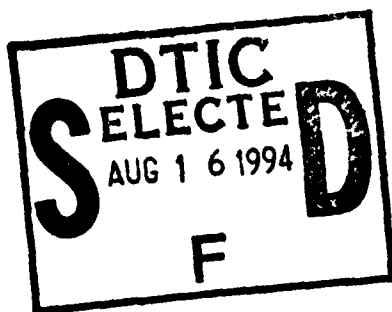




APPENDIX I

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β -ENDORPHIN-INDUCED CARDIORESPIRATORY DEPRESSION IS INHIBITED

BY GLYCYL-L-GLUTAMINE, A DIPEPTIDE DERIVED

FROM β -ENDORPHIN PROCESSING¹

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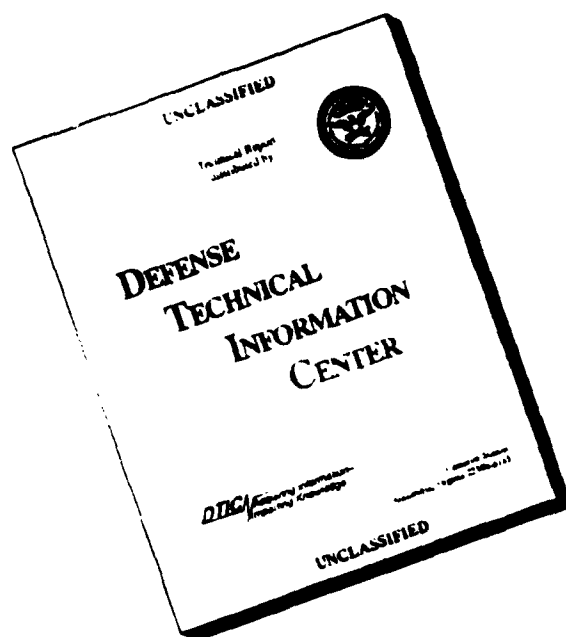
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Running Title: Gly-L-Gln Inhibits β -Endorphin

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Abbreviations: Gly-L-Gln, glycyl-L-glutamine; β -End, β -endorphin; POMC, proopiomelanocortin; MAP, mean arterial pressure; HR, heart rate; i.c.v., intracerebroventricular; i.c., intracisternal; NTS, nucleus of the solitary tract; CSF, cerebrospinal fluid.

ABSTRACT

Glycyl-L-glutamine [β -endorphin-(30-31)] is synthesized through the post-translational processing of β -endorphin-(1-31). Evidence that glycyl-L-glutamine is a prominent end-product of β -endorphin-(1-31) processing in cardiorespiratory regions of rat brain prompted us to investigate whether it modulates the cardiorespiratory depression induced by central β -endorphin-(1-31) injection. As shown previously, β -endorphin-(1-31) (0.5 nmol) lowered mean arterial pressure (MAP) and heart rate when administered i.c.v. to pentobarbital anesthetized rats. Glycyl-L-glutamine (0.3, 0.6, 1.0 and 10.0 nmol) produced a dose-related inhibition of β -endorphin-(1-31)-induced hypotension, but not bradycardia, when injected i.c.v. 15 min after β -endorphin-(1-31). This effect was not attributable to hydrolysis because equimolar amounts of L-glycine and L-glutamine were ineffective. A comparable response was observed when glycyl-L-glutamine was administered to urethane anesthetized rats or when it was injected prior to β -endorphin-(1-31). Glycyl-L-glutamine also attenuated the respiratory depressant effect of β -endorphin-(1-31), significantly inhibiting β -endorphin-(1-31)-induced hypoxia and hypercapnia. Glycyl-L-glutamine (1, 10 or 100 nmol) was inactive when injected alone, however, producing no significant variation from baseline MAP or heart rate values. These results demonstrate that glycyl-L-glutamine inhibits β -endorphin-(1-31)-induced cardiorespiratory depression consistent with accumulating evidence that glycyl-L-glutamine functions as a neuromodulator.

β -Endorphin-(1-31) generates severe hypotension and bradycardia when injected into the cerebrospinal fluid (CSF) of rats and other species (Laubie et al, 1977; Bolme et al., 1978; Sitsen et al., 1982). A comparable response ensues when β -endorphin-(1-31) is microinjected directly into the nucleus of the solitary tract (NTS) (Petty and de Jong, 1982; Mosqueda-Garcia and Kunos, 1987) or the vasopressor region of the ventrolateral medulla (Punnen and Sapru, 1986) suggesting that β -endorphin-(1-31) acts at multiple loci within the brainstem. Like morphine and other opiates, β -endorphin-(1-31) is thought to produce these effects by activating mu opioid receptors (Petty and de Jong, 1982; Mosqueda-Garcia and Kunos, 1987) localized in the NTS and other brainstem cardio regulatory sites (Sales et al., 1985; Dashwood et al., 1988). These findings are consistent with immunohistochemical evidence that the same nuclei contain a dense plexus of β -endorphin immunoreactive axons emanating from neuronal cell bodies in the commissural NTS and the medial basal hypothalamus (Palkovits et al., 1987; Joseph and Michael, 1988). Collectively, these observations support the hypothesis that β -endorphin-(1-31) plays an important role in cardiovascular homeostasis.

This conclusion must be tempered by the consideration that β -endorphin-(1-31) is but one of several structurally related peptides localized in brainstem neurons (Zakarian and Smyth, 1982; Smith and Funder, 1988; Loh, 1992). These include two C-terminally shortened analogs, β -endorphin-(1-27) and β -endorphin-(1-26), and the α -N-acetylated derivatives of all three peptides. Unlike β -endorphin-(1-31), these N- and C-terminally modified β -endorphin

peptides display little or no affinity for opioid receptors and are essentially inactive in standard assays for antinociception (Deakin, et al., 1980; Akil et al., 1981; Nicolas and Li, 1985). To fully understand the role of β -endorphin neurons in cardiovascular homeostasis it is therefore essential to identify the specific β -endorphin peptides localized within cardioregulatory brain regions and to evaluate their independent and interactive effects on cardiovascular function.

