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β-Endorphin-Induced Cardiorespiratory Depression is Inhibited by Glycyl-L-Glutamine, a Dipeptide Derived from β-Endorphin Processing[1]

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ABSTRACT

Glycyl-L-glutamine (Gly-L-Gln), or β-endorphin-(30–31) [β-End-(30–31)], is synthesized through the post-translational processing of β-End-(1–31). Evidence that gly-L-gln is a prominent end product of β-End-(1–31) processing in cardioregulatory regions of rat brain prompted us to investigate whether it modulates the cardiorespiratory depression induced by central β-End-(1–31) injection. As shown previously, β-End-(1–31) (0.5 nmol) lowered mean arterial pressure (MAP) and HR when administered i.c.v. to pentobarbital-anesthetized rats. Gly-L-gln (0.3, 0.6, 1.0 and 10.0 nmol) produced a dose-related inhibition of β-End-(1–31)-induced hypotension, but not bradycardia, when injected i.c.v. 15 min after β-End-(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 gly-L-gln was administered to urethane-anesthetized rats and when it was injected before β-End-(1–31). Gly-L-gln also attenuated the respiratory depressant effect of β-End-(1–31), significantly inhibiting β-End-(1–31)-induced hypoxia and hypercapnia. Gly-L-gln (1, 10 and 100 nmol) was inactive when injected alone, however, and produced no significant variation from base-line MAP or HR values. These results demonstrate that gly-L-gln inhibits β-End-(1–31)-induced cardiorespiratory depression, consistent with accumulating evidence that gly-L-gln functions as a neuromodulator.

β-End-(1–31) generates severe hypotension and bradycardia when injected into the cerebrospinal fluid of rats and other species (Laubie *et al.*, 1977; Bolme *et al.*, 1978; Sitsen *et al.*, 1982). A comparable response ensues when β-End-(1–31) is microinjected directly into the NTS (Petty and de Jong, 1982; Mosqueda-Garcia and Kunos, 1987) or the vasopressor region of the ventrolateral medulla (Punnen and Sapru, 1986), which suggests that β-End-(1–31) acts at multiple loci within the brainstem. Like morphine and other opiates, β-End-(1–31) is thought to produce these effects by activating μ opioid receptors (Petty and de Jong, 1982; Mosqueda-Garcia and Kunos, 1987) localized in the NTS and other brainstem cardioregulatory 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-End 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-End-(1–31) plays an important role in cardiovascular homeostasis.

This conclusion must be tempered by the consideration that β-End-(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-End-(1–27) and β-End-(1–26), and the α-N-acetylated derivatives of all three peptides. Unlike β-End-(1–31), these N- and C-terminally modified β-End 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-End neurons in cardiovascular homeostasis, it is therefore essential to identify the specific β-End peptides localized within cardioregulatory brain regions and to evaluate their independent and interactive effects on cardiovascular function.

Regional analysis of β-End-(1–31) processing has revealed that β-End-(1–31) predominates throughout much of the brain (Zakarian and Smyth, 1982) but that it is a relatively minor component of the β-End peptides localized in the

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ABBREVIATIONS: Gly-L-Gln, glycyl-L-glutamine; β-End, β-endorphin; POMC, proopiomelanocortin; MAP, mean arterial pressure; i.c., intracisternal; NTS, nucleus of the solitary tract.

brainstem (Zakarian and Smyth, 1982) and caudal medulla (Dores *et al.*, 1986). In the brainstem, β-End-(1–31) accounts for less than 25% of total β-End immunoreactivity; α-N-acetyl-β-Endorphin-(1–27), α-N-acetyl-β-Endorphin-(1–26) and β-End-(1–26) are the predominant forms (Zakarian and Smyth, 1982). Structure-activity studies have shown that, in contrast with β-End-(1–31), these post-translationally derived β-End 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-End peptides localized in the brainstem thus have no known cardioregulatory function.

