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The effect of a paracetamol and morphine combination on dynorphin A levels in the rat brain

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Abstract

The purpose of this study was to find out whether the combination of inactive doses of paracetamol (PARA) and morphine was able to change dynorphin (DYN) A levels, evaluated by radioimmunoassay, and whether naloxone or [(−)-2-(3 furylmethyl)-normetazocine] (MR 2266), a κ-opioid antagonist, modifies or prevents the activity of this combination on nociception and on DYN levels. The work was suggested by our previous findings which demonstrated that inactive doses of PARA and morphine, when given in combination, share an antinociceptive effect, and that PARA, at antinociceptive doses, decreases DYN levels in the frontal cortex, thus indicating a selective action within the CNS. Our present results demonstrate that the combination of inactive doses of PARA (100 mg/kg) and morphine (3 mg/kg) is just as effective in decreasing the levels of DYN A as full antinociceptive doses of PARA or morphine alone in the frontal cortex of the rat. The values, expressed in pmol/g tissue, were: control = 2.83 ± 0.20; paracetamol (100) = 2.60 ± 0.23; morphine (3) = 2.73 ± 0.24; paracetamol + morphine = 1.34 + 0.16 ($P < 0.05$). The decrease was partially antagonised by MR 2266, but not by naloxone, suggesting that the activity of PARA and morphine in combination on DYN A levels could be mediated, at least in part, through κ-receptors, although other systems may be involved. On the other hand, both naloxone and MR 2266 prevented the antinociceptive effect of the combination in the hot plate test. All our experimental data suggest that PARA and morphine in combination exert their antinociceptive effect through the opioidergic system, which in turn may cause a decrease in DYN levels in the CNS of the rat.

Keywords: Antinociception; Paracetamol and morphine; ir-Dynorphin A levels; Brain; Rat

1. Introduction

Many reports suggest that PARA exerts its analgesic activity both at peripheral and central levels in animals and humans through an inhibitory action on the synthesis of prostaglandins with the pre-eminent action of cyclo-oxygenase 1 [1]. However, increasing evidence suggests that the inhibition of cyclo-oxygenase may not be solely responsible for the central analgesic effect of PARA and NSAIDs [2,3]. It has been suggested that a number of neurotransmitter systems may be involved in the central analgesic effect of NSAIDs and PARA; in particular, serotonergic pathways may play a pivotal role in the central antinociceptive mechanism of NSAIDs [4,5] and PARA [6,7]. In particular, paracetamol possesses a central antinociceptive effect that is accompanied by an increase in brain serotonin content in cortical membranes. On the other hand, it has been shown that morphine stimulates serotonin release via a supraspinal action [8,9], the effect being regionally selective [10].

Pretreatment with naloxone abolished the antinociceptive effect and the increase in 5-hydroxytryptamine (5-HT) concentration induced by PARA or morphine in cortical membranes [11]. The possibility that morphine and PARA may involve interconnected pathways in their mechanism of action prompted us to investigate the effect of a combination of inactive doses of the two drugs on nociception in rats. Our previous data demonstrated that inactive doses of

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Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; DYN, dynorphin; PARA, paracetamol; and ir-DYN, immunoreactive dynorphin.

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PARA and morphine, given in combination, have an antinociceptive activity that is associated with an increase in 5-HT levels in the cerebral cortex. Both the antinociceptive and the biochemical effects were prevented by naloxone [12].

Morphine exerts its analgesic effect through a direct activation of central opioid receptors in the CNS, namely μ and, to a lesser extent, κ and δ, and an indirect action influencing the release of opioid peptides or other neurotransmitters [13]. Few reports are available on the effect of an acute treatment with morphine, but it is documented that chronic treatment decreases methionine-enkephalin, pro-enkephalin, and β-endorphin in some brain areas [14]. In several CNS areas, peptides were unaffected by an acute injection of morphine, although DYN peptides were decreased in the brainstem and hippocampus (DYN A and DYN B).