Regional analysis of β -endorphin-(1-31) processing has revealed that β -endorphin-(1-31) predominates throughout much of the brain (Zakarian and Smyth, 1982) but it is a relatively minor component of the β -endorphin peptides localized in the brainstem (Zakarian and Smyth, 1982) and caudal medulla (Dores et al., 1986). In the brainstem, β -endorphin-(1-31) accounts for less than 25% of total β -endorphin immunoreactivity; α -N-acetyl- β -endorphin-(1-27), α -N-acetyl- β -endorphin-(1-26) and β -endorphin-(1-26) are the predominant forms (Zakarian and Smyth, 1982). Structure-activity studies have shown that, in contrast to β -endorphin-(1-31), these post-translationally derived β -endorphin analogs have no effect on peripheral hemodynamics when injected centrally (Hirsch and Millington, 1991; van Giersbergen et al., 1991). The major portion of the β -endorphin peptides localized in the brainstem thus have no known cardioregulatory function.

The endoproteolytic conversion of β -endorphin-(1-31) to β -endorphin-(1-27) also generates a dipeptide, glycyl-L-glutamine [β -endorphin-(30-31)]. Glycyl-L-glutamine is a major end-product of β -endorphin-(1-31) processing in the brainstem and other brain

regions because it is produced in amounts equivalent to the combined concentrations of β -endorphin-(1-27), β -endorphin-(1-26), α -N-acetyl- β -endorphin-(1-27) and α -N-acetyl- β -endorphin-(1-26) (Parish et al., 1982). Consequently, brainstem glycyl-L-glutamine concentrations substantially exceed β -endorphin-(1-31) levels. This raises the possibility that glycyl-L-glutamine may be an important product of β -endorphin-(1-31) processing, not only quantitatively, but functionally as well. Despite its quantitative significance, glycyl-L-glutamine's physiological functions have not been thoroughly evaluated and its role in cardiovascular homeostasis, if any, is unknown.

These considerations prompted us to examine whether glycyl-L-glutamine modulates the cardiorespiratory depression produced by central β -endorphin-(1-31) administration. We found that glycyl-L-glutamine is, indeed, a potent inhibitor of β -endorphin-(1-31)-induced hypotension and respiratory depression. When injected i.c.v. following β -endorphin-(1-31), glycyl-L-glutamine attenuated the subsequent fall in arterial pressure that occurred in saline treated controls at doses that, when injected alone, had no effect on peripheral hemodynamics. These results demonstrate that glycyl-L-glutamine is an endogenously synthesized antagonist of β -endorphin-(1-31)-induced cardiorespiratory depression, consistent with accumulating evidence that glycyl-L-glutamine functions as a neuromodulator.

METHODS

Animals and Surgical Procedures:

Male Sprague-Dawley rats (250-350 g; Sasco, Inc., Omaha, NE) were housed under a 12:12 h light:dark cycle with free access to food and water. Rats were anesthetized with either pentobarbital (50 mg/kg i.p.) or urethane (1.5 g/kg i.p.) and the left common carotid artery was cannulated with PE-50 tubing filled with heparinized saline (150 U/ml) and attached to a volumetric pressure transducer (Statham P23). Blood pressure and heart rate were recorded using a Grass model 7D polygraph (Grass Instruments, Quincy, MA).

Peptides were injected i.c.v. through a 20-gauge stainless steel guide cannula implanted in the right lateral ventricle 1.5 mm lateral to the midline, 1.0 mm posterior to bregma and 4.0 mm below the skull surface. The peptides were dissolved in 10 μ l isotonic saline and injected through a 26-gauge stainless steel cannula connected by PE-20 tubing to a 50 μ l Hamilton syringe. The tip of the injection cannula extended 0.5 mm below the end of the guide cannula and the injection volume was monitored by observing the movement of an air bubble placed in the tubing.

For blood gas measurements, 0.3 ml arterial blood was collected into heparinized syringes and immediately replaced with saline. Blood samples were placed on ice and analyzed within 20 min using a Corning model 178 pH/Blood Gas Analyzer.

Receptor Binding Assay:

Rats were sacrificed by decapitation, each brain was rapidly

removed and, after detaching the cerebellum, homogenized in 50 mM Tris-HCl (pH 7.4) at 4 °C with a Teflon homogenizer. The homogenate was centrifuged for 10 min at 3,000 x g and 4 °C, the pellet was discarded and the supernatant was centrifuged at 48,000 x g for 20 min at 4 °C. The pellet was washed with 50 mM Tris-HCL (pH 7.4), re-centrifuged for 20 min at 48,000 x g and 4 °C and resuspended in sufficient 50 mM Tris-HCl (pH 7.4) to achieve a protein concentration of 2 mg/ml. Protein was analyzed with a bicinchoninic acid (BCA) Protein Assay Reagent (Pierce, Rockford, IL).

Receptor binding assays were performed by incubating brain homogenates (200 µg protein) with 1 nM [³H]naloxone (46 Ci/mmol; Amersham Corp., Arlington Heights, IL) at 27 °C for 30 min in 0.5 ml 50 mM Tris-HCl buffer (pH 7.4). At the end of incubation period, bound [³H]naloxone was separated from free by vacuum filtration through polyethylenimine-coated Whatman GF/B filters using a Brandel receptor binding harvester (Brandel, Inc., Gaithersburg, MD). Non-specific binding was estimated using 1 µM naloxone. Km and Bmax values were determined by Scatchard analysis and Ki values and Hill coefficients were determined using a non-linear curve fitting program.

Drugs and Peptides:

Rat β-endorphin-(1-31) and camel β-endorphin-(1-27) were obtained from Peninsula Laboratories (Belmont, CA), glycyl-L-glutamine and glycyl-D-glutamine were purchased from Bachem California (Torrance, CA), and glycyl-L-glutamate, L-glycine and L-glutamine from Sigma Chemical Co. (St. Louis, MO). α-N-Acetyl-

control values but its effect was neither statistically significant nor dose-dependent.

We next examined whether glycyl-L-glutamine could produce independent cardiovascular effects when administered to rats that had not received β -endorphin-(1-31). Glycyl-L-glutamine proved to be ineffective, producing no significant variation from baseline MAP or heart rate at doses of 1, 10 or 100 nmol (Fig. 3). Glycyl-L-glutamine thus antagonizes β -endorphin-(1-31)-induced hypotension without affecting cardiovascular function when administered alone.