The endoproteolytic conversion of β-End-(1–31) to β-End-(1–27) also generates a dipeptide, gly-L-gln [β-End-(30–31)]. Gly-L-gln is a major end product of β-End-(1–31) processing in the brainstem and other brain regions, because it is produced in amounts equivalent to the combined concentrations of β-End-(1–27), β-End-(1–26), α-N-acetyl-β-Endorphin-(1–27) and α-N-acetyl-β-Endorphin-(1–26) (Parish *et al.*, 1983). Consequently, brainstem gly-L-gln concentrations substantially exceed β-End-(1–31) levels. This raises the possibility that gly-L-gln may be an important product of β-End-(1–31) processing, not only quantitatively, but functionally as well. Despite its quantitative significance, gly-L-gln'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 gly-L-gln modulates the cardiorespiratory depression produced by central β-End-(1–31) administration. We found that gly-L-gln is indeed a potent inhibitor of β-End-(1–31)-induced hypotension and respiratory depression. When injected i.c.v. after the administration of β-End-(1–31), gly-L-gln 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 gly-L-gln is an endogenously synthesized antagonist of β-End-(1–31)-induced cardiorespiratory depression, consistent with accumulating evidence that gly-L-gln functions as a neuromodulator.

Materials and Methods

Animals and surgical procedures. Male Sprague-Dawley rats (250–350 g; Sasco, Inc., Omaha, NE) were housed under a 12:12 hr 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 HR 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 μl isotonic saline and injected through a 26-gauge stainless steel cannula connected by PE-20 tubing to a 50-μl 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 of 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 3000 × *g* and 4°C, the pellet was discarded and the supernatant was centrifuged at 48000 × *g* for 20 min at 4°C. The pellet was washed with 50 mM Tris-HCl (pH 7.4), recentrifuged for 20 min at 48000 × *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 protein assay reagent (Pierce, Rockford, IL).

Receptor-binding assays were performed by incubating brain homogenates (200 μg protein) with 1 nM [^{3}H]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 the incubation period, bound [^{3}H]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). Nonspecific binding was estimated using 1 μM naloxone. K_m and B_{max} values were determined by Scatchard analysis, and K_i values and Hill coefficients were determined using a nonlinear curve fitting program.

Drugs and peptides. The following drugs and peptides were used in this study: Rat β-End-(1–31) and camel β-End-(1–27) (Peninsula Laboratories, Belmont, CA), gly-L-gln and glycyl-D-glutamine (Bachem California, Torrance, CA), glycyl-L-glutamate, L-glycine and L-glutamine, morphine sulfate, naloxone HCl, sodium pentobarbital and urethane (Sigma Chemical Co., St. Louis, MO). α-N-Acetyl-glycyl-L-glutamine was obtained through solid-phase synthesis and its identity verified by amino acid analysis and mass spectroscopy.

Statistical analysis. Data are reported as the arithmetic mean ± S.E. Statistical differences between treatment groups were determined by two-tailed Student's *t* test for paired data or analysis of variance followed by Student-Newman-Keuls[1] test for multiple comparisons.

Results

Effects on MAP and HR. Gly-L-gln inhibited β-End-(1–31)-induced hypotension, but not bradycardia, when administered i.c.v. to pentobarbital-anesthetized rats 15 min after β-End-(1–31) injection. β-End-(1–31) (0.5 nmol) followed by saline injection produced a rapid and sustained reduction in arterial blood pressure, lowering MAP by 42.3 ± 6.2 mm Hg within 60 min (fig. 1) [$F(6,108) = 8.26$, $P < .001$]. Gly-L-gln (0.3, 0.6, 1.0 or 10.0 nmol) dose-dependently inhibited β-End-(1–31)-elicited hypotension; the lowest significant inhibitory dose was 1.0 nmol (fig. 1) [$F(24,108) = 2.92$, $P < .001$]. In contrast, gly-L-gln had no significant effect on β-End-(1–31)-induced bradycardia. β-End-(1–31) reduced HR by 154 ± 14 beats/min within 60 min from a base line of 389 ± 15 beats/min (fig. 2). Gly-L-gln partially restored HR to control values, but its effect was neither statistically significant nor dose-dependent.

We next examined whether gly-L-gln could produce independent cardiovascular effects when administered to rats that had not received β-End-(1–31). Gly-L-gln proved to be ineffective, producing no significant variation from base-line MAP or HR at doses of 1, 10 or 100 nmol (fig. 3). Gly-L-gln thus antagonizes β-End-(1–31)-induced hypotension without affecting cardiovascular function when administered alone.