Dynorphins, a family of endogenous opioids related to the opioid precursor prodynorphin, are potential modulatory peptides [15] having a role in opiate analgesia [16,17]. DYN (1–13), which is known to be a selective endogenous ligand for κ-receptors, is widely distributed in the CNS, peripheral nervous system (PNS), and pituitary gland tissues [18]. The stimulation of κ-receptors in the CNS seems to produce analgesia [19]. On the other hand, DYN (1–13) attenuated opiate analgesia when i.c.v. injected in rats naive to morphine [20]. The role of DYN in analgesia is controversial; while Tseng and co-workers found that i.c.v. injection of bremazocine, a κ-receptor agonist, induced the release of DYN for the production of antinociception [21,22], other authors hypothesised that DYN is released in response to certain i.c.v. agonists, including morphine, for the production of antianalgesia, and proposed a mediator role for DYN in a descending antianalgesic system [23].

Our decision to determine the DYN (1–13) level in the cortex was suggested by data indicating that acute and chronic pain changes the levels of the peptide in this and other areas of the brain [24], as does morphine treatment [25]. We focused our attention on the cerebral cortex, because it is a major site of projections from the ventral thalamus and takes part in one of the multiple neural systems responsible for pain processing [26]. Furthermore, opioid receptor subtypes are widely distributed in the cerebral cortex where they modulate noxious stimuli [27]. Finally, in our previous experiments, we demonstrated that a full antinociceptive dose of PARA reduced DYN A levels only in the cerebral cortex among the several areas investigated.[1]

The aim of this work was to investigate the possible involvement of an opioidergic mechanism in the antinociceptive effect of the combination of inactive doses of PARA and morphine; this was explored by studying the influence of the pretreatment of naloxone or MR 2266, [(−)-2-(3 furylmethyl)-normetazocine], a κ-opioid receptor antagonist, on the drug combination. To elucidate whether the analgesic effect of the combination may be correlated with changes in the opioid levels, we chose to evaluate DYN A (1–13) levels in the frontal and temporal–parietal cortex in the rat brain and the effect of a pretreatment with the same antagonists thereon. We also evaluated the effect of an analgesic dose of PARA or morphine alone on DYN A levels in the same areas.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (Harlan-Nossan) weighing 180–190 g at the beginning of the experiments, were housed in Plexiglas cages, four per cage, with free access to food and water, and were maintained on a 12-hr dark/light cycle (light on at 7 a.m.) under controlled environmental conditions (temperature 22 ± 1°; humidity 60%). The ethical guidelines for investigation of experimental pain in conscious animals were followed in all tests, and all the procedures were carried out according to the EEC ethical regulations for animal research (EEC Council 86/609; D.L. 27/01/1992, No. 116).

2.2. Treatment schedule

The rats were randomly divided into groups of 8 animals each. Naloxone (1 mg/kg in 2 mL of sterile saline), MR 2266 (5 mg/kg dissolved in saline by adding an equivalent amount of HCl 0.1 M/H_2O 1:20) or saline (2 mL/kg) was i.p. injected; PARA (100 or 400 mg/kg, dissolved in vehicle, which consisted of 12.5% 1,2-propanediol in sterile saline) or vehicle (2 mL/kg) was injected i.p.; and morphine (3 or 8 mg/kg in sterile saline) or saline was s.c. injected. Morphine (3 mg/kg s.c.) or saline was injected 10 min after PARA (100 mg/kg i.p.). Groups of rats were pretreated with naloxone or MR2266 or saline 10 min before the PARA and morphine combination. The effectiveness of the doses of the antagonists used to block the respective opioid receptors was verified in other experiments in which the same doses of the antagonists, resulting from dose–effect curves, significantly blocked the antinociception induced by its own opioid receptor agonist.[2] Thus, in the present research, we used the lower dose able to antagonize the antinociceptive effect of the PARA and morphine combination using the hot plate test [12]. The rats were subjected to the hot plate test 20 min after the last treatment. Two additional groups of animals were injected with either PARA (400 mg/kg) or morphine (8 mg/kg) or vehicle. Immediately after the pain

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[2] Sandrini M, Vitale G, Pini LA. Unpublished observations.

threshold assessment, rats were anaesthetised by ethyl ether, and decapitated; brains were removed, and areas were dissected according to the method of Glowinsky and Iversen [28] and stored at −80° until required for analysis. As control experiments showed no significant difference in response to saline and 1,2-propanediol (vehicle) at the concentration used, the data were pooled.

