Naloxone-Reversible Antinociception by Paracetamol in the Rat

L. A. PINI, G. VITALE, A. OTTANI and M. SANDRINI

Department of Internal Medicine, Clinical Pharmacology Unit (L.A.P., G.V.), and Department of Biomedical Sciences, Section of Pharmacology (A.O., M.S.), University of Modena, Italy

Accepted for publication October 7, 1996

ABSTRACT

Paracetamol at the dose of 400 mg/kg i.p. displayed antinociceptive activity in the hot-plate test and the formalin test. Moreover, it induced a significant increase in brain serotonin (5-HT) concentration and a reduction in the number of 5-HT2 receptors in cortical membranes. Pretreatment with naloxone abolished this antinociceptive activity both in the hot-plate test and in the first phase of the formalin test without affecting the serum concentration of paracetamol. At the same time, naloxone prevented the increase in 5-HT concentration in the central nervous system and the reduction in 5-HT2 receptors in cortical membranes. Competition experiments demonstrated that paracetamol possesses affinity for [3H]naloxone binding sites. The action of morphine on nociception and on the serotonergic system was similar to that of paracetamol; all morphine-induced effects were blocked by naloxone. These data provide further evidence for a central antinociceptive effect of paracetamol and support the hypothesis that paracetamol exerts its antinociceptive activity through the serotonergic system. Moreover, our results point to the relationship between serotonergic and opiateergic systems in the antinociceptive activity of paracetamol.

Paracetamol is often classified as an NSAID, because it possesses analgesic activity against pain of mild to moderate severity but has few anti-inflammatory properties and exerts its analgesic effect via a central action. Its inhibitory activity on the synthesis of prostaglandin is more evident on cyclooxygenase 1 than on cyclooxygenase 2 (Vane and Botting, 1995), both peripherally and within the CNS, even though the exact antinociceptive mechanism of action of this drug is still not completely clear (Clissold, 1986).

Its biochemical properties, such as its weak inhibitory activity on the synthesis of peripheral prostaglandins, its low plasma-protein binding, its liposolubility and its ability to cross the blood-brain barrier suggest a central activity, which has been reported in several studies both in animals (Carlsson et al., 1988) and in humans (Chen and Chapman, 1980). It has been postulated that this central effect might be linked to the ability of PARA to inhibit central cyclooxygenase (Clissold, 1986; Björkman, 1995). On the other hand, it has been demonstrated that tissue cyclooxygenase in rat brain homogenates is not inhibited in doses of PARA up to 100 mg/kg (Abdel-Alim et al., 1978). Thus, the inhibition of cyclooxygenase may not be solely responsible for the central antinociceptive effect of NSAIDs (Malmberg and Yaksh, 1992).

There is evidence to suggest that the serotonergic system may play a role in the antinociceptive mechanism of NSAIDs (Sandrini et al., 1995; Warner et al., 1990) and of PARA (Pelissier et al., 1995), whereas it has been proposed that other neurotransmitter systems, including opiate pathways, may be involved in the central analgesic effect of this class of drugs (Björkman, 1995). Accordingly we decided to conduct a study on both neurotransmitter systems, serotonergic and opiateergic, to gain further insight into the mechanism of the analgesic action of PARA.

MORP stimulates 5-HT release via a supraspinal action (Bineau-Thurottes et al., 1984), and 5-HT depletion in the CNS decreases the analgesic effect of MORP (Bodnar et al., 1981); thus, this drug exerts its analgesic effect, at least in part, through the serotonergic system (Yang et al., 1994; Drissen and Reiman, 1992). Moreover, there is evidence that the antinociceptive effects of opiates are potentiated by some NSAIDs (Poggioli et al., 1980; Maves et al., 1994) and by PARA (Pircio et al., 1978), whereas naloxone is able to revert antinociception by dicyclomenac in the rat (Björkman et al., 1990).

