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Short communication

Delta opioid receptors contribute to the cardiorespiratory effects of biphalin in anesthetized rats

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Abstract:
Biphalin expresses almost equal affinity for μ- and δ-opioid receptors. The aim of this study was to delineate a possible role of δ-opioid receptors in the cardio-respiratory effects of systemic injection of biphalin in anesthetized, spontaneously breathing rats. In control animals, an intravenous bolus of biphalin (0.3 μmol/kg) evoked apnea, followed by a decreased breathing rate and increased tidal volume, hypotension and bradycardia. Blockade of δ-opioid receptors with naltrindole (4.2 μmol/kg) significantly reduced the duration of apnea, slowdown of respiration, immediate post-challenge hypotension and bradycardia induced by biphalin administration. These results indicate that the activation of δ-opioid receptors adds to the depressive response produced by biphalin.

Key words:
biphalin, respiration, opioid receptors, naltrindole

Introduction

It is generally accepted that respiratory depression induced by the central or systemic injection of opioid peptides is mediated by μ-opioid receptors. The involvement of δ-receptors in the hypoventilatory effects of opioids remains unclear. There are reports on the effectiveness of a selective non-peptide δ-opioid receptor antagonist, naltrindole, in attenuating hypercapnia and hypoxia evoked by systemic sufentanil in conscious dogs and rats [6, 24]. Furthermore, the reversal of post-alfentanil depression of breathing in rats was affected by the systemic injection of the non-peptide δ-opioid receptor agonist BW 373U86, which did not directly influence respiration [22]. It can then be assumed that δ-opioid receptors modulate respiratory impairment induced by the activation of μ-receptors *via* an indefinite interaction. As mentioned earlier, little is known about the effects of δ-receptor activation on ventilatory parameters.

Biphalin, $(Tyr\text{-}D\text{-}Ala\text{-}Gly\text{-}Phe\text{-}NH)_2$, synthesized by Lipkowski et al. [10], expresses equal affinity to μ- and δ-opioid receptors [13]. In our study of the effects of systemic injection of biphalin in anesthetized rats [25], we observed that cardiorespiratory vagal reflexes might be potentiated by the activation of δ-opioid receptors. It is of note that naloxone prevented all the effects of biphalin. Although the antagonist activity of naloxone is 20-fold weaker against δ-opioid receptors compared to μ-opioid receptors [12], it is still capable of blocking them.

In general, the activation of peripheral opioid receptors results in cardiovascular responses, namely, hypotension and bradycardia. Systemic administration of δ-opioid receptor agonists deltorphin II and DPDPE in anesthetized rats were described to induce either hypotension with no change in heart rate [21] or bradycardia [11].

The exact role of peripheral δ-opioid receptors in mediating respiratory and cardiovascular reflexes is still unclear. Therefore, in the present study, we blocked δ-opioid receptors with naltrindole to expose their contribution to the depressant effects of biphalin.

Material and Methods

Animals

All animal procedures complied with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the 4th Local Ethics Committee for Animal Experimentation. Eighteen adult male Wistar rats weighing 199.6 ± 4.14 g were anesthetized with an intraperitoneal (*ip*) injection of 750 mg/kg urethane (Sigma-Aldrich, Poland) and 150 mg/kg α-chloralose (Sigma-Aldrich, Poland). Supplementary doses of the anesthetic were administered as needed based on response(s) to nociceptive test stimuli.

Surgical procedures

Anesthetized animals were placed in the supine position and were allowed to spontaneously breathe room air. The trachea of each animal was exposed in the neck, sectioned below the larynx and cannulated with 1.8–2.4 mm gauge polyethylene tubing. A catheter (0.5–0.8 mm gauge) was inserted into the femoral vein for drug administration. A second catheter was inserted into the femoral artery to monitor blood pressure. A rectal temperature of 38°C was maintained with a heating pad. The rats were neurally intact in both experimental groups.