2.3. Hot plate test

The hot plate consisted of an electrically heated surface (Socrel DS-35, Ugo Basile) kept at a constant temperature of 54 ± 0.4°. The latencies for paw licking or jumping were recorded for each animal. The baseline latencies in the hot plate test ranged from 6.3 ± 0.4 to 6.7 ± 0.5 sec. (Analysis of variance, ANOVA, $P > 0.5$). The analgesic efficacy of the drug was evaluated as a percentage of the maximum possible effect (% MPE), according to the formula (TL − BL)/(45 − BL) × 100, where TL = test latency, BL = baseline latency, 45 = cut-off time, in seconds.

2.4. Radioimmunoassay (RIA)

Immunoreactive dynorphin A (ir-dynorphin A) levels were measured in the frontal cortex and parietal-temporal cortex. Samples were weighed, homogenised in 10 mL/g of 0.1 M acetic acid at 95°, and held at this temperature for 10 min. Homogenates were then centrifuged at 10,000 × *g* at 4° for 20 min, and the supernatants were removed and stored at −24° until assay for ir-DYN by an already described RIA [29].

For the opioid peptide immunoreactive ir-DYN A content, tissues were obtained from eight rats in each treatment group and analysed for ir-DYN A detection. ^{125}I-Tyr^{1} DYN A (1–13) (Peninsula Laboratories) was utilised in our studies. The antiserum employed ("Lucia") was kindly supplied by Prof. B.M. Cox. Raised against DYN A (1–13), it displays full cross-reactivity to DYN A (1–17), partial cross-reactivity to the COOH-extended form [e.g. DYN A (1–32)], and does not recognise DYN A (1–8), DYN B, or any other opioid peptides [29]. In the light of these properties, the total immunoreactivity detected by the antiserum is referred to as ir-DYN A. It was used at the appropriate dilution to give about 30% binding of ^{125}-I-DYN A (1–13) added (5000 cpm). In a typical assay, the IC_{50} was 10 fmol/assay tube (intra- and interassay coefficient of variations were 5% and 7%).

RIA evaluates a steady-state level of the peptide: this statement is normally accepted in measuring any peptide level by this technique. Although the antibody used is selective enough to assure the presence of DYN A (1–13) peptide, the retention time was assessed by means of HPLC analysis to validate the results, as described elsewhere [30]. Radioimmunoassays were carried out in 0.15 M sodium phosphate buffer (pH 7.4), with incubation for 18–24 hr at 4°. The reaction was terminated by the addition of 1.0 mL of buffer containing 3.0% charcoal and 0.3% dextran. Bound peptide was separated by centrifugation at 5000 × *g* at 4° and 1-mL samples of the supernatant were counted for 1 min with a gamma counter.

Table 1
Influence of naloxone (NAL) and MR 2266 on the antinociceptive effect of paracetamol (PARA) + morphine (MORP) in the hot plate test

Treatment	% MPE
SAL + VEH + SAL	1.6 ± 2.8
SAL + PARA + SAL	1.8 ± 1.7
SAL + VEH + MORP	2.5 ± 2.0
SAL + PARA + MORP	32.9 ± 10.2*
NAL + VEH + SAL	2.1 ± 2.2
NAL + PARA + MORP	9.2 ± 4.9
MR 2266 + VEH + SAL	3.2 ± 2.7
MR 2266 + PARA + MORP	3.9 ± 4.0

NAL (1 mg/kg i.p.), MR 2266 (5 mg/kg i.p.), or saline (2 mL/kg) was administered 10 min before PARA (100 mg/kg, i.p.) and 20 min before MORP (3 mg/kg, s.c.). The hot plate was started 20 min after the last treatment. % MPE = percentage of the maximum possible effect. Values are expressed as means ± SEM for 8 rats for each group.

* $P < 0.05$ vs SAL + VEH + SAL values (ANOVA followed by Student–Newman–Keuls test).

2.5. Drugs

Paracetamol, morphine hydrochloride, and naloxone were purchased from Sigma Chemical Co. MR 2266 was a gift from Boehringer Ing. Formalin was obtained through Bracco Chemical Co.