One caveat to this conclusion is the possibility that glycyl-L-glutamine's inhibitory action may have been indirect, resulting from enzymatic hydrolysis to its constituent amino acids. L-Glycine also modulates cardiovascular function, either raising (Talman, 1988) or lowering (Persson, 1980; Talman and Robertson, 1989) MAP depending upon the site of injection. To test this, we injected equimolar amounts of L-glycine and L-glutamine (1.0 nmol each amino acid) i.c.v. 15 min after β -endorphin-(1-31). L-Glycine and L-glutamine co-injection had no effect whatsoever on the hypotension (Fig. 4) or bradycardia (not shown) produced by β -endorphin-(1-31). A considerably higher dose (1.0 μ mol each amino acid) potentiated, rather than inhibited, β -endorphin-(1-31)'s hypotensive effect (data not shown). When administered to rats that had not received β -endorphin-(1-31) pretreatment, 1.0 μ mol L-glycine or equivalent amounts of L-glycine combined with L-glutamine also lowered MAP (data not shown), consistent with a previous report (Persson, 1980). Thus, hydrolysis does not account for glycyl-L-glutamine's inhibitory activity.

Effects in Urethane Anesthetized Rats:

The cardiovascular effects of β -endorphin-(1-31) and other opioid peptides are influenced by several variables, including the type of anesthesia (Feuerstein, 1985). To determine whether glycyl-L-glutamine was effective only under pentobarbital anesthesia we tested it in urethane anesthetized rats. β -Endorphin-(1-31) produced a biphasic response under urethane anesthesia, generating a transient rise in MAP followed by a dose-related hypotension (Fig. 5), as reported previously (Bolme et al., 1976; Laubie et al., 1977). β -Endorphin-(1-31) was less potent in urethane anesthetized rats; 3.0 nmol β -endorphin-(1-31) produced a smaller MAP reduction than did 0.5 nmol under pentobarbital anesthesia. Nonetheless, glycyl-L-glutamine produced a dose-dependent inhibition of β -endorphin-(1-31)-induced hypotension under urethane anesthesia. The hypotensive response to 1.0 nmol β -endorphin-(1-31) was almost entirely blocked by an equimolar glycyl-L-glutamine dose injected 15 min later (Fig. 6) ($F(3,12) = 9.23$, $P < 0.01$); bradycardia was not significantly affected (data not shown).

Next, we reversed the peptide injection sequence by pre-treating rats with glycyl-L-glutamine 5 min before they received β -endorphin-(1-31). Glycyl-L-glutamine (6.0 nmol) pretreatment was also effective; it inhibited the hypotension induced by subsequent β -endorphin-(1-31) (3.0 nmol) injection but did not significantly influence the initial pressor response (Table 1). These data indicate that glycyl-L-glutamine's modulatory activity is not dependent on a specific anesthetic agent or the temporal

sequence of peptide injection.

Respiratory Depression:

Like morphine, β -endorphin-(1-31) produces respiratory depression when injected centrally (Flórez et al., 1980; Moss and Scarpelli, 1981; Shook et al., 1990). To determine if glycyl-L-glutamine inhibits the respiratory depressant effect of β -endorphin-(1-31), we measured blood gases immediately before and 45 min after β -endorphin-(1-31) injection; as in previous experiments, glycyl-L-glutamine or saline was administered 15 min after β -endorphin-(1-31). As expected, β -endorphin-(1-31) followed by saline injection increased $p\text{CO}_2$ and lowered $p\text{O}_2$ and pH although HCO_3^- (Table 2) and base excess concentrations (not shown) did not change significantly.

Glycyl-L-glutamine attenuated the hypercapnia and hypoxia induced by β -endorphin-(1-31) (Table 2). Both the rise in plasma $p\text{CO}_2$ and fall in $p\text{O}_2$ elicited by β -endorphin-(1-31) were significantly diminished by subsequent glycyl-L-glutamine injection; indeed, $p\text{CO}_2$ and $p\text{O}_2$ were not significantly different than baseline values following sequential β -endorphin-(1-31) and glycyl-L-glutamine administration (Table 2). Glycyl-L-glutamine did not influence the reduction in plasma pH caused by β -endorphin-(1-31), however. When administered i.c.v. to rats that had not been pretreated with β -endorphin-(1-31), glycyl-L-glutamine (1.0 nmol) had no effect on $p\text{CO}_2$; (baseline = 32.5 ± 2.1 mm Hg; final = 35.8 ± 4.6 mm Hg; $n = 3$), $p\text{O}_2$ (baseline = 87.0 ± 2.8 mm Hg; final = 84.8 ± 3.5 mm Hg) or pH (baseline = 7.42 ± 0.01 ; final = 7.41 ± 0.01)

measured immediately before and 30 min post-injection. These data indicate that glycyl-L-glutamine attenuates β -endorphin-(1-31)-induced hypercapnia and hypoxia but has no effect on blood gases when administered independently.

Glycyl-L-Glutamine Related Dipeptides:

To further define the structural requirements for glycyl-L-glutamine's inhibitory activity, we tested whether glycyl-D-glutamine, α -N-acetyl-glycyl-L-glutamine or glycyl-L-glutamate inhibited β -endorphin-(1-31)-induced hypotension. The glycyl-L-glutamine stereoisomer, glycyl-D-glutamine, was completely inactive, and produced no significant inhibition when injected at the same dose and time interval at which glycyl-L-glutamine was maximally effective (Table 3). α -N-Acetyl-glycyl-L-glutamine significantly inhibited β -endorphin-(1-31)-induced hypotension ($F(4,25) = 4.59$, $P < 0.01$) indicating that α -N-acetylation did not abolish the dipeptide's biological activity; in contrast, α -N-acetylation essentially eliminates the analgetic and hypotensive potency of β -endorphin-(1-31) (Deakin et al., 1980; Hirsch and Millington, 1991).

The human β -endorphin-(1-31) sequence terminates in glycyl-L-glutamate, rather than glycyl-L-glutamine, unlike the rat and virtually every other species examined thus far (Yamashiro and Li, 1984). Glycyl-L-glutamate partially blocked β -endorphin-(1-31)-induced hypotension, inhibiting the maximal response to β -endorphin-(1-31) by approximately fifty percent. Together, these data indicate that glycyl-L-glutamine's cardiovascular effects appear

to be stereospecific but that modifications to the dipeptide's N- or C-terminus do not entirely eliminate its inhibitory activity.

[³H]-Naloxone Binding:

The cardiorespiratory effects of β -endorphin-(1-31) are thought to be mediated by mu opioid receptors (Petty and de Jong, 1982; Mosqueda-Garcia and Kunos, 1987; Shook et al., 1990) raising the possibility that glycyl-L-glutamine inhibits β -endorphin-(1-31)'s effects by acting as a mu receptor antagonist. To test this, we conducted receptor binding experiments using [³H]naloxone as a ligand. [³H]Naloxone binding to rat brain homogenates was saturable with K_d and B_{max} values of 1.5 nM and 138 fmol/mg protein, respectively, consistent with earlier reports (Wood et al., 1981; Schnittler et al., 1990). Non-specific binding was consistently less than 25% total binding.