One caveat to this conclusion is the possibility that gly-L-gln'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 Rob-

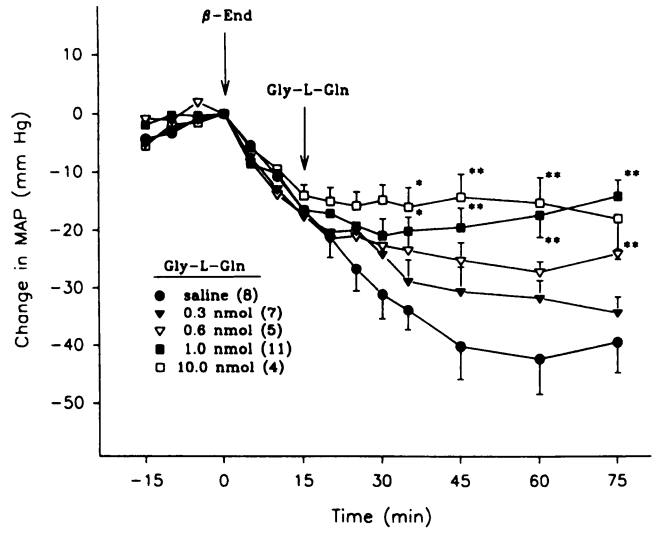

Fig. 1. Gly-L-gln inhibits β-End-(1–31)-induced hypotension. β-End-(1–31) (0.5 nmol i.c.v.) was administered to pentobarbital-anesthetized rats, followed 15 min later by either saline or the indicated dose of gly-L-gln, and MAP was recorded at 5- to 15-min intervals. Data represent the mean ± S.E. change in MAP from base-line values and were analyzed by ANOVA followed by Student-Newman-Keuls[1] test. The numbers in parentheses indicate the number of animals in each treatment group. Base-line MAP was 117.6 ± 2.5 mm Hg. * $P < .05$; ** $P < .01$ differs from saline-treated controls.

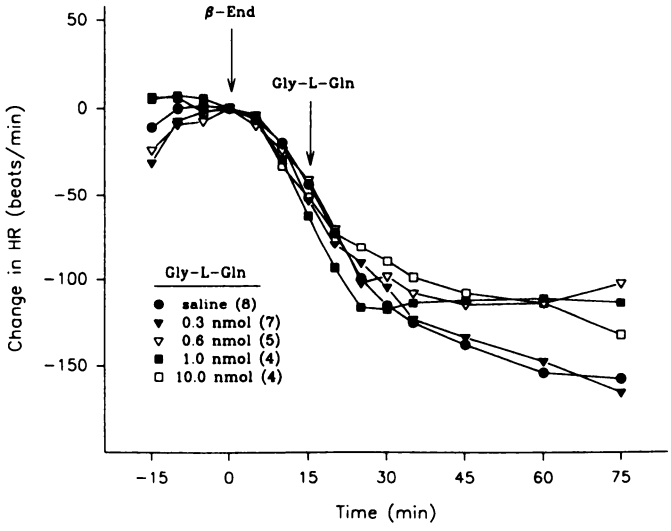

Fig. 2. Gly-L-gln does not significantly inhibit β-End-(1–31)-induced bradycardia. Pentobarbital-anesthetized rats were treated with β-End-(1–31) (0.5 nmol i.c.v.), followed 15 min later by either saline or the indicated gly-L-gln dose. Data were analyzed by ANOVA followed by Student-Newman-Keuls[1] test. Base-line HR was 389 ± 7 beats/min (mean ± S.E.).

ertson, 1989) MAP, depending on the site of injection. To test this, we injected equimolar amounts of L-glycine and L-glutamine (1.0 nmol of each amino acid) i.c.v. 15 min after administration of β-End-(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-End-(1–31). A considerably higher dose (1.0 μmol of each amino acid) potentiated, rather than inhibited, β-End-(1–31)'s hypotensive effect (data not shown). When administered to rats that had not received β-End-(1–31) pretreatment, 1.0 μmol L-glycine or equivalent amounts of L-glycine combined with L-glutamine