The study of the effect of PARA on the serotonergic and opiateergic systems, therefore, might throw some light on the complex antinociceptive activity of this widely used drug. The purpose of this study was twofold. First, we wanted to find out whether naloxone was able to modify or prevent the

ABBREVIATIONS: CNS, central nervous system; NSAIDs, nonsteroidal anti-inflammatory drugs; 5-HT, serotonin; PARA, paracetamol (acetaminophen); MORP, morphine; ANOVA, analysis of variance; %MPE, percentage of maximum possible effect.
antinociceptive effect of PARA in the hot-plate and formalin tests and the changes to 5-HT levels and central 5-HT_2 receptor numbers, and the influence of naloxone this activity.

Materials and Methods

Animals

Adult male Wistar rats (Morini, S.Polo d’Enza, Reggio Emilia, Italy), weighing 180 to 190 g at the beginning of the experiments, were housed in Plexiglas cages, four per cage, with free access to food and water, and maintained on a 12 h dark/light cycle (light on at 7:00 A.M.) under controlled environmental conditions (temperature, 22 ± 1°C; humidity, 60%). The ethical guidelines for investigation of experimental pain in conscious animals were followed in all tests, and the procedures were carried out according to the EEC ethical regulations for animal research (EEC Council 86/609; D.L. 27/01/1992, A.M.) under controlled environmental conditions (temperature, 22 ± 1°C; humidity, 60%). The reason for testing at different times after PARA administration was to obtain serum and brain tissue from rats sacrificed after an identical time lapse from the first injection, so that we could compare both the behavioral and the biochemical parameters.

Two additional groups of animals were tested regarding their behavioral profile by use of either PARA (400 mg/kg) or vehicle. Four other groups were intraperitoneally pretreated with naloxone or saline and then subcutaneously injected with MORP (8 mg/kg in a volume of 2 ml/kg of saline) or vehicle, i.p. injected 30 min after pretreatment with naloxone. This dose of PARA was scheduled as being effective in our experimental condition, on the basis of dose-response experiments in both the hot-plate and the formalin tests performed in our laboratory under identical experimental conditions (Pini et al., 1996).

The rats were tested 30 and 90 min after the final treatment in the formalin (50 µl of 5% formalin solution injected s.c. into the right hind paw) and hot-plate (54 ± 0°C) tests, respectively, and were sacrificed 2 h after naloxone treatment. The reason for testing at different times after PARA administration was to obtain serum and brain tissue from rats sacrificed after an identical time lapse from the first injection, so that we could compare both the behavioral and the biochemical parameters.

Drug Treatment

The rats were randomly divided into groups of eight animals each. Naloxone (1 mg/kg in 2 ml/kg of sterile saline) or saline were injected i.p., and PARA (400 mg/kg, dissolved in a volume of 10 ml/kg of vehicle, which consisted of 12.5% of 1,2-propanediol in sterile saline) or vehicle were i.p. injected 30 min after pretreatment with naloxone. This dose of PARA was scheduled as being effective in our experimental condition, on the basis of dose-response experiments in both the hot-plate and the formalin tests performed in our laboratory under identical experimental conditions (Pini et al., 1996).

The rats were tested 30 and 90 min after the final treatment in the formalin (50 µl of 5% formalin solution injected s.c. into the right hind paw) and hot-plate (54 ± 0°C) tests, respectively, and were sacrificed 2 h after naloxone treatment. The reason for testing at different times after PARA administration was to obtain serum and brain tissue from rats sacrificed after an identical time lapse from the first injection, so that we could compare both the behavioral and the biochemical parameters.

Drug Assay

After the hot-plate and formalin tests, PARA serum levels were measured 90 min after drug administration and were assayed by fluorescent polarized immunoassay, which uses the concept of fluorescence detection with polarized light emission. Fluorescein-tagged drugs and unlabeled drugs are incubated with antibody and then excited with polarized light; as the drug concentration increases, there is an increase in unbound fluorescein-labeled molecules which tumble free in solution and cause the light to be depolarized upon emission. A TDX analyzer was used for drug determination (Abbott Laboratories, Chicago, IL).