[³H]Naloxone binding was displaced by morphine and β -endorphin-(1-31) with K_i values in the nM range (Table 4), comparable to previously published data (Wood, et al., 1981); Hill coefficients did not differ significantly from unity for any inhibitor (data not shown). β -Endorphin-(1-27) displayed considerably lower affinity for [³H]naloxone binding sites, less than one-tenth that of β -endorphin-(1-31), similar to its potency ratio for [³H]-morphine binding (Akil et al., 1981).

Glycyl-L-glutamine failed to displace [³H]naloxone binding at concentrations ranging from 1 pM to 10 mM (Table 4). For example, [³H]naloxone binding in the presence of 10 mM glycyl-L-glutamine, the highest concentration tested, was essentially the same as

control values (102.5 ± 6.7 % control; $n = 3$). Glycyl-L-glutamine is therefore unlikely to inhibit β -endorphin-(1-31)-induced hypotension by acting as an opioid receptor antagonist.

DISCUSSION

Glycyl-L-glutamine is a major end-product of β -endorphin-(1-31) processing in the brainstem yet its role in cardiovascular regulation has not been previously investigated (Zakarian and Smyth, 1982; Parish et al., 1983). Here we report that glycyl-L-glutamine inhibits the characteristic hypotension and respiratory depression induced by i.c.v. β -endorphin-(1-31) injection. Glycyl-L-glutamine is a relatively potent β -endorphin-(1-31) antagonist; 1.0 nmol inhibited the reduction in MAP induced by 0.5 nmol β -endorphin-(1-31). The glycyl-L-glutamine effect is not attributable to hydrolysis because it was not reproduced by equimolar amounts of L-glycine and L-glutamine, nor was it dependent on the type of anesthesia or the temporal sequence of peptide administration. Glycyl-L-glutamine was inactive when given alone, however, and did not influence arterial pressure or heart rate at doses up to 100-fold higher than required to inhibit β -endorphin-(1-31). Collectively, these findings demonstrate that glycyl-L-glutamine is a potent antagonist of β -endorphin-(1-31)-induced cardiorespiratory depression but lacks hemodynamic activity when given alone.

These data extend previous structure-activity studies showing that post-translational processing substantially alters β -endorphin-(1-31)'s central cardioregulatory activity. These studies

revealed that most post-translationally derived β -endorphin peptides, including α -N-acetyl- β -endorphin-(1-31), α -N-acetyl- β -endorphin-(1-27), α -N-acetyl- β -endorphin-(1-26) and β -endorphin-(1-26), are essentially inactive in tests of central cardiovascular potency (Hirsch and Millington, 1991; van Giersbergen et al., 1991). β -Endorphin-(1-27), on the other hand, is a potent hypotensive and bradycardic agent when centrally injected; indeed, it is even more potent than β -endorphin-(1-31) (Hirsch and Millington, 1991; van Giersbergen et al., 1991). β -Endorphin-(1-27) may thus play a role in the central regulation of cardiovascular function, although quantitatively, it is a relatively minor end-product of brain β -endorphin-(1-31) processing (Zakarian and Smyth, 1982; Dores et al, 1986; Emeson and Eipper, 1986).

These findings underscore the difficulties involved in predicting the physiological role of endogenous β -endorphin peptides from pharmacological data. Hypotheses regarding their endogenous function must take into account the anatomical pathways in which β -endorphin is synthesized, the specific β -endorphin peptides expressed within these pathways and their independent and interactive effects on cardiovascular function. In the forebrain, these relationships are relatively uncomplicated. The forebrain is innervated solely by proopiomelanocortin (POMC) neurons projecting from the medial basal hypothalamus (Khachaturian et al., 1985) which primarily synthesize β -endorphin-(1-31) and small amounts of β -endorphin-(1-27) and β -endorphin-(1-26); α -N-acetylation occurs to only a limited extent (Zakarian and Smyth, 1982; Emeson and Eipper, 1986).

In contrast, β -endorphin-(1-31) is a relatively minor end-

product in the brainstem, where it accounts for only about 25% of β -endorphin immunoreactivity (Zakarian and Smyth, 1982; Dores et al., 1986). The predominant forms are α -N-acetylated and C-terminally truncated, hence inactive (Deakin et al, 1980; Hirsch and Millington, 1991). Based on structure-activity data, it is difficult to discern why POMC neurons convert β -endorphin-(1-31) to peptide derivatives lacking cardiovascular, or for that matter, any other identified physiological function in brain. But these studies failed to consider that glycyl-L-glutamine is also a prominent end-product of β -endorphin-(1-31) processing. The present data indicate that glycyl-L-glutamine is an important β -endorphin-(1-31) derivative, not only quantitatively, but functionally as well, and further suggest that it may serve as an antagonist of β -endorphin-(1-31)'s hypotensive activity. The concept that agonist and antagonist β -endorphin peptides are co-released from the same neuron is somewhat paradoxical, yet not unprecedented. β -Endorphin-(1-27) is a potent opioid receptor antagonist that inhibits β -endorphin-(1-31)-induced antinociception (Nicolas and Li, 1985; Suh et al., 1988; Hong et al., 1993). Together these data support the concept that β -endorphin-(1-27) and glycyl-L-glutamine modulate the antinociceptive and cardioregulatory actions of the parent peptide in a regionally specific manner.

In addition to its cardiovascular effects, central β -endorphin-(1-31) injection produces respiratory depression (Flórez et al., 1980; Moss and Scarpelli, 1981; Sitsen et al., 1982). Respiratory depression is often a complicating factor when evaluating the cardiovascular effects of opioid peptides, particularly in

finding that glycyl-L-glutamine fails to displace [3 H]naloxone binding at concentrations as high as 10 mM indicates that blockade of mu receptors is unlikely to account for its inhibitory interaction with β -endorphin-(1-31). Nevertheless, the conclusion that mu receptors mediate β -endorphin-(1-31)'s cardiovascular effects has not met with universal agreement because a number of investigations have shown that, in contrast to β -endorphin-(1-31), mu selective agonists elevate, rather than lower MAP when injected centrally (Hassen and Feuerstein, 1987; Petty and Sitsen, 1989). This evidence has led to the alternative hypothesis that β -endorphin-(1-31)'s cardiovascular activity is attributable to activation of the putative epsilon receptor or other non-classical β -endorphin-(1-31) binding sites (Petty and Sitsen, 1989; Millington and Hirsch, 1993). Thus, it is conceivable that glycyl-L-glutamine acts as an antagonist at a β -endorphin-(1-31) binding site other than the mu opioid receptor.