Apparatus and measurements

Tidal volume signals were recorded with a pneumotachograph head connected to the tracheal cannula, which was linked to a Research Pneumotach System (RSS 100HR, Hans Rudolph Inc., USA) and a computerized recording system (Windows software ver. 3.07.02, KORR Medical Technologies Inc., USA) for measuring and recording tracheal air flow, respiratory frequency (f), tidal volume (V_T), respiratory minute volume (V_E), inspiratory (T_I) and expiratory (T_E) times. The electromyogram of the costal diaphragm was recorded with bipolar electrodes connected to an NL 104 amplifier (Digitimer Ltd, England), filtered and measured with an AS 101 (Asbit, Poland) leaky integrator (time constant, 100 ms). Arterial blood pressure and heart rate were measured with a BP-2 monitor (Columbus Instruments, USA).

The recordings were registered with an Omnilight 8M 36 apparatus (Honeywell, Japan).

Drugs and treatments

Biphalin was synthesized in our laboratory, and naltrindole, a δ-opioid receptor antagonist, was purchased from Tocris (UK). Analytical properties of biphalin [1, 5], as well as its cardio-respiratory effects, have already been described [25]. Both drugs were freshly prepared from powder prior to each experiment. Biphalin and naltrindole were dissolved in isotonic saline (0.9% w/v aqueous sodium chloride) and injected intravenously (*iv*) in volumes of 1 ml/kg and flushed with 0.2 ml aliquots of normal saline.

Animals were divided into two groups:

Group 1. Control animals were given a single dose of biphalin (0.3 μmol/kg, *iv* bolus) (n = 9).

Group 2. Animals were treated by *iv* injections of 4.2 μmol/kg (2 mg/kg) naltrindole. Five minutes later, they were exposed to a biphalin bolus (n = 9). Each animal received a single dose of the compounds.

Naltrindole dose selection was based on initial experimentation. We also tested the effectiveness of 2.1 μmol/kg and 8.4 μmol/kg naltrindole in four animals at each dose (data not shown) and found that the lower dose did not have an impact on biphalin response, whereas the higher dose precluded the effect of biphalin. This indicates that the degree of inhibition corresponds to bound μ and δ sites by naltrindole.

Measurements

The baselines of each individual value of V_T, minute ventilation (V_E), respiratory rate (f), mean arterial pressure (MAP) and heart rate (HR) were determined by averaging the variables measured for five respira-