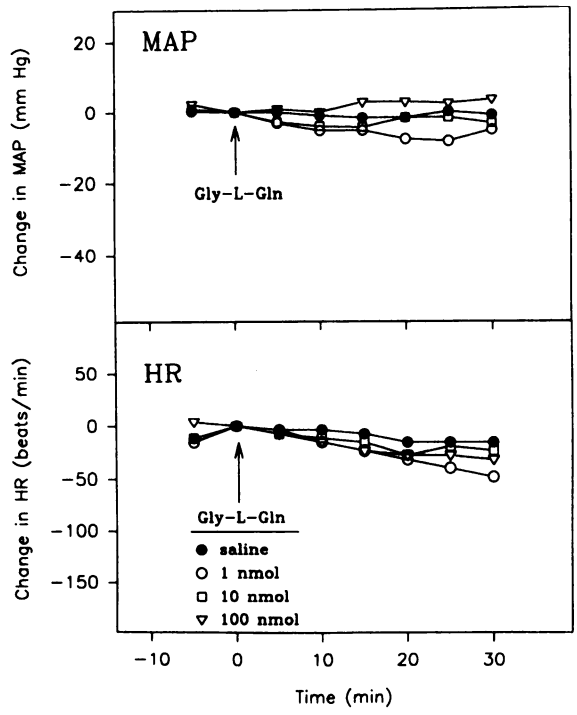

Fig. 3. Gly-L-gln does not affect MAP or HR when injected alone. Pentobarbital-anesthetized rats were treated with the indicated dose of gly-L-gln or saline i.c.v., and MAP and HR were recorded at 5-min intervals for 30 min ($n = 3$). Base-line MAP and HR values were 98.4 ± 4.1 mm Hg and 373 ± 15 beats/min (mean ± S.E.).

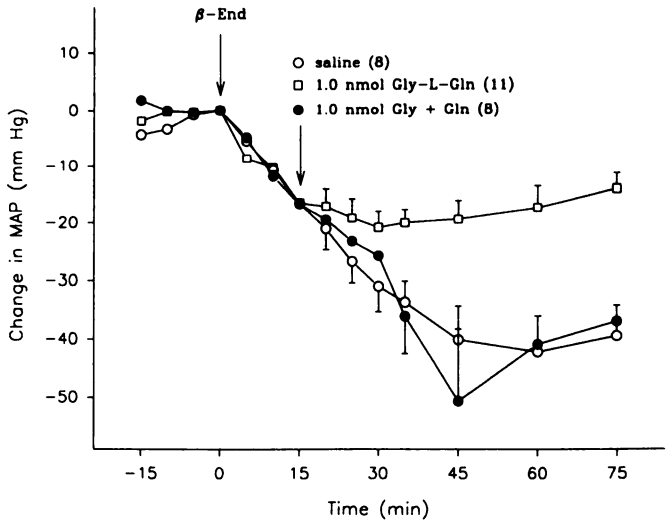

Fig. 4. Co-injection of L-glycine and L-glutamine does not influence the hypotensive response to β-End-(1–31). Pentobarbital-anesthetized rats received 0.5 nmol β-End-(1–31) i.c.v., followed 15 min later by either saline, L-glycine combined with L-glutamine (L-Gly + L-Gln; 1.0 nmol each amino acid) or gly-L-gln (1.0 nmol), and MAP was recorded at 5- to 15-min intervals. Data are presented as the mean ± S.E. The numbers in parentheses indicate the number of animals in each treatment group.

also lowered MAP (data not shown), consistent with a previous report (Persson, 1980). Thus hydrolysis does not account for gly-L-gln's inhibitory activity.

Effects in urethane-anesthetized rats. The cardiovascular effects of β-End-(1–31) and other opioid peptides are influenced by several variables, including the type of anes-

thesia (Feuerstein, 1985). To determine whether gly-L-gln was effective only under pentobarbital anesthesia, we tested it in urethane-anesthetized rats. β-End-(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.*, 1978; Laubie *et al.*, 1977). β-End-(1–31) was less potent in urethane-anesthetized rats; 3.0 nmol β-End-(1–31) produced a smaller MAP reduction than did 0.5 nmol under pentobarbital anesthesia. Nonetheless, gly-L-gln produced a dose-dependent inhibition of β-End-(1–31)-induced hypotension under urethane anesthesia. The hypotensive response to 1.0 nmol β-End-(1–31) was almost entirely blocked by an equimolar gly-L-gln dose injected 15 min later (fig. 6) [$F(3,12) = 9.23$, $P < .01$]; bradycardia was not significantly affected (data not shown).