5-HT Determination

After the hot-plate test, the cerebral cortex and the pons were rapidly removed and frozen; 24 h later the areas were homogenized in 0.1 N HCl (100 µl/mg wet wt.) and centrifuged for 10 min at 1500 x g at 4°C. 5-HT was measured in 25 µl supernatant by means of radioimmunoassay (Manz et al., 1986). A commercial kit (IBL, Immunological Laboratories, Hamburg, Germany; intraassay coefficient of variation, 10%; sensitivity, 12 pg/tube) was used. The data were expressed as nanograms per gram of brain tissue.
Binding Assay
The characteristics of 5-HT₂ binding sites were evaluated according to Leysen et al. (1982) with minor modifications. Brain regions were homogenized in 5 ml of ice-cold 0.25 M sucrose (12 strokes of a Teflon pestle at 120 rpm) and centrifuged at 1300 × g for 10 min at 4°C. This procedure was repeated, then the combination of sucrose supernatants was diluted with 10 ml of 50 mM Tris-HCl, pH 7.7, and the suspension was centrifuged at 3500 × g for 10 min. The pellet was resuspended in 20 ml of Tris-HCl buffer and centrifuged once at 50,000 × g for 10 min. The pellet was then homogenized and diluted in Tris-HCl (about 300 mg protein/ml). Aliquots of membranes (800 µl) were placed in plastic test tubes containing [³H]ketanserin (six increasing concentrations in 10% ethanol), methysergide (10 µM, dissolved in Tris-HCl buffer to define nonspecific binding) or Tris buffer at 37°C for 15 min. The mixture was filtered under reduced pressure through Whatman GF/B glass fiber filters, previously soaked for 5 min in 0.5% polyethyleneimine with use of a Millipore vacuum pump and rapidly rinsed twice with 5 ml ice-cold Tris buffer. The filters were transferred to plastic vials containing 6 ml of Packard Optifluor and shaken. The vials were stored for 20 h at 4°C in the dark.

The following concentrations were used: 0.05 to 4 nM [³H]ketanserin, a 5-HT₂ receptor ligand (specific activity, 87.5 Ci/mmol). The specific binding was 65 to 70% of the total binding for [³H]ketanserin.

Competition binding studies were performed to assess opiate receptors and PARA activity. Binding assay was performed according to the method of Windh and co-workers (1995) with minor modifications. Brain areas were homogenized in 10 volumes of 50 mM Tris buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid with Brickman polytron (setting, 7 × 15 sec), then centrifuged at 18,000 × g for 15 min. After suspension in 20 volumes of buffer, the membranes were incubated at room temperature for 1 h, centrifuged again, resuspended in 20 volumes of buffer and frozen at −70°C until required for use. Thereupon, the membranes were centrifuged at 38,000 × g and resuspended in assay buffer: 50 mM Tris-HCl, pH 7.4, containing (in mM): MgCl₂, 5; NaCl, 100; ethylenediaminetetraacetic acid, 1. Binding was performed by incubating the membrane suspension in a final volume of 1 ml of assay buffer for 90 min at room temperature, 10 µM MORP being used to define the specific binding. The reaction was stopped by filtration under reduced pressure through Whatman GF/B glass filters and rapid rinsing twice with 5 ml ice-cold buffer. The filters were processed following the same procedure as above. Saturation curves were performed with concentrations of [³H]naloxone between 0.2 and 10 nM. Competition studies used 10 concentrations of between 0.1 nM and 100 µM unlabeled PARA to displace binding of 1 nM [³H]naloxone (specific activity, 58.2 Ci/mmol). Inhibition constant (Kᵢ) values were evaluated according to the formula: IC₅₀/[1 + Free/Kᵢ].

Drugs
PARA, morphine sulfate and naloxone were purchased from Sigma Chemical Co. (St. Louis, MO); [³H]ketanserin and [³H]naloxone from Du Pont NEN, Co. Ltd (Milan, Italy). Formalin was obtained through Bracco Chemical Co. (Milan, Italy). PARA was dissolved in 12.5% 1,2-propanediol in sterile saline, and MORP was dissolved in sterile saline.

Statistical Analysis
The results obtained from the behavioral tests were analyzed with Student’s t test and the Mann-Whitney U test for motor activity and sensorimotor score, respectively.