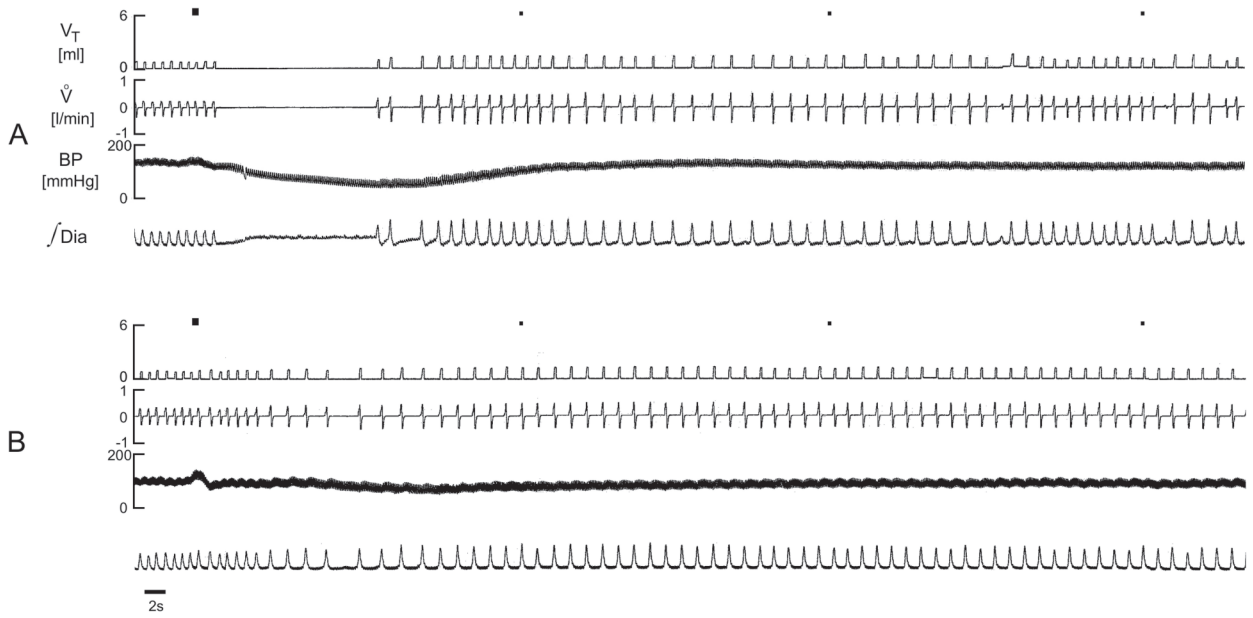

Fig. 1. Responses to biphalin *iv* injection in control rats (**A**) and those pre-treated with naltrindole (**B**). The squares above the top traces show the moment of injection and the following 30 s intervals. (**A**) An immediate apneic spell, and a fall in arterial blood pressure after injection. Recovered breathing occurs at a reduced rate and increased tidal volume. (**B**) Naltrindole blocks δ-opioid receptors and prevents apnea, lessens the drop in blood pressure and reduces decreased respiration rate. V_T – tidal volume; V – airflow; BP – blood pressure; DIA – integrated electromyogram of the diaphragm

tory cycles, before and after injection of biphalin in control rats and those pre-treated with naltrindole. The ventilatory data were derived from integrated pneumotachograph signals just before drug challenge, immediately after the post-challenge apnea (if present) and at 15 s (0.25 min), 1, 2, 10 and 20 min post challenge. The mean arterial pressure was calculated, and the heart rate was recorded at these time intervals. The duration of the apneic period, as indicated by the diaphragm electromyographic activity, was used as an index of respiratory inhibition.

Statistical analysis

The experimental data were analyzed by two-way ANOVA following repeated measurements of post-biphalin challenge time (pre-challenge and defined time points after the challenge) and naltrindole pre-treatment. Differences between individual time points and experimental conditions were evaluated by *post-hoc* Tukey *t*-test. In all cases, $p < 0.05$ was considered statistically significant. The results are shown as the mean ± SEM.

Results

In the current study, the effects of biphalin on the basal respiratory values were similar to those reported previously [25] and are summarized briefly. In neurally intact rats, systemic *iv* injection of biphalin evoked uniform respiratory effects, including immediate apnea followed by a decreased respiratory rate and increased tidal volume.

Pretreatment with the selective δ-opioid receptor antagonist naltrindole did not change the baseline respiratory value but did significantly increase the MAP at the early phase after injection up to 1 min ($p < 0.05$, $n = 9$).

The time interval between naltrindole injection and the subsequent administration of biphalin was 5 min. Figure 1 illustrates the typical response to biphalin injection (0.3 μmol/kg) into the femoral vein in neurally intact rats before (A) and after (B) pretreatment with naltrindole. There were no significant differences between the baseline variables in these two groups of animals. It is of note that the respiratory depression evoked by biphalin and manifested by the apnea was

attenuated in rats treated with naltrindole (Fig. 1B). The post-biphalin drop in the MAP appeared to be less apparent after naltrindole treatment.

As shown in Figure 2, post-biphalin apnea occurred in all control rats, and the mean duration was 13.2 ± 1.1s (n = 9). In five out of nine rats treated with naltrindole, the apnea induced by biphalin was significantly shorter, with a duration of 4.4 ± 1.1 s ($p < 0.001$), compared with control rats.