2.6. Data analysis

The data were expressed as means ± SEM. The data obtained from the hot plate test as well as the values of DYN cerebral levels were evaluated by one-way ANOVA followed by Student–Newman–Keuls test when appropriate. A two-way analysis of variance followed by a 2 × 2 factorial analysis by means of orthogonal comparison [31] was used to analyse the effect of the paracetamol and morphine combination, the effects of naloxone or MR 2266 pretreatment, and their interaction. The data were evaluated with the Student-Newman-Keuls test when the effects of naloxone, MR 2266, and PARA + morphine were being evaluated separately.

3. Results

The antinociceptive effect of the PARA and morphine combination was completely prevented by pretreatment with naloxone (1 mg/kg i.p.) or MR 2266 (5 mg/kg i.p.) (Table 1); the factorial analysis showed a negative interaction [$F_{(1-28)} = 6.8$; $P < 0.01$]; [$F_{(1-28)} = 6.2$; $P < 0.01$]. The two opioid antagonists alone did not affect the % MPE

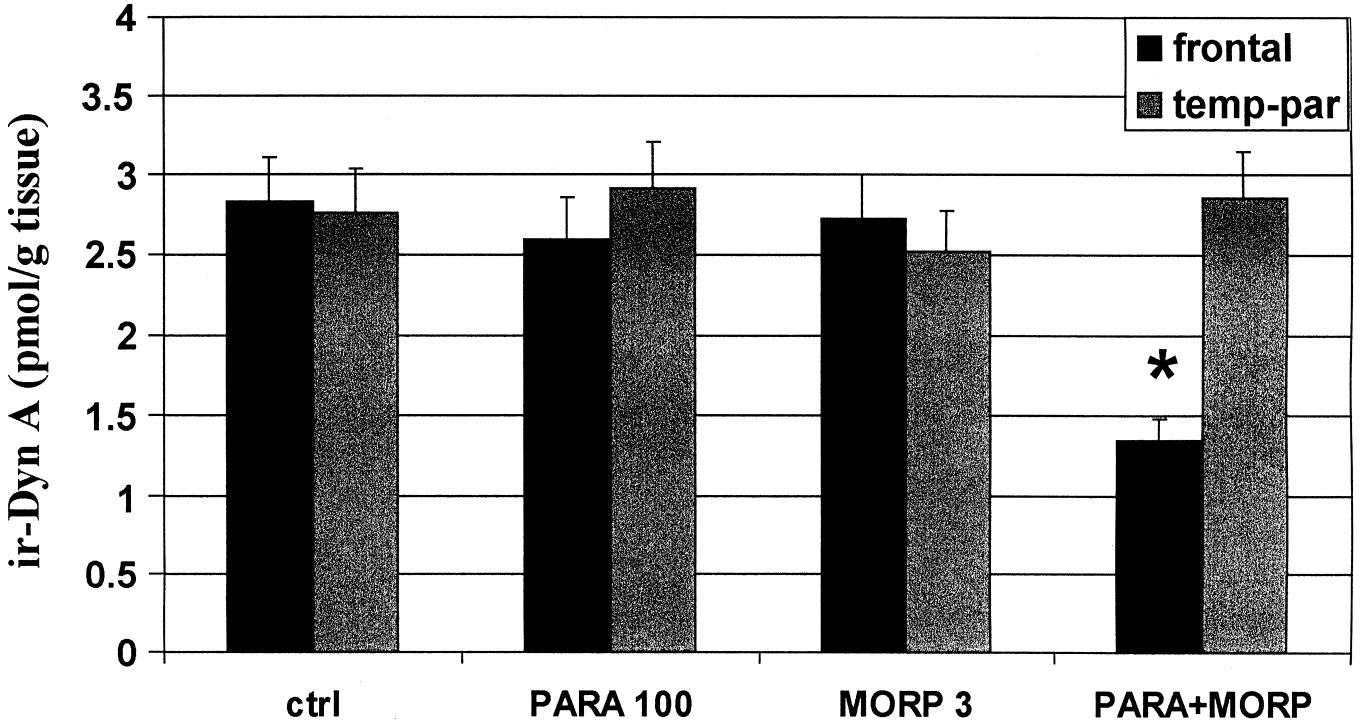

Fig. 1. The effect of the combination of paracetamol (PARA, 100 mg/kg, i.p.) and morphine (MORP, 3 mg/kg, s.c.) on ir-DYN A content in the frontal and temporal–parietal (temp–par) cortex. PARA was administered 10 min before MORP. Rats were killed 20 min after the last treatment and brain areas were weighed and frozen at −80° until assayed. Values are expressed as means ± SEM for 8 rats for each group. *$P < 0.05$ vs control (ctrl) values (ANOVA followed by Student–Newman–Keuls test).