Several lines of evidence argue against this conclusion, however. We recently reported, for example, that glycyl-L-glutamine inhibits the hyperthermia generated by α -melanocyte-stimulating hormone (α -MSH) microinjection into thermoregulatory sites in the medial preoptic area (Resch and Millington, 1993). As in the present study, glycyl-L-glutamine was inactive when injected alone. Glycyl-L-glutamine also inhibits the characteristic behavioral effects produced by i.c.v. α -MSH injection, the grooming and stretching and yawning syndromes, without influencing these behavioral repertoires when injected alone (Hirsch and O'Donohue, 1986). These findings demonstrate that glycyl-L-

glutamine inhibits the action of at least one other POMC-derived peptide, an effect which is difficult to ascribe to blockade of opioid receptors or other β -endorphin-(1-31) binding sites.

Glycyl-L-glutamine also produces independent effects unrelated to inhibition of POMC peptides (Parish et al., 1983; Koelle et al., 1988; Lotwick et al., 1990; Haynes, 1991; Nyquist-Battie et al., 1993). This was first demonstrated by Parish et al. (1983) who showed that glycyl-L-glutamine reduces the firing rates of brain-stem neurons when applied iontophoretically. Glycyl-L-glutamine's inhibitory electrophysiological activity was unaffected by naloxone, indicating that opioid receptors are not involved in the response. Strychnine was also ineffective, implying that glycyl-L-glutamine does not produce its modulatory effects by activating glycine receptors (Parish et al., 1982). Our data support this conclusion, showing that glycyl-L-glutamine's cardiomodulatory effects are not reproduced by equimolar amounts of L-glycine; in fact, higher L-glycine doses have the opposite effect, lowering arterial pressure when injected i.c.v. (Persson, 1980; unpublished data). We further showed that glycyl-L-glutamine's inhibitory interaction with β -endorphin-(1-31) was stereospecific, to the extent that it was not reproduced by glycyl-D-glutamine, and that it was not abolished by N-terminal acetylation or by substituting glutamate for glutamine. These findings support the hypothesis that glycyl-L-glutamine acts through a stereospecific receptor but whether this represents a unique glycyl-L-glutamine binding site remains to be investigated.

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FOOTNOTES

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Table 2. Glycyl-L-glutamine attenuates β -endorphin-(1-31)-induced respiratory depression.

Treatment	Baseline	Final	Change
<u>pCO₂ (mm Hg):</u>			
Saline	40.5 $\pm$ 3.0	59.6 $\pm$ 6.5*	19.1 $\pm$ 4.3
Gly-L-Gln	42.0 $\pm$ 2.2	50.5 $\pm$ 3.2	8.5 $\pm$ 2.5 [#]
<u>pO₂ (mm Hg):</u>			
Saline	80.1 $\pm$ 3.9	56.3 $\pm$ 6.1**	-23.8 $\pm$ 4.9
Gly-L-Gln	73.2 $\pm$ 2.9	66.9 $\pm$ 3.3	-6.4 $\pm$ 5.2 [#]
<u>HCO₃⁻ (mmol/L):</u>			
Saline	23.4 $\pm$ 1.3	25.6 $\pm$ 2.4	2.2 $\pm$ 1.9
Gly-L-Gln	23.9 $\pm$ 0.9	23.6 $\pm$ 1.0	-0.3 $\pm$ 1.2
<u>pH:</u>			
Saline	7.37 $\pm$ 0.01	7.24 $\pm$ 0.03**	-0.13 $\pm$ 0.02
Gly-L-Gln	7.36 $\pm$ 0.01	7.28 $\pm$ 0.02*	-0.08 $\pm$ 0.02

Pentobarbital anesthetized rats were treated i.c.v. with 0.5 nmol β -endorphin-(1-31) followed 15 min later by either saline (n = 6) or 1.0 nmol glycyl-L-glutamine (Gly-L-Gln; n = 7). Arterial blood samples were drawn 5 min prior to β -endorphin-(1-31) (baseline) and 30 min after saline or glycyl-L-glutamine injection (final). Data are presented as the mean $\pm$ S.E. and were analyzed by two-tailed t-test. *P < 0.05 and **P < 0.01 differs from the corresponding baseline values; [#]P < 0.05 differs from β -endorphin-(1-31) + saline treated animals.

Table 3. The effect of glycyl-L-glutamine related dipeptides on the hypotensive response to β -endorphin-(1-31).

Treatment	Change in MAP (mm Hg)
Saline	-21.1 $\pm$ 4.1
Gly-L-Gln (8)	-2.8 $\pm$ 3.1**
Gly-D-Gln (6)	-19.3 $\pm$ 4.5
Gly-L-Glu (4)	-10.5 $\pm$ 4.0
Ac-Gly-L-Gln (4)	-7.0 $\pm$ 3.1*

Pentobarbital anesthetized rats were treated i.c.v. with β -endorphin-(1-31) (0.5 nmol), followed 15 min later, by glycyl-L-glutamine (Gly-L-Gln; 1.0 nmol), glycyl-D-glutamine (Gly-D-Gln; 1.0 nmol), glycyl-L-glutamate (Gly-L-Glu; 1.0 nmol) or α -N-acetylglycyl-L-glutamine (Ac-Gly-L-Gln; 10 nmol). Data represent the mean change in MAP ($\pm$ S.E.) recorded immediately before and 30 min following dipeptide administration and were analyzed by ANOVA followed by Newman-Keuls test. The numbers in parentheses indicate the number of animals in each treatment group. *P < 0.05; **P < 0.01 differs from control.

Table 4. Inhibition of [^3H]naloxone binding by morphine, β -endorphin-(1-31) and β -endorphin-(1-27) but not glycyl-L-glutamine.

Inhibitor	K_i (nM)
Naloxone	6.7 ± 1.4
Morphine	34.2 ± 9.9
β -End-(1-31)	10.7 ± 3.2
β -End-(1-27)	155.2 ± 26.5
Gly-L-Gln	$> 10,000,000$

[^3H]Naloxone (1 nM) binding was determined in the presence of inhibitor concentrations ranging between 1 pM and 10 μM or, for glycyl-L-glutamine, 10 mM. Data represent the mean $\pm$ S.E. of three separate experiments.