In both groups of rats, biphalin increased tidal volume (V_T), and there was no significant difference in its size between control and naltrindole-treated animals at

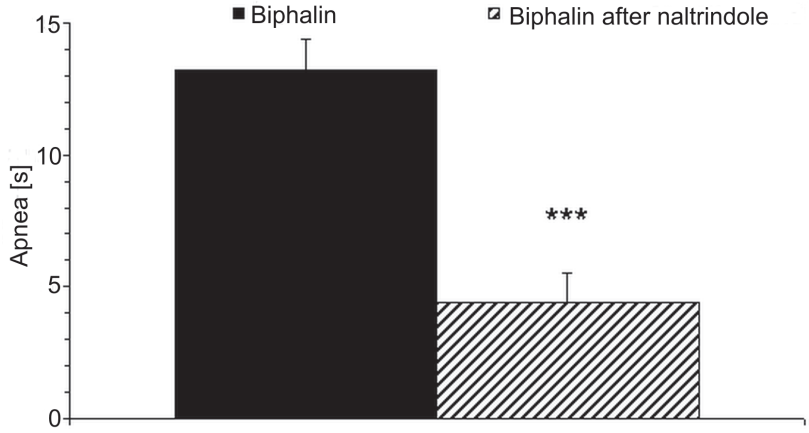

Fig. 2. Duration of post-biphalin apnea in control and naltrindole-treated rats. One-way ANOVA reveals significant differences between both groups of animals (ANOVA, $F_{1, 12} = 25.56$, $p < 0.001$).Values are the means ± SEM, *** $p < 0.001$ between the two conditions

