. This might also explain the reduced intrinsic heart rate observed in these animals after cholinergic blockade.

The parasympathetic activation began immediately after the start of morphine administration, and augmented sympathetic activity was clearly evident when first tested 2 days later. This suggests a complex series of systemic and cellular compensations in which the temporal sequence and cause-effect relationships remain to be determined. Finally, this study focused on the heart as the target end organ, but the compensations observed in vivo certainly also involve cholinergic and opiate targets observed within the central and peripheral nervous systems.

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References