values; moreover, neither PARA (100 mg/kg) alone nor a single dose of morphine (3 mg/kg) was able to modify the levels of ir-DYN A in the frontal or temporal–parietal cortex. The combination of the same doses of PARA and morphine, on the other hand, significantly decreased the peptide levels in the frontal cortex, but not in the temporal–parietal cortex (Fig. 1). The combination of the two drugs showed a potentiation effect on ir-DYN A, demonstrated by the factorial analysis [$F_{(1–28)} = 9.4$; $P < 0.01$].

Naloxone (1 mg/kg) was not able to prevent the decrease in DYN levels induced by the combination. MR 2266 significantly prevented the effect of the PARA and morphine combination, but the interaction test showed no significance [$F_{(1–28)} = 0.92$; $P > 0.05$]. Naloxone or MR 2266, when given alone, significantly increased DYN levels in the frontal cortex, but not in the temporal–parietal cortex (Fig. 2). The incomplete prevention exerted by MR 2266 may be due to the activity of this drug, as the values of the peptide levels were significantly different from those of the combination, but also significantly different from control values (controls, 2.80 ± 0.19; saline + PARA + morphine, 1.27 ± 0.14; MR 2266 + PARA + morphine, 1.95 ± 0.25, pmol/g tissue).

Fig. 3 shows the effect of fully antinociceptive doses of PARA (400 mg/kg) or morphine (8 mg/kg) on ir-DYN A levels: PARA significantly decreased the values in the frontal cortex, but not in the temporal–parietal cortex, while morphine showed a significant decrease in both areas.

4. Discussion

The combination of inactive doses of PARA and MORP provoked an antinociceptive effect in the hot plate test that was prevented by pretreatment with naloxone or MR 2266. This result agrees with the suggestion that the analgesic action of NSAIDs and PARA is carried out, at least in part, through the opioidergic system [32]. To confirm the possible involvement of an opioidergic mechanism in the antinociceptive effect of the paracetamol and morphine combination, we evaluated the levels of DYN A in two brain areas under the above experimental conditions. This choice was suggested by the findings that both acute and chronic treatment with morphine affect DYN levels in several CNS nuclei [24,25]. Another result supporting this hypothesis is the decrease, shown by our group, in DYN levels in the frontal cortex by high doses of PARA.[3] Data obtained in the present study show that the PARA and morphine combination provoked a decrease in DYN levels in the frontal cortex but not in the temporal–parietal cortex, one that is of the same order as that observed when PARA or morphine was given alone in full antinociceptive doses. This result suggests that the two drugs, given in combination at inactive