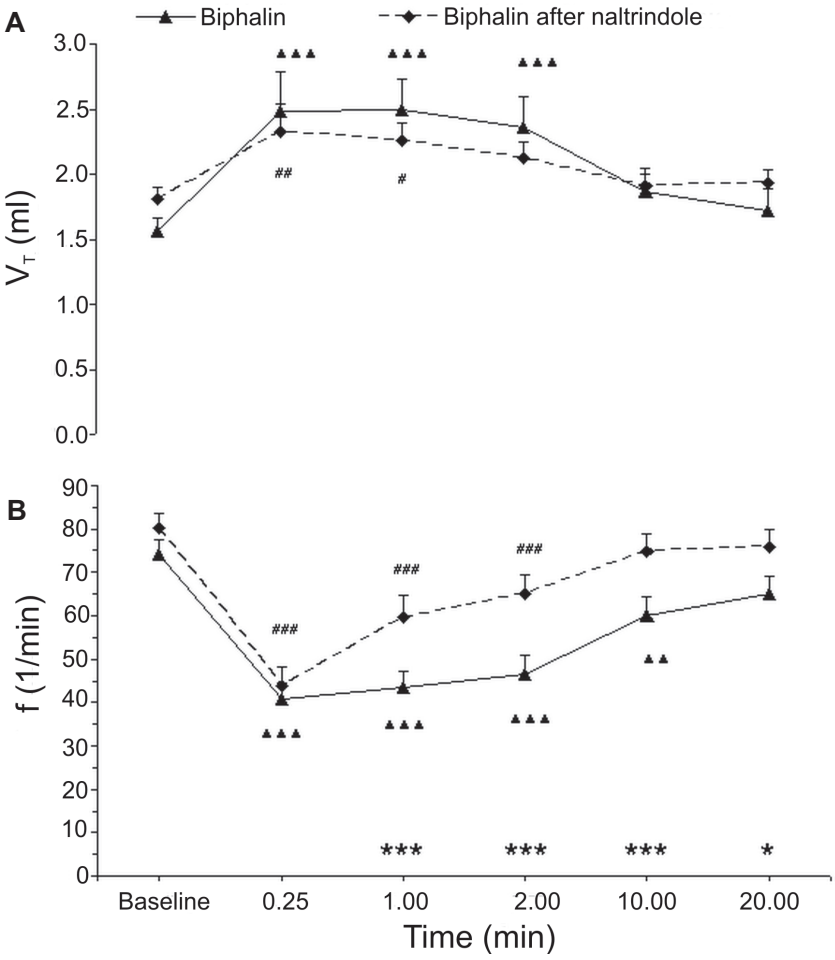

Fig. 3. Mean changes in (**A**) tidal volume (V_T) and (**B**) respiratory rate (f) evoked by biphalin in control (▲, continuous line) and naltrindole-treated rats (◆, dashed line). Two-way ANOVA reveals significant effects of biphalin ($F_{5, 80} = 23.17$, $p < 0.0000001$) and biphalin × δ-opioid receptor blockade interaction ($F_{5, 80} = 2.93$, $p < 0.02$) but shows no effect of δ-opioid receptor blockage ($F_{1, 16} = 0.01$, $p = 0.92$) on tidal volume. Significant effects on respiratory rate induced by biphalin ($F_{5, 80} = 69.56$, $p < 0.0000001$) were diminished by δ-opioid receptor blockage ($F_{1, 16} = 5.12$, $p = 0.038$) and also interaction between biphalin × δ-opioid receptor blockage occurred ($F_{5, 80} = 3.62$, $p < 0.01$). All values are the means ± SEM; ▲▲ $p < 0.01$, ▲▲▲ $p < 0.001$, and # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, *vs.* the baseline values in each condition, respectively; * $p < 0.05$, *** $p < 0.001$, between two protocols