Next we reversed the peptide injection sequence by pretreating rats with gly-L-gln 5 min before they received β-End-(1–31). Gly-L-gln (6.0 nmol) pretreatment was also effective; it inhibited the hypotension induced by subsequent β-End-(1–31) (3.0 nmol) injection but did not significantly influence the initial pressor response (table 1). These data indicate that gly-L-gln's modulatory activity is not dependent on a specific anesthetic agent or on the temporal sequence of peptide injection.

Respiratory depression. Like morphine, β-End-(1–31) produces respiratory depression when injected centrally (Flórez *et al.*, 1980; Moss and Scarpelli, 1981; Shook *et al.*, 1990). To determine whether gly-L-gln inhibits the respiratory depressant effect of β-End-(1–31), we measured blood gases immediately before and 45 min after β-End-(1–31) injection; as in previous experiments, gly-L-gln or saline was administered 15 min after β-End-(1–31). As expected, β-End-(1–31) followed by saline injection increased pCO_2 and lowered pO_2 and pH, although HCO_3^- (table 2) and base excess concentrations (not shown) did not change significantly.

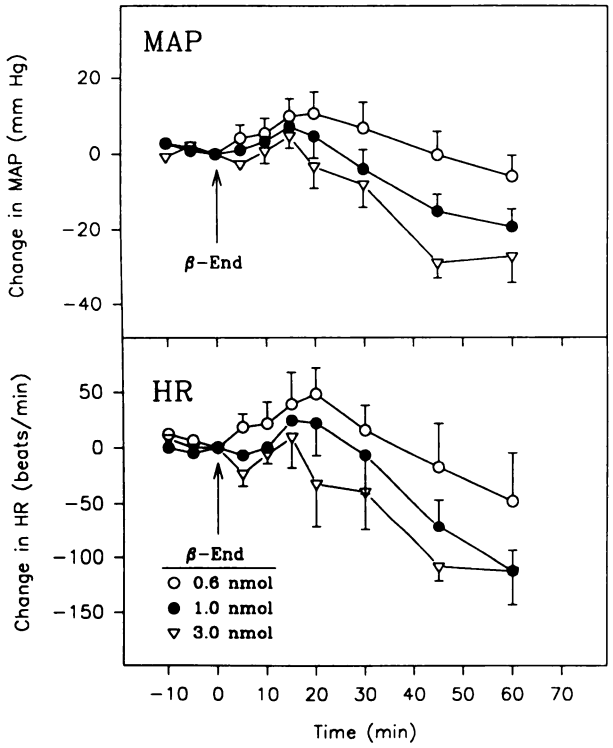

Fig. 5. Dose-response effects of β-End-(1–31) on MAP and HR in urethane-anesthetized rats. Groups of four to five rats were treated i.c.v. with the indicated dose of β-End-(1–31), and MAP and HR were recorded at 5- to 15-min intervals. Base-line MAP and HR values were 85.0 ± 3.9 mm Hg and 350 ± 8 beats/min (mean ± S.E.).

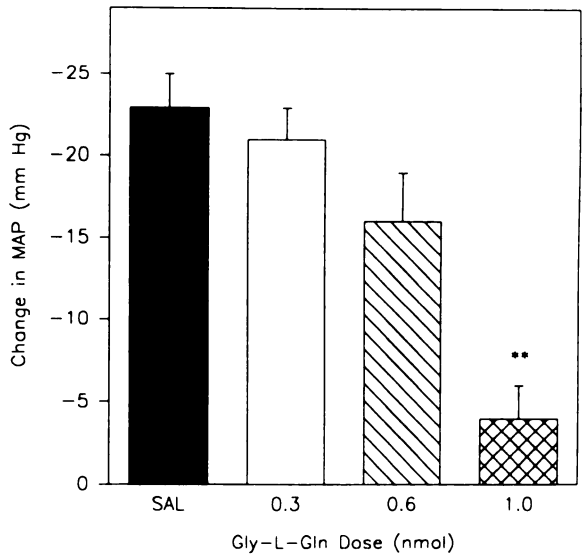

Fig. 6. Gly-L-gln inhibits β-End-(1–31)-induced hypotension in urethane-anesthetized rats. Groups of four rats were treated i.c.v. with 1.0 nmol β-End-(1–31), followed 15 min later by either saline or the indicated gly-L-gln dose. The data represent the mean ± S.E. change in MAP 20 min after the administration of gly-L-gln and were analyzed by ANOVA followed by the Newman-Keuls[1] test. Base-line MAP values were 118.8 ± 6.7 mm Hg. ** $P < .01$ differs from saline-treated controls.