The results of the binding experiments were analyzed according to the method of Rosenthal (1967). The equilibrium dissociation constant (Kᵢ) and maximum number of binding sites (Bₘₐₓ) were evaluated individually for each sample with six concentrations of labeled drug. A two-way analysis of variance was used to analyze the effects of naloxone treatment, PARA treatment and their interaction, followed by a 2 × 2 factorial analysis by means of orthogonal comparisons (Snedecor and Cochran, 1980).

The data were examined by ANOVA followed by Student-Newman-Keuls’ test when the effects of naloxone and PARA were being evaluated separately.

Results
Figures 1 and 2 show that naloxone is able to antagonize the analgesic effect exerted by PARA or MORP both in the hot-plate test and in the formalin test. Naloxone (1 mg/kg) alone did not affect either algesimetric test; the %MPE values in the hot-plate test and the total number of flinches in all the phases of the formalin test were similar to those obtained in saline + vehicle-treated rats. Naloxone significantly prevented the antinociceptive action of PARA (400 mg/kg) on both %MPE in the hot-plate test and the total number of flinches in the first phase of the formalin test [F(1,28) = 8.44; P < .01 and F(1,28) = 4.48; P < .05, respectively]. The same results were obtained for MORP (8 mg/kg) [F(1,28) = 19.3; P < .01 and F(1,28) = 13.2; P < .01, for the hot-plate test and the first phase of the formalin test, respectively]. Moreover, the effect of PARA on phase 2A and 2B was not affected by pretreatment with naloxone, whereas the same pretreatment was also able to reverse the effect of MORP on phase 2A and 2B.

The behavioral profile was not modified by the treatment with 400 mg/kg of PARA. No significant differences were observed between experimental and control rats either in motor activity [1403 ± 48 and 1383 ± 56 movements (mean ± S.E.), respectively, n = 8, P > .05, t test] or in sensorimotor score [1.01 ± 0.3 and 0.48 ± 0.4 (mean ± S.E.), respectively, n = 8, P > .05].

Treatment with naloxone (1 mg/kg) did not change the serum levels of PARA assayed 90 min after drug administration; the concentrations of PARA were 278 ± 23 and 320 ± 16 mg/kg i.p.

Fig. 1. Influence of naloxone (NAL) pretreatment on the antinociceptive action of PARA or MORP in the hot-plate test. PARA (400 mg/kg i.p.) or MORP (8 mg/kg s.c.) were administered 30 min after naloxone (1 mg/kg i.p.), and the rats were tested 30 and 90 min after MORP and PARA treatment, respectively. Values are mean ± S.E. of eight rats for each group. SAL, saline; VEH, vehicle (12.5% 1,2-propanediol in sterile saline). *P < .05 vs. SAL + VEH (ANOVA followed by Student-Newman-Keuls test).
**Discussion**

Naloxone significantly prevents the action of PARA in the hot-plate test and in the first phase of the formalin test, but it does not affect PARA activity in the second phase. The injection of formalin causes an immediate and intense increase in the spontaneous activity of C fiber afferent (Heapy et al., 1987) and evokes a distinct quantifiable behavior indicative of pain, flinching/shaking of the injected paw (Wheeler-Aceto et al., 1990). The behavioral response is biphasic, with an initial acute phase followed by a quiescent period and then a prolonged response between 20 and 60 min (Malmberg and Yaksh, 1992). It has been suggested that the early phase is caused by a direct effect of formalin on nociceptors, whereas the late phase is a tonic response in which inflammatory processes are involved and neurons in the dorsal horns of the spinal cord are activated (Tjølsen et al., 1991). Indeed, there is no obvious inflammatory state in the injected paw of the animal during the first 5 to 10 min after the injection of formalin. Thus, antinociceptive non-anti-inflammatory properties of drugs can be evaluated after the formalin injection has been applied and for 5 to 10 min thereafter (Hunskaar et al., 1985). On the other hand, the hot-plate test measures the complex response to an acute, not inflammatory, nociceptive input and can be considered a good model for studying central antinociceptive activity.