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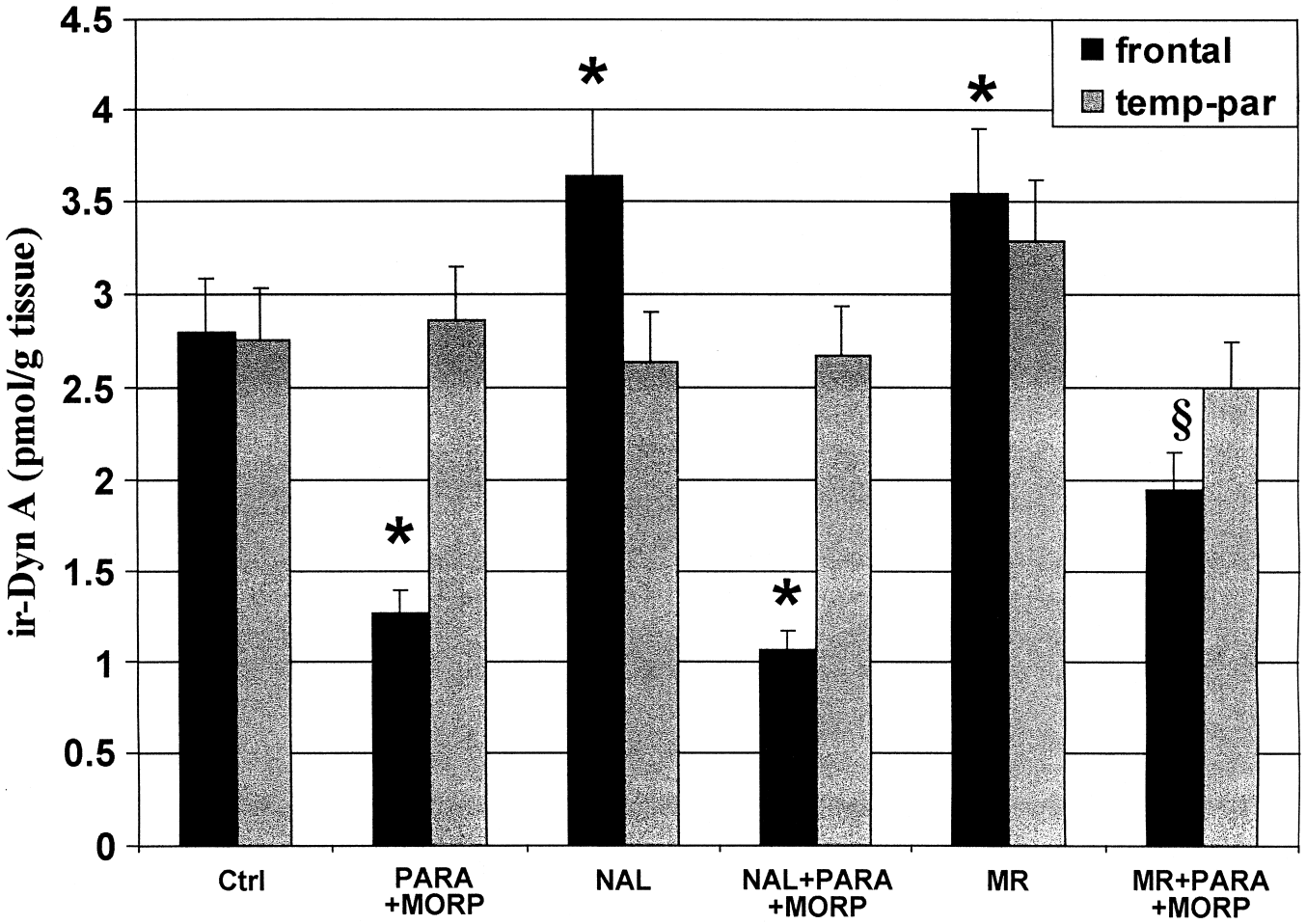

Fig. 2. Influence of the pretreatment with naloxone or MR 2266 on the effect of the paracetamol and morphine combination on ir-DYN A content in the frontal and temporal–parietal (temp–par) cortex. Naloxone (NAL, 1 mg/kg, i.p.), MR 2266 (MR, 5 mg/kg, i.p.), or saline (2 mL/kg) was administered 10 min before paracetamol (PARA, 100 mg/kg, i.p.) and 20 min before morphine (MORP, 3 mg/kg, s.c.). Rats were killed 20 min after the last treatment and brain areas were weighed and frozen at −80° until assayed. Values are expressed as means ± SEM for 8 rats for each group. *$P < 0.05$ vs control (ctrl) values, §$P < 0.05$ vs both control and PARA + MORP (ANOVA followed by Student–Newman–Keuls test).

doses, may act, at least in part, through similar mechanisms involving the opioidergic system.

DYN expression in discrete areas of brain has been amply documented by Simonato and Romualdi [33], who reported the synthesis of this family of peptides in the frontal cortex, as well as in other areas. Prodynorphin gene expression is controlled by dopaminergic [34], serotonergic [35], and opioidergic [36] systems, and by morphine [37].

The slight action of low doses of PARA and morphine on the opioidergic system seems to be sufficient to modify peptide levels when the drugs are given in combination. The changes in DYN A levels do not fully explain the antinociceptive effect exerted by the combination, but the two effects proceed jointly, suggesting a role for this peptide in the behavioural effect investigated. DYN A is the main endogenous ligand for κ-receptors in the spinal cord and in the brain [19,38]. Moreover, some authors have demonstrated that the activation of κ-opioid receptors by i.c.v. injection of a κ-opioid receptor agonist, bremazocine or U50,488H, influences DYN release, producing analgesia [21,39].