TABLE 1

Gly-L-Gln pretreatment inhibits β-End-(1–31)-induced hypotension

Groups of four urethane-anesthetized rats were treated i.c.v. with Gly-L-Gln (6.0 nmol) followed 5 min later by β-End-(1–31) (3.0 nmol). Data are presented as the mean change in MAP (±S.E.) recorded immediately before Gly-L-Gln and at the indicated time point after β-End-(1–31) injection and were analyzed by two-tailed *t* test.

Treatment	Base-line MAP (mm Hg)	Change in MAP (mm Hg)	
		15 Min	45 Min
Saline + β-End	86.2 ± 5.1	4.5 ± 3.1	−28.0 ± 4.0
Gly-L-Gln + β-End	85.8 ± 6.2	18.7 ± 7.6	−10.2 ± 1.5*

*$P < .05$ differs from saline and β-End-(1–31)-treated animals.

Gly-L-gln attenuated the hypercapnia and hypoxia induced by β-End-(1–31) (table 2). Both the rise in plasma pCO_2 and the fall in pO_2 elicited by β-End-(1–31) were significantly diminished by subsequent gly-L-gln injection; indeed, pCO_2 and pO_2 were not significantly different from base-line values after sequential β-End-(1–31) and gly-L-gln administration (table 2). Gly-L-gln did not influence the reduction in plasma pH caused by β-End-(1–31), however. When administered i.c.v. to rats that had not been pretreated with β-End-(1–31), gly-L-gln (1.0 nmol) had no effect on pCO_2; (base line = 32.5 ± 2.1 mm Hg; final = 35.8 ± 4.6 mm Hg; $n = 3$), pO_2 (base line = 87.0 ± 2.8 mm Hg; final = 84.8 ± 3.5 mm Hg) or pH (base line = 7.42 ± 0.01; final = 7.41 ± 0.01) measured immediately before and 30 min after injection. These data indicate that gly-L-gln attenuates β-End-(1–31)-induced hypercapnia and hypoxia but has no effect on blood gases when administered independently.

TABLE 2

Gly-L-Gln attenuates β-End-(1–31)-induced respiratory depression

Pentobarbital-anesthetized rats were treated i.c.v. with 0.5 nmol β-End-(1–31) followed 15 min later by either saline (n = 6) or 1.0 nmol (Gly-L-Gln (n = 7). Arterial blood samples were drawn 5 min before β-End-(1–31) (base line) and 30 min after saline or Gly-L-Gln injection (final). Data are presented as the mean ± S.E. and were analyzed by two-tailed t test.

Treatment	Base line	Final	Change
pCO_2 (mm Hg):			
Saline	40.5 ± 3.0	59.6 ± 6.5*	19.1 ± 4.3
Gly-L-Gln	42.0 ± 2.2	50.5 ± 3.2	8.5 ± 2.5#
pO_2 (mm Hg):			
Saline	80.1 ± 3.9	56.3 ± 6.1**	−23.8 ± 4.9
Gly-L-Gln	73.2 ± 2.9	66.9 ± 3.3	−6.4 ± 5.2#
HCO_3^- (mmol/l):			
Saline	23.4 ± 1.3	25.6 ± 2.4	2.2 ± 1.9
Gly-L-Gln	23.9 ± 0.9	23.6 ± 1.0	−0.3 ± 1.2
pH:			
Saline	7.37 ± 0.01	7.24 ± 0.03**	−0.13 ± 0.02
Gly-L-Gln	7.36 ± 0.01	7.28 ± 0.02*	−0.08 ± 0.02

*P < .05 and ** P < .01 differs from the corresponding base-line values.
#P < .05 differs from β-End-(1.31) and saline-treated animals.

Gly-L-Gln-related dipeptides. To further define the structural requirements for gly-L-gln's inhibitory activity, we tested whether glycyl-D-glutamine, α-N-acetyl-glycyl-L-glutamine or glycyl-L-glutamate inhibited β-End-(1–31)-induced hypotension. The gly-L-gln stereoisomer glycyl-D-glutamine was completely inactive, and produced no significant inhibition when injected at the same dose and time interval at which gly-L-gln was maximally effective (table 3). α-N-Acetyl-glycyl-L-glutamine significantly inhibited β-End-(1–31)-induced hypotension [$F(4,25) = 4.59$, $P < .01$], which indicated 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-End-(1–31) (Deakin *et al.*, 1980; Hirsch and Millington, 1991).

