Glycyl-glutamine inhibits the respiratory depression, but not the antinociception, produced by morphine

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Owen, Medge D., Can B. Unal, Michael F. Callahan, Kavita Trivedi, Catherine York, and William R. Millington. Glycyl-glutamine inhibits the respiratory depression, but not the antinociception, produced by morphine. Am J Physiol Regulatory Integrative Comp Physiol 279: R1944–R1948, 2000.—Glycyl-glutamine (Gly-Gln; β-endorphin30–31) is an endogenous dipeptide that is synthesized through the posttranslational processing of β-endorphin in brain stem regions that control respiration and autonomic function. This study tested the hypothesis that Gly-Gln administration to conscious rats will prevent the respiratory depression caused by morphine without affecting morphine antinociception. Rats were administered Gly-Gln (1–100 nmol) or saline (10 μl) intracerebroventricularly followed, 5 min later, by morphine (40 nmol icv). Arterial blood gases and pH were measured immediately before Gly-Gln and 30 min after morphine injection. Gly-Gln pretreatment inhibited morphine-induced hypercapnia, hypoxia, and acidosis significantly. The response was dose dependent and significant at Gly-Gln doses as low as 1 nmol. In contrast, Gly-Gln (1–300 nmol) had no effect on morphine-evoked antinociception in the paw withdrawal test. When given alone to otherwise untreated animals, Gly-Gln did not affect nociceptive latencies or blood gas values. These data indicate that Gly-Gln inhibits morphine-induced respiratory depression without compromising morphine antinociception.

MORPHINE AND OTHER OPIATE drugs are the mainstay of pain therapy, but the respiratory depression they produce can be a serious liability (3, 4, 6). Morphine-induced respiratory depression is often treated with naloxone, an opioid receptor antagonist, but naloxone also inhibits morphine analgesia (4). In this study, we investigated whether glycyl-glutamine (Gly-Gln; β-endorphin30–31), an endogenous dipeptide synthesized from β-endorphin, prevents morphine-induced respiratory depression in conscious rats without inhibiting morphine antinociception.

The hypothesis that Gly-Gln will inhibit morphine-induced respiratory depression selectively at first seems paradoxical, because Gly-Gln is synthesized from an opioid peptide, β-endorphin. Gly-Gln is produced from β-endorphin when β-endorphin is posttranslationally processed (15, 22). β-Endorphin is processed to at least five other peptides, Nα-acetyl-β-endorphin1–31, and Nα-acetylated and des-acetyl β-endorphin1–27, and β-endorphin1–26, which display considerably lower affinity for opioid receptors and markedly reduced antinociceptive potency compared with the parent peptide (9, 15). One exception is β-endorphin1–27; although a weak agonist, β-endorphin1–27 is a potent opioid receptor antagonist that blocks the antinociception and other effects produced by β-endorphin in vivo (1, 17, 18, 23, 24). β-Endorphin is almost entirely converted to these Nα-acetylated and truncated derivatives and Gly-Gln in the brain stem (22, 27) and caudal medulla (7), but it is not extensively modified in the midbrain periaqueductal gray region (2), hypothalamus (10, 16, 27), and most forebrain regions (27). The hypothesis that Gly-Gln will inhibit morphine toxicity selectively is thus consistent with evidence that it is synthesized through a posttranslational processing pathway that converts β-endorphin to opioid receptor antagonist and inactive derivatives in brain regions that govern respiratory and autonomic function.

The basic observation that Gly-Gln is a biologically active peptide was first made in the early 1980s (22).
These studies showed that Gly-Gln inhibits the firing frequencies of neurons in the nucleus reticularis gigantocellularis of rat brain stem without affecting neuronal activity in normally quiescent neurons (22). The response was not affected by naloxone or strychnine, which indicates that Gly-Gln does not interact with opioid or glycine receptors. Its localization and biological actions in rat brain stem prompted us to test whether Gly-Gln influences cardiovascular function. We found that central Gly-Gln administration potently inhibited the hypotension produced by morphine or β-endorphin in anesthetized rats, although it did not affect cardiovascular homeostasis when given alone to normotensive animals that did not receive opioids (25, 26). The response was dose related and stereospecific and did not result from Gly-Gln hydrolysis, because equimolar amounts of Gly-Gln’s constituent amino acids were ineffective. Gly-Gln thus prevents the hypotension evoked by opioids without affecting cardiovascular function in normotensive animals.