Figure 1

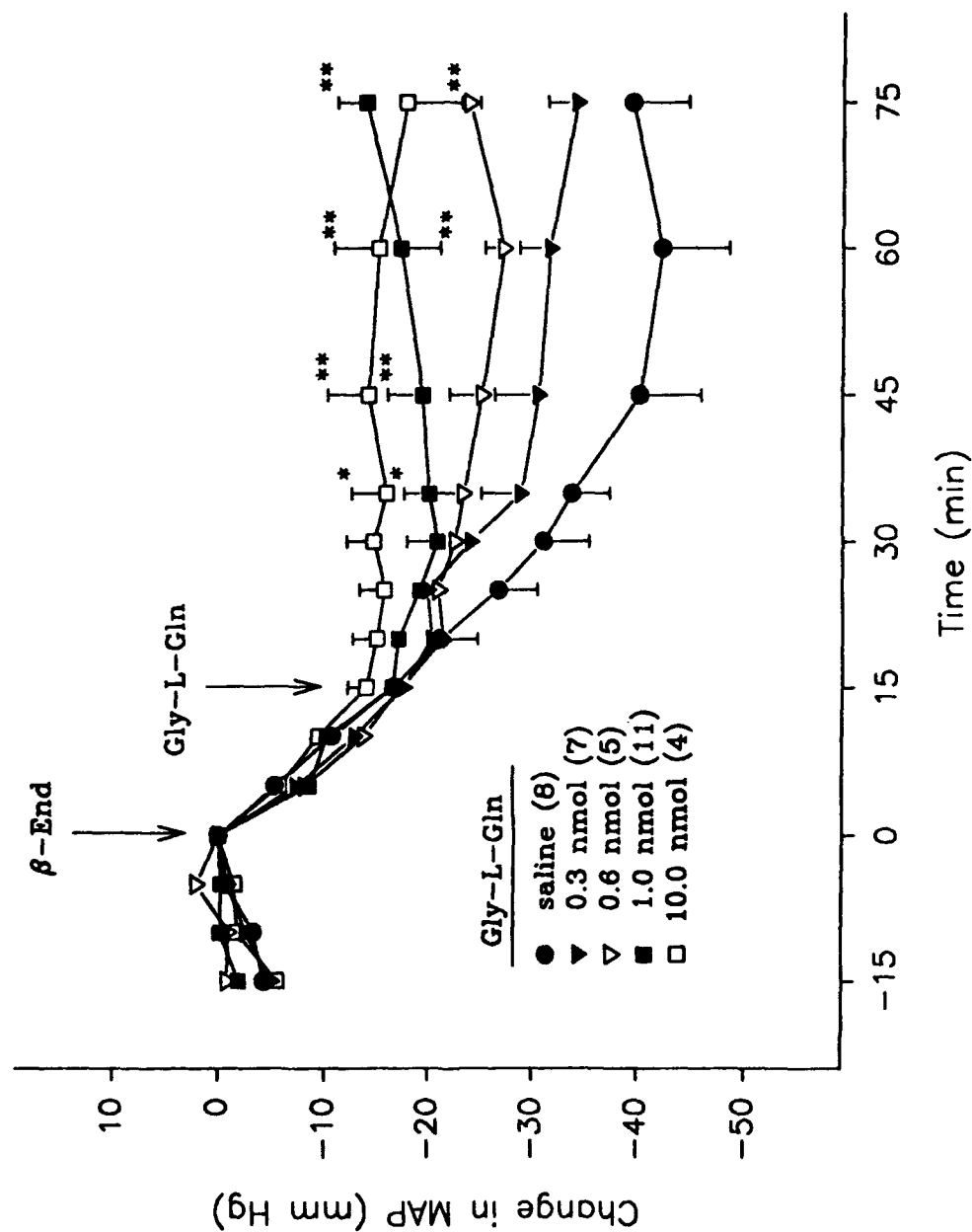


Figure 2

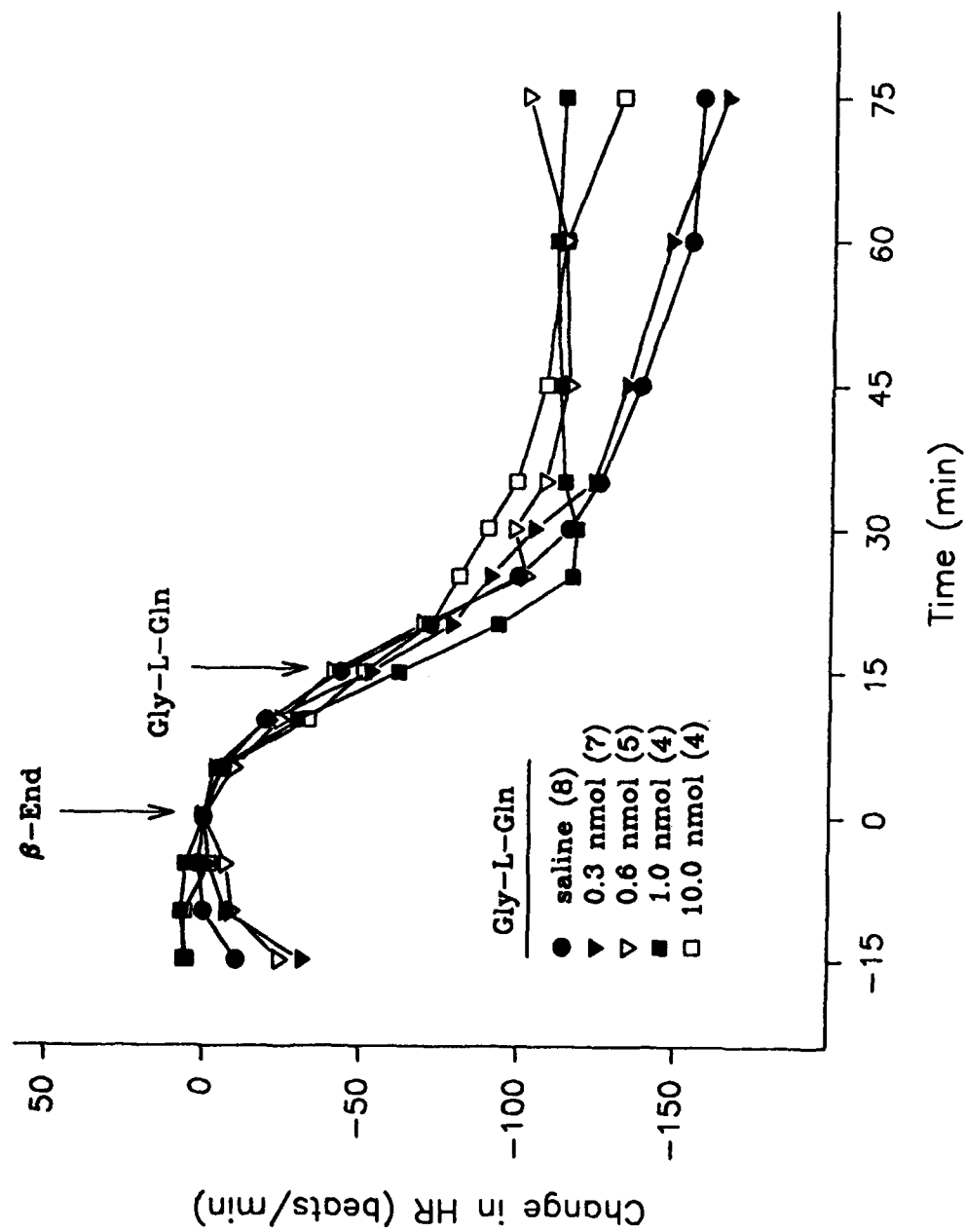