The human β-End-(1–31) sequence terminates in glycyl-L-glutamate, rather than gly-L-gln, unlike that in the rat and virtually every other species examined thus far (Yamashiro and Li, 1984). Glycyl-L-glutamate partially blocked β-End-(1–31)-induced hypotension, inhibiting the maximal response to β-End-(1–31) by approximately 50%. Together, these data indicate that gly-L-gln'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.

TABLE 3

The effect of Gly-L-Gln related dipeptides on the hypotensive response to β-End-(1–31)

Pentobarbital-anesthetized rats were treated i.c.v. with β-End-(1–31) (0.5 nmol), followed 15 min later by 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-acetyl-glycyl-L-glutamine (Ac-Gly-L-Gln 10 nmol). Data represent the mean change in MAP (±S.E.) recorded immediately before and 30 min after dipeptide administration and were analyzed by ANOVA followed by Newman-Keuls[1] test. The numbers in parentheses indicate the number of animals in each treatment group.

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

* P < .005; ** P < .01 differs from control.

[^{3}H]-Naloxone binding. The cardiorespiratory effects of β-End-(1–31) are thought to be mediated by μ opioid receptors (Petty and de Jong, 1982; Mosqueda-Garcia and Kunos, 1987; Shook *et al.*, 1990), which raises the possibility that gly-L-gln inhibits β-End-(1–31)'s effects by acting as a μ receptor antagonist. To test this, we conducted receptor-binding experiments using [^{3}H]naloxone as a ligand. [^{3}H]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). Nonspecific binding was consistently less than 25% of total binding.

[^{3}H]Naloxone binding was displaced by morphine and β-End-(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-End-(1–27) displayed considerably lower affinity for [^{3}H]naloxone binding sites, less than one-tenth that of β-End-(1–31), similar to its potency ratio for [^{3}H]-morphine binding (Akil *et al.*, 1981).

Gly-L-gln failed to displace [^{3}H]naloxone binding at concentrations ranging from 1 pM to 10 mM (table 4). For example, [^{3}H]naloxone binding in the presence of 10 mM gly-L-gln, the highest concentration tested, was essentially the same as control values (102.5 ± 6.7% of control; n = 3). Gly-L-gln is therefore unlikely to inhibit β-End-(1–31)-induced hypotension by acting as an opioid receptor antagonist.

Discussion

Gly-L-gln is a major end product of β-End-(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 gly-L-gln inhibits the characteristic hypotension and respiratory depression induced by i.c.v. β-End-(1–31) injection. Gly-L-gln is a relatively potent β-End-(1–31) antagonist; 1.0 nmol inhibited the reduction in MAP induced by 0.5 nmol β-End-(1–31). The gly-L-gln 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 on the temporal sequence of peptide administration. Gly-L-gln was inactive when given alone, however, and did not influence arterial pressure or HR at doses up to 100-fold higher than required to inhibit β-End-(1–31). Collectively, these findings demonstrate that gly-L-gln is a potent antagonist of β-End-(1–31)-induced cardiorespiratory depression but that it lacks hemodynamic activity when given alone.

These data extend previous structure-activity studies that showed that post-translational processing substantially al-

TABLE 4

Inhibition of [^{3}H]naloxone binding by morphine, β-End-(1–31) and β-End-(1–27) but not by Gly-L-Gln

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

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

ters β-End-(1–31)'s central cardioregulatory activity. These studies revealed that most post-translationally derived β-End 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-End-(1–27), on the other hand, is a potent hypotensive and bradycardic agent when centrally injected; indeed, it is even more potent than β-End-(1–31) (Hirsch and Millington, 1991; van Giersbergen *et al.*, 1991). β-End-(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-End-(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-End peptides from pharmacological data. Hypotheses regarding their endogenous function must take into account the anatomical pathways in which β-End is synthesized, the specific β-End 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 POMC neurons projecting from the medial basal hypothalamus (Khachaturian *et al.*, 1985), which primarily synthesize β-End-(1–31) and small amounts of β-End-(1–27) and β-End-(1–26); α-N-acetylation occurs to only a limited extent (Zakarian and Smyth, 1982; Emeson and Eipper, 1986).