Our dose-response results (Pini et al., 1996) show that PARA, at the dose of 300 mg/kg, is able to affect the behavioral response in both phase 1 and phase 2A of the formalin test, whereas at the dose of 400 mg/kg it is active in all the phases. These data disagree with those of Malmberg and Yaksh (1994), who showed that some NSAIDs reduce the first period of the second phase, with no or minimal effect on the first phase, which suggests that the mechanism of action for NSAIDs is related to the inhibition of exaggerated spinal processing. On the other hand, MORP suppresses all the phases of the formalin test in our experimental conditions, as previously shown for specific mu receptor agonists (Malmberg and Yaksh, 1993).
The antinociceptive activity in the two pain tests examined, devoid of any inflammatory process, provides further evidence for a central action of PARA, as suggested by several authors (Carlsson et al., 1988; Chen and Chapman, 1980; Filetta et al., 1991; Vitale et al., 1995).

The lack of antagonistic effect of naloxone in the second phase of the formalin test is not caused by the absence of its pharmacological activity, for it has been shown that its effect lasts for more than 2 h (Granados-Soto et al., 1995). Moreover, we demonstrated that it antagonizes the effect of MORP in all the phases of the test. At the same time, naloxone failed to antagonize the effect of PARA in the writhing test induced by ethacridine acid (Björkman, 1995), which suggests that the mechanisms of action of PARA involved in antinociception differ in the two phases of the formalin test.

Interestingly, we noticed the failure of naloxone to exert an antagonistic effect on the action of PARA in phase 2 of the formalin test; the modulation of central mechanisms, such as the antagonism of opioid receptors by naloxone, may therefore be less relevant to the overall behavioral reaction. Naloxone-induced inhibition of PARA activity in the first phase of the formalin test, and in the hot-plate test as well, strongly suggests that the PARA-induced antinociceptive effect is mediated via a mechanism related to opioid receptor activation, although the subtype and the location within the CNS remain unclear. Recent studies have indicated that some NSAIDs exert a central opioid receptor-mediated effect (Björkman, 1995), although the exact mechanism has not been fully elucidated. Indeed, indirect action on opioid receptors with release of endorphins or enkephalins has already been proposed for ketorolac and diclofenac (Domer, 1990; Sacerdote et al., 1983).

To find out whether the interaction of PARA in the opioid mechanism is of a direct or indirect nature, we investigated the possible interaction of PARA with naloxone binding sites. Competition experiments demonstrated that PARA competes for \(^{3}H\)naloxone binding sites. This surprising result prompted us to conclude that PARA may behave like MORP regarding not only its analgesic effect but also its action on mu receptors. These results of in vitro experiments indicate, however, a low affinity of PARA for mu receptors and suggest a dose-related effect in which PARA may bind directly to opioid receptors only at high concentrations. On the other hand, the micromolar concentrations of PARA used for naloxone binding experiments are lower than the serum levels achieved after the administration of 400 mg/kg in the rat (Pini et al., 1996), but comparable with therapeutic levels in humans (Clissold, 1986). Moreover, the distribution of PARA is homogeneous in all tissues, with a brain/serum ratio close to 1, the concentration in the serum thus reflecting the concentration in the brain (Ochs et al., 1985).

Another result of our present experiments is that naloxone blocks both the increase in 5-HT levels in the brain areas examined and the decrease in the cortical 5-HT\(_2\) receptor number induced by PARA. MORP induces changes in the serotonergic system similar to those obtained with PARA, which are also reversed by naloxone.

Our hypothesis is that PARA, in acting on opiate receptors, may release 5-HT that provokes an analgesic effect; this is supported by many findings which indicate that 5-HT takes part in the complex nociceptive pathways and plays a pivotal role in antinociception (Malmsgren, 1990; Richardson, 1990).

It has been demonstrated that 5-HT\(_2\) receptors are involved in pain transmission (Eide and Hole, 1991), and the reduction in the number of these receptors occurs concomitantly with pain perception, as shown by the present data. 5-HT\(_2\) receptors reveal a specific distribution in many brain areas including the frontal cortex (Leysen and Pawels, 1990), and the cortex is one of the most important axonal projection targets for neurons derived from brainstem, where 5-HT\(_{1A}\) receptors have high density (Türk, 1990). 5-HT\(_{1A}\) receptors are also found in the hippocampus, septum, raphe nuclei and spinal cord (Millan and Colpaert, 1991). We therefore decided to study those brain areas, such as the cerebral cortex and the pons, that have a high density of 5-HT\(_{1A}\) and 5-HT\(_{2}\) receptors.