Figure 3

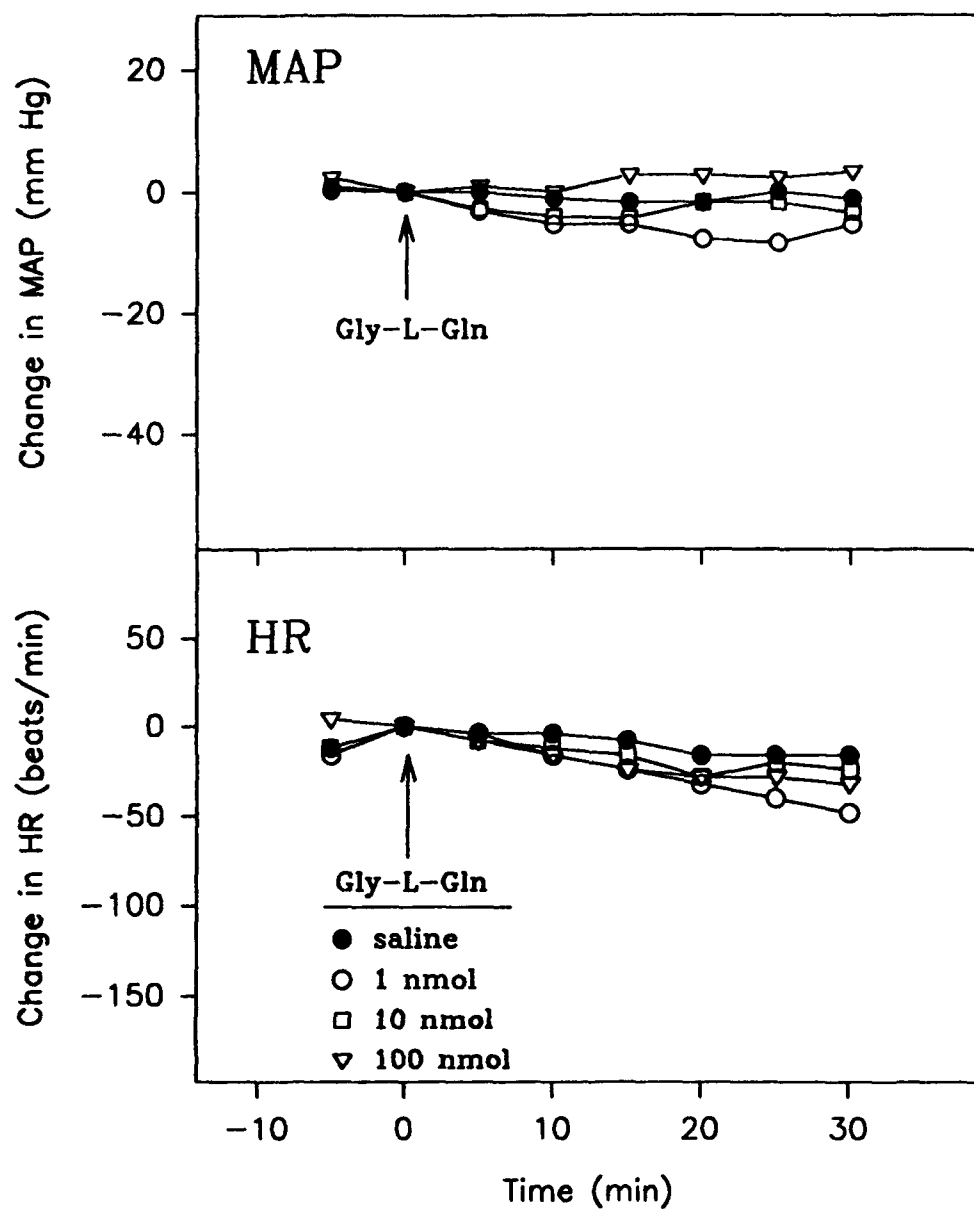


Figure 4