The present study has two objectives. First, to determine if Gly-Gln inhibits morphine-induced respiratory depression in conscious rats. In an earlier study, we found that Gly-Gln attenuated the respiratory depression caused by morphine in anesthetized rats (26), but anesthesia complicates interpretation of these results. The second and principal objective is to determine whether Gly-Gln inhibits morphine antinociception. The results show that Gly-Gln produced a dose-related inhibition of morphine-evoked hypercapnia, hypoxia, and acidosis in conscious rats but did not affect morphine-induced antinociception, even at doses >100-fold higher than required to inhibit respiratory depression. These data support the hypothesis that Gly-Gln selectively inhibits morphine toxicity without compromising morphine analgesia.

METHODS

Animals and surgery. Male Sprague-Dawley rats (260–400 g; Zivic-Miller, Pittsburgh, PA) were housed in a light- and temperature-controlled room with free access to food and water. The rats were anesthetized with ketamine (7.5 mg/kg im) and xylazine (3 mg/kg im), and a 23-gauge cannula was implanted into the right lateral ventricle as described previously (20). For respiratory studies, a PE-10 cannula filled with heparinized saline (100 U/ml) was inserted into the left femoral artery, exteriorized at the neck, and sealed until use. Rats were handled daily after surgery. The experimental protocols were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Respiratory depression. Four days after surgery, rats were allowed to habituate for 30 min in a quiet environment after which the arterial cannula was flushed with heparinized saline (50 U/ml) and 0.4 ml arterial blood was withdrawn into a heparinized syringe, placed on ice, and replaced with 0.4 ml saline. Immediately thereafter, the animals were treated with Gly-Gln (1, 3, 10, 30, or 100 nmol) or saline (10 μl) intracerebroventricularly followed by morphine sulfate (40 nmol icv) 5 min later. The morphine dose and treatment duration were selected from preliminary dose- and time-response experiments. Thirty minutes after morphine injection, a second arterial blood sample (0.4 ml) was withdrawn through the femoral artery. Arterial blood PO2, P CO2, and pH were analyzed within 20 min of sampling using a Corning model 178 pH/blood gas analyzer.

Nociception. Antinociception was investigated by using the paw withdrawal and tail flick reflex tests, which measure the time required for a rat to remove its hindpaw (11) or tail (14) from a beam of light. For the paw withdrawal test, rats were treated with morphine (30 nmol icv) and Gly-Gln (1, 3, 10, 30, 100, or 300 nmol icv) or saline (10 μl), and paw withdrawal latencies were measured at 15-min intervals for 1 h. Baseline paw withdrawal latencies were determined by averaging the last three of five baseline determinations. Left and right hindpaw responses were averaged at each time point. The cut-off time was set at 20 s to prevent tissue damage. The tail flick test was conducted similarly, except that the cut-off time was set at 8 s. Paw withdrawal and tail flick latencies were converted to percent maximal possible effect (%MPE) by using the formula: %MPE = (postdrug latency – baseline latency)/cutoff latency – baseline latency.

Statistical analyses. Data were analyzed by analysis of variance followed by the Bonferroni multiple-comparisons test.

RESULTS

Central morphine administration produces severe hypercapnia, hypoxemia, and acidosis (8). To test whether Gly-Gln inhibits morphine-induced respiratory depression, rats were pretreated with Gly-Gln intracerebroventricularly and then given morphine sulfate (40 nmol) 5 min later by the same route of administration. Blood (0.4 ml) was withdrawn through a femoral artery cannula immediately before Gly-Gln and 30 min after morphine injection for blood gas and pH determinations. Morphine produced significant respiratory depression (Fig. 1). Within 30 min after morphine injection, PCO2 rose from 34.8 ± 6.1 mmHg, PO2 fell from 86.4 ± 3.2 mmHg, and pH was reduced from 7.47 ± 0.03 to 7.30 ± 0.01.

Gly-Gln pretreatment (1–100 nmol icv) inhibited the hypercapnia, hypoxia, and acidosis evoked by morphine significantly (Fig. 1). Analyses of variance demonstrated significant effects of Gly-Gln treatment on P CO2 [F(5,48) = 6.17, P < 0.001], P O2 [F(5,48) = 6.06, P < 0.001], and pH [F(5,48) = 4.21, P < 0.01]. The response was dose dependent; the minimum significantly inhibitory Gly-Gln dose was 1 nmol for pH and P O2 and 10 nmol for P CO2, and EC50 values were 0.7 nmol for pH, 1.1 nmol for P O2, and 1.5 nmol for P CO2. Gly-Gln (1, 10, or 100 nmol) did not change blood gases or pH when given alone to animals that did not receive morphine. Thirty-five minutes after 100 nmol Gly-Gln administration, for example, there were no significant differences in P CO2 (saline = 33.2 ± 1.5 mmHg; Gly-Gln = 32.2 ± 0.8 mmHg), P O2 (saline = 82.0 ± 1.8 mmHg; Gly-Gln = 80.4 ± 2.1 mmHg), or pH (saline = 7.48 ± 0.03; Gly-Gln = 7.48 ± 0.01) compared with saline-treated controls. These data show that Gly-Gln attenuates the respiratory depression evoked by morphine but does not act as a respiratory stimulant when given alone to otherwise untreated animals.