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

In addition to its cardiovascular effects, central β-End-(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 anesthetized animals (Feuerstein, 1985). Indeed, β-End-(1–31) reduces arterial pressure at considerably lower doses in anesthetized rats than in conscious animals, which suggests that respiratory depression potentiates its hemodynamic activity (Sitsen *et al.*, 1982). Consistent with this observation, we found that β-End-(1–31) is considerably less potent in mechanically ventilated rats than in spontaneously breathing animals under pentobarbital anesthesia (unpublished data). Thus 0.5 nmol β-End-(1–31), a dose that markedly lowered MAP in spontaneously breathing rats (fig. 1), failed to produce any MAP reduction whatsoever in ventilated animals, and a substantially higher β-End-(1–31) dose (10 nmol) generated only a small decrement in MAP (-9.5 ± 5.5 mm Hg; $n = 6$). These findings suggest that the hypotension β-End-(1–31) produces in anesthetized rats may be secondary to the induction of respiratory depression. They also raise the possibility that gly-L-gln restores blood pressure by inhibiting β-End-(1–31)-induced respiratory depression.

The mechanism responsible for gly-L-gln's inhibitory interaction with β-End-(1–31) remains to be elucidated. One possibility is that gly-L-gln simply blocks the opioid receptors that mediate β-End-(1–31)'s central cardiorespiratory effects. Previous investigations have shown that β-End-(1–31)-induced hypotension is inhibited by pretreatment with either naloxone (Petty and de Jong, 1982; Mastrianni *et al.*, 1989; Hirsch and Millington, 1991) or the selective μ receptor antagonist β-funaltrexamine (Mosqueda-Garcia and Kunos, 1987), suggesting that μ receptors mediate the response. The present finding that gly-L-gln fails to displace [^{3}H]naloxone binding at concentrations as high as 10 mM indicates that blockade of μ receptors is unlikely to account for its inhibitory interaction with β-End-(1–31). Nevertheless, the conclusion that μ receptors mediate β-End-(1–31)'s cardiovascular effects has not met with universal agreement, because a number of investigations have shown that, in contrast to β-End-(1–31), μ-selective agonists elevate MAP, rather than lower it, when injected centrally (Hassen and Feuerstein, 1987; Petty and Sitsen, 1989). This evidence has led to the alternative hypothesis that β-End-(1–31)'s cardiovascular activity is attributable to activation of the putative ε receptor or other nonclassical β-End-(1–31) binding sites (Petty and Sitsen, 1989; Millington and Hirsch, 1994). Thus it is conceivable that gly-L-gln acts as an antagonist at a β-End-(1–31) binding site other than the μ opioid receptor.

Several lines of evidence argue against this conclusion, however. We recently reported, for example, that gly-L-gln inhibits the hyperthermia generated by the microinjection of α-melanocyte-stimulating hormone into thermoregulatory sites in the medial preoptic area (Resch and Millington, 1993). As in the present study, gly-L-gln was inactive when injected alone. Gly-L-gln also inhibits the characteristic behavioral effects produced by i.c.v. injection of α-melanocyte-stimulating hormone (the grooming syndrome and the stretching and yawning syndrome) without influencing these behavioral repertoires when injected alone (Hirsch and O'Donohue, 1986). These findings demonstrate that gly-L-gln inhibits the action of at least one other POMC-derived peptide, an effect that is difficult to ascribe to blockade of opioid receptors or other β-End-(1–31) binding sites.

Gly-L-gln 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 gly-L-gln reduces the firing rates of brainstem neurons when applied iontophoretically. Gly-L-gln's inhibitory electrophysiological activity was unaffected by nal-

oxone, which indicates that opioid receptors are not involved in the response. Strychnine was also ineffective, implying that gly-L-gln does not produce its modulatory effects by activating glycine receptors (Parish *et al.*, 1983). Our data support this conclusion, showing that gly-L-gln'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 gly-L-gln's inhibitory interaction with β-End-(1–31) was stereospecific, to the extent that it was not reproduced by gly-D-gln and that it was not abolished by N-terminal acetylation or by substituting glutamate for glutamine. These findings support the hypothesis that gly-L-gln acts through a stereospecific receptor, but whether this represents a unique gly-L-gln binding site remains to be investigated.

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