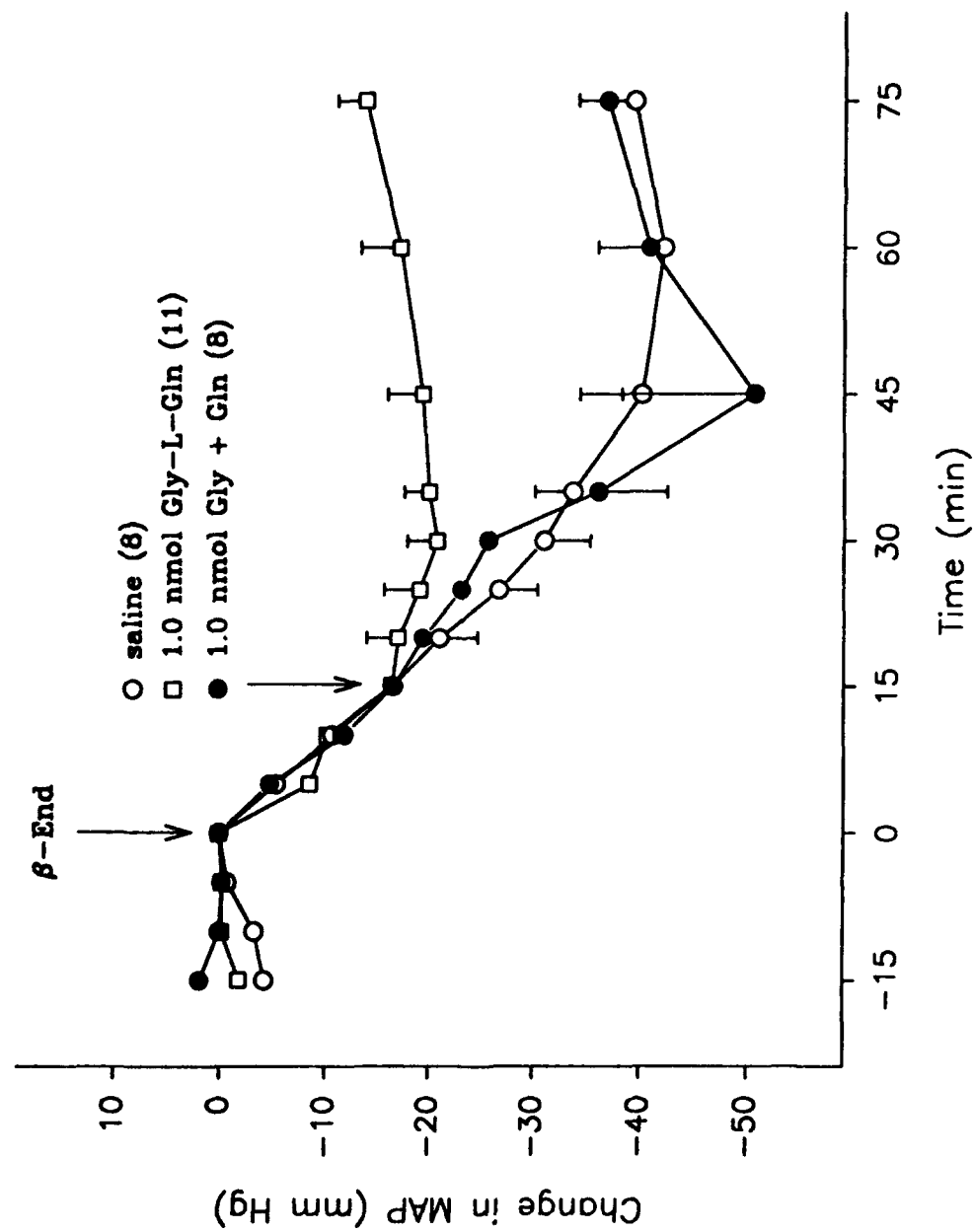


Figure 5

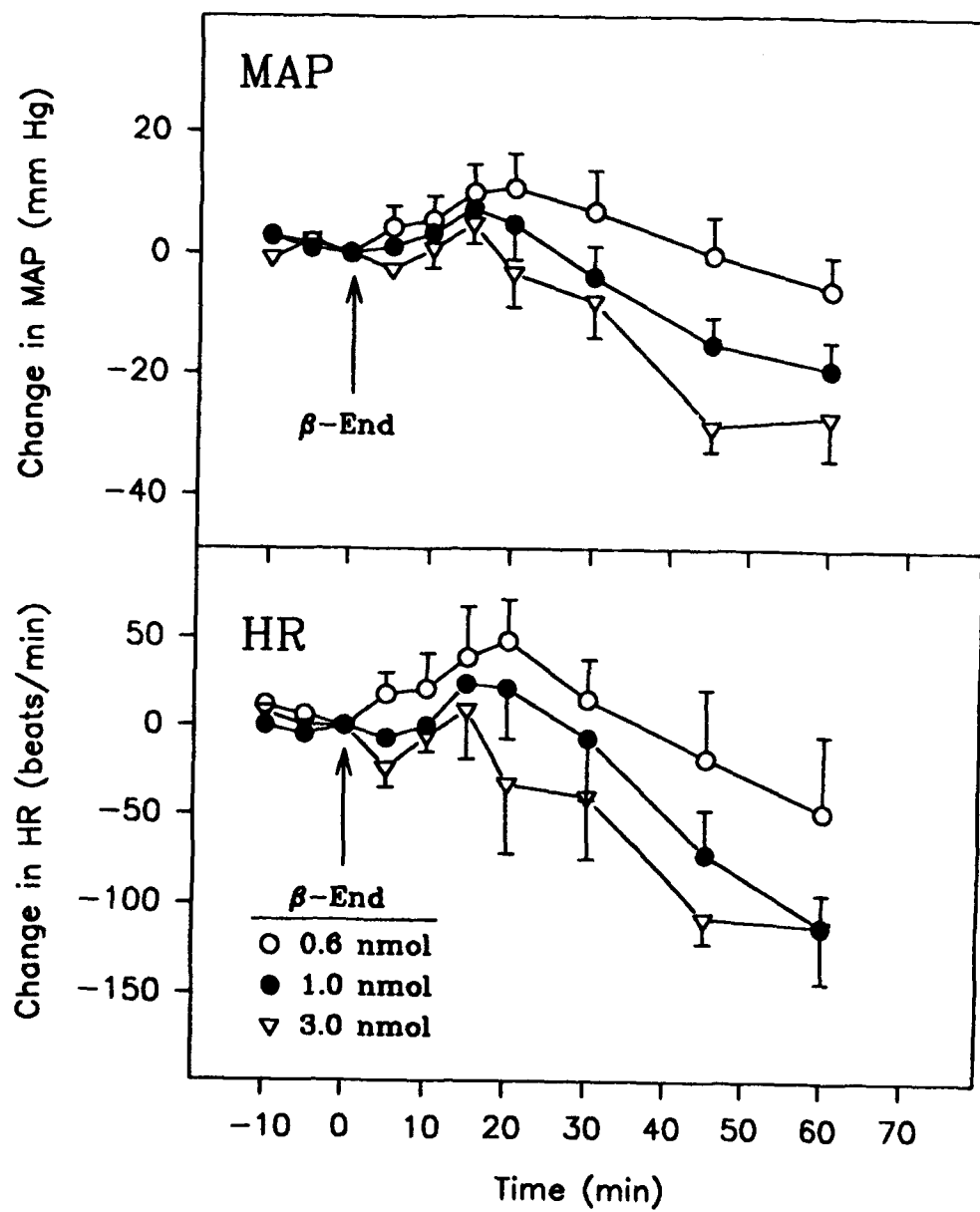


Figure 6