The ability to prevent morphine-induced respiratory depression does not make Gly-Gln unique, of course.
This property is shared by opioid receptor antagonists, but opioid receptor antagonists also inhibit morphine analgesia. To determine if Gly-Gln inhibits respiratory depression selectively, we tested whether it blocks the antinociception produced by morphine. Nociception was investigated by using the paw withdrawal test (11). Morphine (3, 10, 30, or 100 nmol icv) produced a long-lasting prolongation of paw lift latencies. Response latencies were maximally elevated within 15 min and remained at essentially the same response duration for at least 60 min. The half-maximal effective dose was ~30 nmol.

Gly-Gln had no effect on morphine-induced antinociception. Figure 2 shows that 30 min after Gly-Gln (1–300 nmol) and morphine (30 nmol) were administered to conscious rats intracerebroventricularly, paw lift response latencies were not significantly different than when morphine was given alone, even after a Gly-Gln dose 300-fold higher than that required to inhibit morphine-induced respiratory depression significantly. Response latencies measured 15, 45, and 60 min after Gly-Gln and morphine treatment were also no different than those of rats treated with morphine alone (data not shown). Gly-Gln administration to rats that did not receive morphine did not change paw lift latencies significantly (saline = −1.2 ± 1.6 %MPE; Gly-Gln 1 nmol = 4.7 ± 1.5 %MPE; Gly-Gln 3 nmol = 1.7 ± 3.6 %MPE; Gly-Gln 10 nmol = 1.9 ± 1.5 %MPE; Gly-Gln 30 nmol = −4.7 ± 1.9 %MPE).

The receptor mechanism responsible for morphine analgesia is thought to be different than for β-endorphin, from which Gly-Gln is derived (17, 24). We therefore tested whether Gly-Gln influences β-endorphin-induced antinociception using the tail flick test. Gly-Gln (1, 3, 10, 30, 100, or 300 nmol) did not influence β-endorphin (0.5 nmol) antinociception and did not change response latencies when administered alone to otherwise untreated animals (data not shown). Together, these data indicate that Gly-Gln does not suppress the antinociception evoked by morphine or β-endorphin and does not influence nociceptive responses when given alone to opioid naive rats.

By way of comparison, we tested naloxone, an opioid receptor antagonist that preferentially blocks μ-opioid receptors. As expected, intracerebroventricular naloxone (3, 10, or 30 nmol) administration produced a dose-related inhibition of morphine analgesia; 30 nmol naloxone abolished the response completely (Fig. 2). The same naloxone doses suppressed morphine-
induced respiratory depression significantly (Table 1). Naloxone thus inhibits both the antinociception and the respiratory depression caused by morphine with comparable potency.

**DISCUSSION**

These data show that Gly-Gln inhibits the respiratory depression evoked by morphine selectively, without suppressing morphine-induced antinociception and without affecting respiratory function or nociceptive response latencies in otherwise untreated animals. This finding extends results from an earlier investigation showing that Gly-Gln pretreatment prevents morphine-induced respiratory depression in anesthetized rats (26). These earlier data are compromised by the use of anesthesia, which potentiates morphine-evoked respiratory depression. The present data thus establish that Gly-Gln effectively inhibits the respiratory depression caused by morphine in conscious rats without the additional complication of concurrent anesthesia. In earlier studies, we also found that Gly-Gln attenuates the hypotension caused by morphine and β-endorphin in anesthetized rats with a potency similar to that required to inhibit respiratory depression (25, 26) and subsequently demonstrated that Gly-Gln inhibits hypovolemic hypotension, consistent with evidence that opioid peptide neurons participate in the central control of arterial pressure during hemorrhage (20). Gly-Gln thus inhibits morphine-induced cardiorespiratory depression and hypovolemia-evoked hypotension but does not influence respiratory or cardiovascular homeostasis in untreated animals.