We used the radioimmunoassay to assess DYN A levels; this method measures the amount of peptide present at the moment of analysis, but not that released or neo-synthesised. Thus, a decrease in DYN A levels can be due to two different mechanisms: an increased release or a decreased biosynthesis.

On the other hand, it is widely accepted that the transmission of the nociceptive information along the CNS is a complex process involving neural structures and a variety of neurotransmitters, including opioid peptides and their receptors [40]. β-Endorphin, methionine-enkephalin, and DYN are released both supraspinally and in the spinal cord, modulating nociception by binding to opioid receptors [41].

Multiple descending pain control pathways are involved in nociception induced by the stimulation of various opioid agonists given supraspinally [42,43]. The antinociception induced by μ-receptor agonists such as morphine and [d-

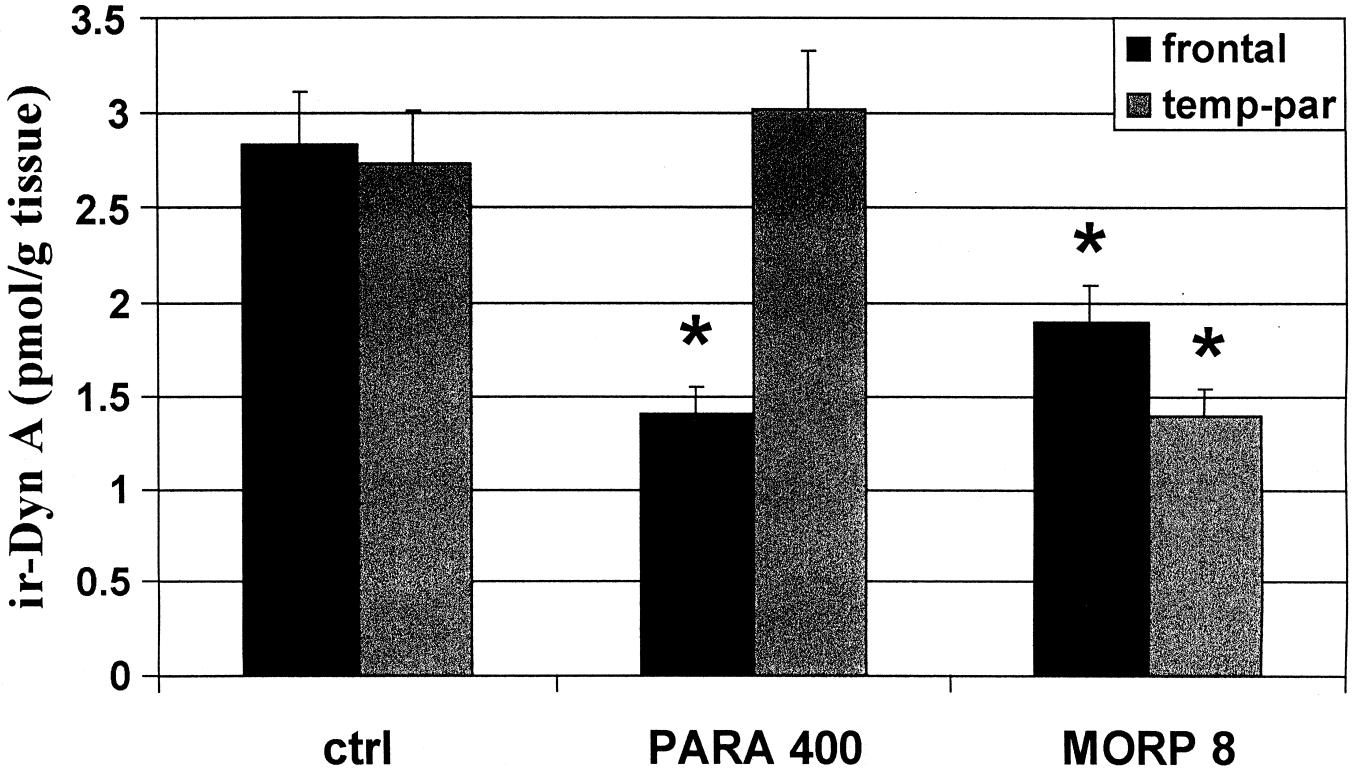

Fig. 3. The effect of paracetamol (PARA, 400 mg/kg, i.p.) or morphine (MORP, 8 mg/kg, s.c.) on ir-DYN A content in the frontal and temporal–parietal (temp–par) cortex. Rats were killed 20 min after treatment and brain areas were weighed and frozen at −80° until assayed. Values are expressed as means ± SEM for 8 rats for each group. *$P < 0.05$ vs control (ctrl) values (ANOVA followed by Student–Newman–Keuls test).

Ala(2), N-Me(4), Gly(5)-ol]-enkephalin (DAMGO) is mediated by the release of noradrenaline and 5-hydroxytryptamine (5-HT), which act on α_2-adrenoceptors and 5-HT receptors, respectively [44], whereas the antinociception induced by κ-opioid receptor agonists is mediated by the release of DYN A acting on κ-opioid receptors [21]. Moreover, it has been suggested that opioid receptors might be involved in the feedback control of the release of endogenous opioids from some opioidergic neurons, but not from others [26]. Therefore, the block of κ-receptors by a specific κ-opioid receptor antagonist, MR 2266, may decrease the effect on DYN A levels, whereas, at the dose used, naloxone (which acts chiefly on μ-receptors) cannot.

The combination of ineffective doses of PARA and morphine may provoke antinociception through the action on the different pathways proposed: in particular, it may somehow influence the release of peptides acting as agonists on μ- and κ-receptors, respectively, producing analgesia. Thus, the ability of both naloxone and MR 2266 to reverse paracetamol plus morphine antinociception may be explained by the existence of these different descending pathways. On the other hand, our data on DYN levels may be correlated with the observation, made by other authors, that κ-opioid receptor antagonists block the changes in DYN levels induced by endomorphin 2 by the activation of a particular pain control pathway [22]. In our experiments, the block of κ-receptors by the κ-antagonist MR 2266 may counteract the change in DYN A levels provoked by the drug combination via a similar mechanism.