Furthermore, the reduction in the number of 5-HT\(_2\) receptors in the cortical, but not in the pontine areas, could depend on the different density of receptors in these areas and would emphasize the role of the cortex as the endpoint for the serotonergic antinociceptive system.

Many findings have highlighted the complexity of the adaptive mechanisms of the 5-HT system, but the monoamine adaptation theory implies that a persistent exposure to agonists or endogenous neurotransmitters results in receptor down-regulation. Darmani and co-workers (1992) showed that, after agonist exposure, the ability of the 5-HT\(_2\) receptor system to induce down-regulation appears in a rel-
atively short period of time. Thus, PARA, by indirectly increasing the concentration of the 5-HT, may induce an adaptive down-regulation of 5-HT<sub>2</sub> receptors. Further, we have previously demonstrated that the depletion of brain 5-HT by parachlorophenylalanine prevents the antinociceptive activity of PARA in phase 1 but not in phase 2 of the formalin test (Vitale et al., 1995), thus confirming the observation that lesion of the bulbospinal serotonergic pathways inhibits the antinociceptive effect of PARA (Tølsen et al., 1991) and pointing to the involvement of 5-HT in the antinociceptive activity of PARA.

In the CNS, 5-HT neurons are involved in nociceptive transmission as well as in pain inhibition induced by opiate agonists (Bensemana and Gascon, 1978; Lin et al., 1980). Moreover, enkephalins and dynorphins have been shown to coexist with 5-HT in some raphe and dorsal horn neurons (Glazer et al., 1981); this would point to an indirect interaction with opioid receptors which triggers an endogenous descending pain-suppression system (Yang et al., 1994).

However, opioids affect other neurotransmitters, including dopamine and norepinephrine; presumably, this altered balance between several different modulatory substances may be responsible for opioid-induced changes in behavioral states including nociception (Tao and Auerbach, 1995). MORP enhances brain 5-HT synthesis and metabolism (Rivot et al., 1988); these effects are regionally selective (Tao and Auerbach, 1995). Microdialysis studies also indicate that extracellular 5-HT is increased after MORP administration (Grauer et al., 1992; Tao and Auerbach, 1994). Furthermore, Bineau-Thurottes and co-workers (1984) have demonstrated that MORP stimulates 5-HT release via a supraspinal site of action.

Our experimental data confirm that MORP enhances 5-HT levels, albeit to a lesser extent than PARA; indeed, our experimental design fails to establish a direct correlation between the potency of the analgesic effect and the degree of 5-HT concentration, probably because of the pain models used and the different pharmacokinetic profiles of the two drugs.

The data presented in this paper suggest that PARA may activate opiate receptors that in turn may increase 5-HT levels, at least in the cerebral cortex and in the pons, thus provoking an analgesic effect. Indeed, in the mechanism of action of PARA, a 5-HT-mediated antinociception is of interest because central 5-HT activation may potentiate the effect of opioids, as observed in rats (Baraldi et al., 1983) and humans (Bentley and Head, 1987). These potentially regulatory and interactive mechanisms between 5-HT and opioid transmission in nociception are supported by the finding that the analgesic effect of PARA depends on an intact 5-HT neurotransmission and is antagonized by the opioid antagonist naloxone.

In conclusion, these data provide further evidence for a central antinociceptive effect of PARA antagonized by naloxone, which suggests that this activity may involve the opiateergic pathways which in turn activate the serotonergic system.

Acknowledgments
The authors would like to thank Professor Alfio Bertolini for his most precious help in supervising the present paper.

References
BENSEMANA, D. AND GASCON, A. L.: Relationship between analgesia and turn-


Pini et al., 940

Send reprint requests to: L-A Pini, MD, Clinical Pharmacology Unit via del Pozzo, 71, I-41100 Modena, Italy.