Gly-Gln’s specificity for the respiratory and cardiovascular effects of morphine is consistent with evidence that it is preferentially synthesized in brain stem regions that regulate respiratory and autonomic function. The brain stem is dually innervated by proopiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus and the commissural nucleus of the nucleus of the solitary tract (NTS) (13, 21). Hypothalamic POMC neurons project axons throughout the forebrain, brain stem, and spinal cord but the innervation pattern of NTS neurons is restricted to the brain stem, including baroreceptor and respiratory control centers in the midline and ventrolateral medulla (13, 21). Chromatographic analysis of β-endorphin peptides present in the hypothalamus (10) and caudal medulla (7) suggests that β-endorphin is converted to Gly-Gln and truncated β-endorphin derivatives to a considerably greater extent by NTS than hypothalamic POMC neurons. These findings are consistent with the hypothesis that Gly-Gln is synthesized and released by NTS POMC neurons that influence respiratory and cardiovascular function but not to a functionally significant extent by hypothalamic POMC neurons that modulate nociception.

The receptor mechanism responsible for Gly-Gln’s pharmacological effects has not been identified, although Gly-Gln’s failure to inhibit morphine or β-endorphin antinociception indicates that it does not interact with opioid receptors. Receptor binding experiments support this conclusion. Gly-Gln fails to displace [3H]naloxone binding to rat brain membranes even at millimolar concentrations (25). Conversely, neither opioid peptides nor opioid receptor-selective ligands inhibit [3H]Gly-Gln binding (unpublished data). Conceivably, Gly-Gln may produce effects in brain by interacting with a previously characterized neurotransmitter receptor, transport protein, or related binding site. However, Gly-Gln did not displace radioligands for a wide variety of neurotransmitter receptors or other binding sites analyzed in single concentration displacement assays conducted by the National Institute of Mental Health NovaScreen program (unpublished data). It is also conceivable that Gly-Gln acts through an independent Gly-Gln receptor, although this has yet to be conclusively established and alternative explanations for its biological activity have been proposed (12).

### Table 1. Naloxone inhibits morphine-induced respiratory depression

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<tr>
<th>Treatment</th>
<th>Change From Baseline</th>
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<td></td>
<td>PO2, mmHg</td>
<td>PCO2, mmHg</td>
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<tr>
<td>Saline (6)</td>
<td>-0.2 ± 2.1</td>
<td>0.2 ± 2.1</td>
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<tr>
<td>Morphine (13)</td>
<td>-32.2 ± 2.4</td>
<td>17.6 ± 2.4</td>
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<tr>
<td>Morphine + naloxone</td>
<td>10 nmol (5)</td>
<td>12.2 ± 1.3†</td>
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<tr>
<td></td>
<td>30 nmol (5)</td>
<td>-2.2 ± 2.6†</td>
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Values are means ± SE. Rats were treated intracerebroventricularly with the indicated dose of naloxone or saline (10 μl) followed, 5 min later, by morphine (40 nmol) or saline. Arterial blood samples were removed immediately before naloxone and 30 min after morphine treatment. The data represent the difference between baseline and 30-min posttreatment arterial PO2, PCO2, and pH values and were analyzed by ANOVA followed by the Bonferroni multiple-comparisons test. The numbers in parentheses represent the number of animals in each group. *P < 0.05 and †P < 0.01 differs from morphine-treated animals.

**Perspectives**

These findings underscore both the complexity and potential utility of multitransmitter signaling in brain. POMC neurons are multitransmitter neurons that synthesize not only β-transmitter, but the melanocortins, α-, β-, and γ-melanocyte-stimulating hormones, and other peptides. The physiological and behavioral effects produced by β-endorphin and melanocortin peptides are complementary in some cases but distinctly antagonistic in others (19). Exactly how POMC or other peptidergic neurons produce unambiguous synaptic messages from multiple peptide neurotransmitters with opposing biological actions is not completely understood. One way may be to selectively inactivate specific peptides presynaptically. POMC neurons inactivate β-endorphin through two mechanisms, NH2-terminal acetylation and carboxy-terminal proteolysis (15). Nα-acetylation essentially eliminates β-endorphin’s affinity for opioid receptors but endoproteolytic cleavage generates two functional opioid antagonists,
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β-endorphin1–27, which blocks opioid receptors, and Gly-Gln, which presumably acts through a nonopioid receptor. The dual inactivation of β-endorphin may thus facilitate the effects of melanocortins. This implies that Gly-Gln is synthesized through a process that converts POMC neurons from an opioid to a non-opioid phenotype. Gly-Gln’s specificity for the respiratory and cardiovascular depression caused by opioids is likely to result simply from the location in which this phenotypic switching takes place. The present findings illustrate that, by understanding the dynamics of peptide processing and by scrutinizing processing pathways for minor end-products with interesting biological properties, it may be possible to identify peptides that produce clinically useful pharmacological actions.

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REFERENCES