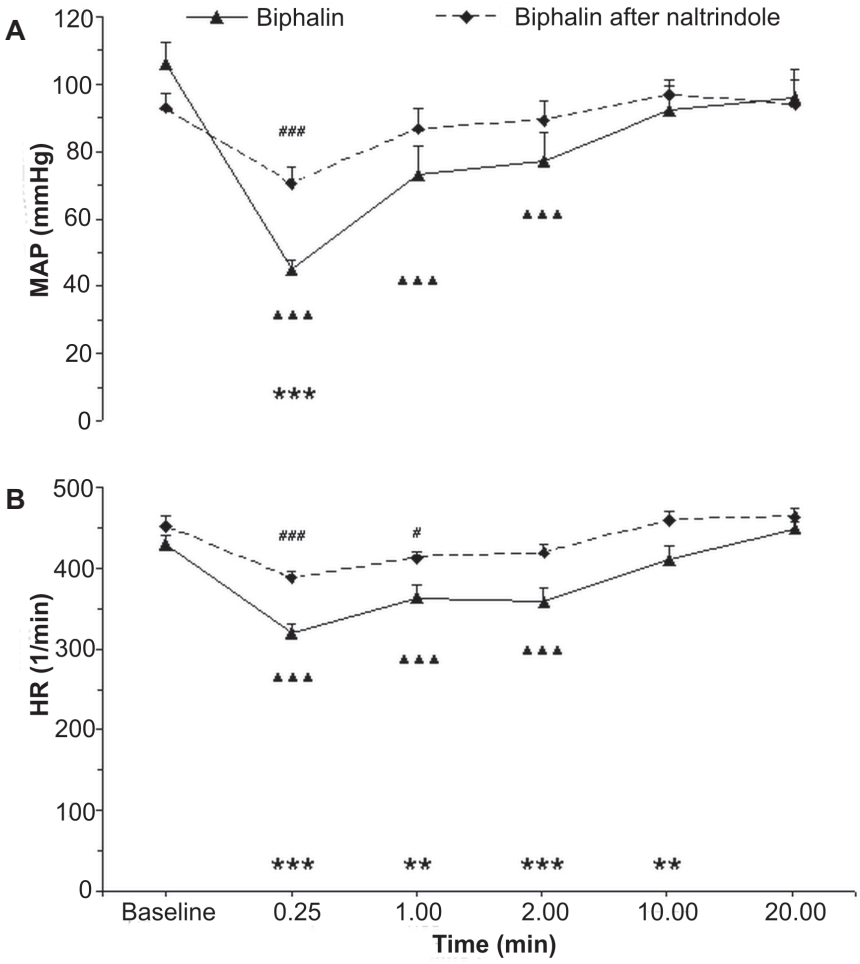

Fig. 4. Mean changes in (**A**) arterial blood pressure (MAP) and (**B**) heart rate (HR) induced by biphalin in control and naltrindole pretreated rats. Two-way ANOVA analysis uncovered significant effects of biphalin ($F_{5, 80}$ = 42.60, $p < 0.0000001$) and biphalin δ-opioid receptor blockade interaction ($F_{5, 80}$ = 8.12, $p = 0.000003$) but not of δ-opioid receptor blockade ($F_{1, 16}$ = 0.72, $p = 0.41$) on MAP and significant effects of biphalin ($F_{5, 80}$ = 45.77, $p < 0.00000001$), δ-opioid receptor blockade ($F_{1, 16}$ = 9.55, $p < 0.01$) and biphalin' δ-opioid receptor blockade interaction ($F_{5, 80}$ = 3.50, $p < 0.01$) on HR. For p-values see Fig. 3

any time point (Fig. 3A). As mentioned above, the blocking of δ receptors reduced the duration of post-biphalin apnea and significantly limited the decrease in respiratory rate compared with the effects in the control rats (Fig. 3B). The decreased rate of respiration was the result of a prolonged expiratory time (data not shown). The blockade of δ-opioid receptors diminished and shortened respiratory inhibition caused by biphalin, which persisted in control conditions from 1 to 10 min following injection (Fig. 3B).

Intravenous administration of biphalin decreased the MAP immediately following drug injection. At 0.25 min, the MAP fell significantly less after the blockade of δ-opioid receptors, compared with untreated rats ($p < 0.001$), and showed an insignificant trend towards an increase by the end of the observation time. In the control rats, post-biphalin hypotension was maintained for 2 min (Fig. 4A).

Bradycardia, evoked by biphalin, was observed during both experimental conditions. As illustrated in Figure 4B, heart rates were significantly less reduced in rats whose δ-opioid receptors were blocked, and the effect lasted for 1 min.

Discussion

This study demonstrates, for the first time, the effects of δ-opioid receptor blockage on cardio-respiratory response to systemic injection of biphalin. Biphalin, a potent enkephalin analogue, binds to both μ- and δ-opioid receptors with high affinity [13]. Although the extremely high antinociception of biphalin has been postulated to be a result of the synergic activation of both μ and δ-opioid receptors [9], such dual agonist action has not been defined in respiratory depression evoked by biphalin.

Earlier experimental evidence pointed to the possibility that tidal volume and respiratory rate are con-

trolled by μ- and δ-opioid receptors, respectively [15]. The literature summarized in the introduction seems to suggest that both types of receptors are involved in the respiratory depressant effects of opioids.

We recently reported [25], and confirmed in the current experiments, that respiratory impairment induced by biphalin results in immediate apnea, followed by a decreased respiratory rate and increased tidal volume. As to the role of the δ-opiate pathway on respiratory depression induced by μ receptor agonist, no study has been devoted to the changes in ventilation and breathing pattern.

To delineate the contribution of δ receptors to biphalin effects, we have used naltrindole, a δ-opioid antagonist that is highly selective for δ sites [17, 18]. In this study, the systemic application of naltrindole (4.2 μmol/kg) did not affect the baseline respiratory variables, which confirms the data reported in rats and mice, with various dosages of naltrindole [14, 20, 22].

These results show that in anesthetized, spontaneously breathing rats, pre-treatment with naltrindole apparently reduces post-biphalin occurrence and duration of apnea, the predominant sign of instability in the respiratory pattern (Figs. 1, 2). It is of note that blockage of δ-opioid receptors did not change the effects of biphalin on tidal volume (Fig. 1B). There was no difference in the profile of tidal volume response within the same time points and between two experimental schemes (Fig. 3A). This suggests that the enhancement of tidal volume is mainly due to the activation of μ-opioid receptors. This assumption is supported by earlier results showing that dermorphin, a highly selective μ-opioid receptor agonist, augments tidal volume in rats [26].