The failure of naloxone to prevent the change in DYN levels provoked by PARA and morphine in combination is partially in line with its failure to antagonise the decrease in DYN levels induced by a high dose of PARA.[1] Our present data show that MR 2266 did not completely prevent the effect of the combination, while fully preventing the effect of a high dose of PARA.[1] MR 2266 alone significantly increased DYN levels, so the interaction between MR 2266 and morphine may be due only to a summation effect, as they have opposite effects on DYN levels. Taken together, these data suggest that the drug combination can influence the opioidergic system and so modify DYN release, although the exact mechanism has not been established. Taking into account the involvement of multiple pathways in nociception, we suggest that PARA + MORP acts on the 5-hydroxytryptamine and DYN pathways, separately and/or in combination. In fact, it has been reported that numerous physiological events, including analgesia, are modulated by an interaction between endogenous opioids and the serotonergic system [45].

The biochemical effect of PARA seems to be regionally

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selective, as it significantly decreases the DYN levels only in the frontal cortex, while it is ineffective in other brain areas studied.[1] In the present paper, we show that morphine alone decreased the levels of the peptide both in the frontal and in the temporal–parietal cortex. We observed a significant positive interaction between PARA and morphine only in the frontal cortex, where a full dose of PARA was active, suggesting that the activity of both drugs, when given at inactive doses, is necessary for the modification of the opioidergic system. If the action of PARA is regionally selective, the potentiation of the effect of morphine may occur only in these areas, but not in other areas of the brain. Moreover, these data confirm the importance of the frontal cortex in the effect of PARA, in particular its antinociceptive activity, as it has been suggested that this area is the end point of the nociceptive system where the noxious information is perceived and processed [27,46]. In conclusion, PARA and morphine in combination seem to exert their antinociceptive effect through a more complex mechanism than a single action on the dynorphinergic system modulating the release of DYN A, since naloxone did not affect the decrease in DYN levels induced by the combination, and MR 2266 only partially reversed it.

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