Our data provide evidence for the involvement of δ receptors in the depressant effects of biphalin on respiratory rate. The post-challenge dip in respiration, which dampened minute ventilation, showed a tendency to rapidly return to the baseline respiratory rate in rats pre-treated with naltrindole (Fig. 3B). The effect of this blockage indicates that the depression of respiratory rate caused by biphalin may be mediated by both the δ-opioid and μ-opioid receptors.

This study showed that the activation of δ-opioid receptors also contributes to the cardiovascular effects of biphalin. Naltrindole-mediated receptor blockage, in part, antagonized the prompt post-challenge fall in arterial blood pressure induced by biphalin and markedly reduced the following hypotension (Fig. 4A). Furthermore, animals pretreated with naltrindole presented an attenuated bradycardia in response to biphalin administration, as illustrated in Figure 4B. Our investigation provides experimental evidence showing the contribution of δ-opioid receptors to the depressive cardio-respiratory effects of biphalin. Blocking these receptors did not prevent, but apparently attenuated, the response. There are reports, addressed in the introduction, that have shown that naltrindole is effective in reversing the respiratory depression induced by the ligands of μ-opioid receptors. In experiments assessing antinociception, carried out with conscious animals, depression of ventilation evoked by sufentanil, reflected in arterial gas blood content, was reversed by an intravenous administration of varying doses of naltrindole in dogs and rats [6, 24]. Also, post-alfentanil hypercapnemia and hypoxemia were effectively averted by this antagonist in rats [22].

Our findings in anesthetized rats are somehow different and support the view that δ-opioid receptors potentiate the depressive effects of biphalin on respiration. However, naltrindole, lacking its own respiratory effects [14, 20, 22, this paper] and having 13-fold higher binding affinity for δ than μ-opioid receptors [4], attenuated biphalin-induced ventilatory changes, probably due to antagonism of δ-opioid receptors. It is noteworthy that naltrindole also shows significant affinity for μ-opioid receptors, being only 5.5-fold less potent than naloxone [3], and may be effective in counteracting the respiratory response to biphalin.

It is generally believed that opioid receptors are involved in the control of the cardiovascular system. In our study, hypotensive and bradycardic responses evoked by systemic administration of biphalin were apparently reduced and attenuated (respectively) after pre-treatment with naltrindole. The data on the effects of δ receptor activation on cardiovascular parameters is limited and controversial. In conscious rats, systemic administration of δ ligands failed to affect arterial blood pressure and heart rate [20], whereas anesthetized rats displayed hypotension [21]. In fact, decreases in blood pressure and heart rate are seen with activation of all three types of opioid receptors [23]. Delta and μ, as well as δ and κ receptors, are able to form heterodimers, and these complexes may be crucial for the regulation of the respiratory and hypotensive responses to opioid ligands [8, 19].

In light of the fact that opioid receptors belong to a class of G-protein coupled receptors as do most neuropeptide and nonpeptide receptors co-expressed within the neuraxis, their interaction may potentiate

or inhibit stimulant action of specific agents to one or another type of receptor. Indeed, opioid peptides were shown to antagonize the effects mediated by β-adrenergic receptors in the heart [16], but reinforced A_1 adenosine and α_2 adrenergic receptor-induced antinociception [2]. Similarly, hyperthermia evoked by two neuropeptides (somatostatin and cholecystokinin) was shown to be enhanced by μ receptors [7]. However, substantial contribution of a simultaneous co-release of endogenous opioids in these processes remains far from being explicitly documented.

The naltrindole dosage used in our study (2 mg/kg) constitutes half of the lowest dose that caused sufentanil-induced antinociception in rats [22]. It has already been reported that in large doses (10 mg/kg), naltrindole might saturate μ-opioid receptors [24]. Therefore, the dose we have applied is likely to antagonize δ-opioid receptors and diminish the cardiorespiratory effects of biphalin, which we have shown in this work.

In conclusion, the data presented here indicate that respiratory depression and cardiovascular inhibition following intravenous injection of biphalin are, in part, blocked by naltrindole. This suggests a subtle contribution of the δ-opioid receptor subtype for triggering the cardiorespiratory effects of biphalin.

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